Use of *P*-Element-Mediated Transformation to Identify the Molecular Basis of Naturally Occurring Variants Affecting *Adh* Expression in *Drosophila melanogaster*

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ABSTRACT

The purpose of the work reported here is to identify the molecular basis of the difference in level of expression between the polymorphic Slow and Fast alcohol dehydrogenase (*Adh*) alleles in *Drosophila melanogaster*. Previous studies have shown that Fast lines typically have a two- to threefold higher activity level than Slow lines and they also have a substantially higher level of ADH-protein (estimated immunologically). The results of a restriction fragment length polymorphism study in relation to ADH activity variation had previously suggested that the difference in *Adh* expression between allozymes might not be due entirely to the amino acid replacement substitution, but could be due in part to linkage disequilibrium with a regulatory site polymorphism. Here we describe an approach that makes use of *P*-element-mediated transformation in order to identify the nucleotide substitution(s) responsible for this difference in ADH level. This approach consists of generating recombinants *in vitro* between *Adh* region clones derived from a typical Slow/Fast pair of alleles and then testing for the effects of particular restriction fragments on expression *in vivo* by transformation. Using this approach, the effect on both ADH activity and ADH-protein level clearly maps to a 2.3-kb restriction fragment that includes all of the *Adh* coding sequence and some intron and 3′ flanking sequence, but excludes all of the 5′ flanking sequence of the distal (adult) transcriptional unit. Comparison of Kreitman's DNA sequences for this fragment from several Slow and Fast alleles showing the typical difference in activity level shows that only three nucleotide substitutions distinguish all Fast from all Slow alleles. Thus, it is likely that one or more of these substitutions causes the major difference in *Adh* expression between allozymic classes. One of these substitutions is, of course, the Slow/Fast amino acid replacement substitution (at 1490) while the other two are nearby third position silent substitutions (at 1443 and 1527). A quantiative analysis of variation among transformant stocks shows that the *P*-element transformation approach can be used to localize even relatively small effects on gene expression (on the order of 20%).

A LARGE body of literature shows that natural populations of Drosophila harbor extensive genetic variability affecting the quantitative level, tissue distribution and developmental pattern of specific enzyme activities (reviewed by LAURIE-AHLBERG 1985). Many of these variants are *cis*-dominant, map very close to or within the structural gene and affect enzyme concentration (*e.g.*, CHOVNICK *et al.* 1980; SHAFFER and BEWLEY 1983; MARONI and LAURIE-AHLBERG 1983; BEWLEY 1981; DICKINSON 1978). These types of variants represent likely candidates for "regulatory" polymorphism (*i.e., not* amino acid substitutions). However, primary structure effects on enzyme turnover rate remain a possible mechanism of altered enzyme level in nearly every case, and in no case involving an enzyme has the molecular basis of the effect been determined. In recent years, the molecular cloning of protein-coding genes and analyses of restriction fragment length polymorphisms have revealed a number of associations between particular DNA-level changes, such as insertion/deletion variants, and altered level of protein expression (SHERMOEN and BECKENDORF 1982; ESTELLE and HODGETTS 1984; AQUADRO *et al.* 1986). However, there is so much polymorphism at the DNA level in Drosophila populations (see KREITMAN 1983) that such associations could be spurious. Identification of the molecular basis of a naturally occurring variant affecting protein expression will require a direct, experimental approach in order to distinguish between the polymorphism causing the effect and other associated polymorphisms.

P-element-mediated transformation in Drosophila provides a powerful method for assessing the effects on expression *in vivo* of DNA sequences that have been manipulated *in vitro* (SPRADLING and RUBIN 1982; RUBIN and SPRADLING 1982). Transformation experiments have been done with several different genes and, although integration occurs at a variety of chromosomal locations, the transduced genes are ex-

pressed with normal tissue and developmental specificity and, in most cases, at approximately normal levels, provided that minimal lengths of flanking sequences are included (*e.g.*, SPRADLING and RUBIN 1983; GOLDBERG, POSAKONY and MANIATIS 1983; SCHOLNICK, MORGAN and HIRSCH 1983). These results suggest a method for determining which of the many substitutions that distinguish two alleles actually affect expression. The approach involves cloning the gene from two lines that differ in expression, construction of recombinants between the sequences *in vitro* and testing for the effects of particular restriction fragments *in vivo* by transformation. After localization of the effect to a relatively small restriction fragment, DNA sequencing and site-directed mutagenesis can be used to identify the particular substitution or insertion/deletion difference responsible. Here we show that even relatively small quantitative effects (on the order of 20%) can be investigated in this way.

The purpose of the work reported here is to identify the molecular basis of the difference in level of expression between the polymorphic Slow (S) and Fast (F) alcohol dehydrogenase (*Adh*) allozymes. Amino acid sequencing has shown that ADH-F differs from ADH-S by a threonine/lysine substitution at residue 192 (FLETCHER *et al.* 1978) and recent DNA sequencing of several alleles of each electrophoretic type indicates that this is generally the only difference in primary structure (KREITMAN 1983). Lines homozygous for F alleles generally have a two- to threefold higher activity level than S lines and they also have a substantially higher concentration of ADH-protein estimated immunologically (GIBSON 1972; DAY, HILLIER and CLARKE 1974; LEWIS and GIBSON 1978; MCDONALD, ANDERSON and SANTOS 1980; BIRLEY, COUCH and MARSON 1981; MARONI *et al.* 1982). The difference in ADH concentration appears to account for a large part, but probably not all, of the activity difference between allozymes (see also WINBERG, HOVIK and MCKINLEY-MCKEE 1985). ANDERSON and MCDONALD (1983) reported the results of a protein turnover study in which they failed to find any difference in the *in vivo* rate of degradation of ADH-protein, whereas F lines had significantly higher rates of synthesis *in vivo*. They also reported evidence suggesting that F lines have a higher level of ADH-mRNA than S lines. Most of this work has been done with whole adult flies, but the activity difference between allozymes is essentially the same for whole larvae and in different larval tissues (MARONI *et al.* 1982).

The results of an extensive study of restriction fragment length polymorphism in the *Adh* region revealed a pattern of strong nonrandom association among ADH activity level, ADH allozyme and several restriction site polymorphisms (AQUADRO *et al.* 1986). One of the strongest associations in the region occurs between the S/F substitution site and a *Bam*HI polymorphic restriction site located about 7 kb upstream from it. Only two of the four possible haplotypes occur at high frequency (+S and −F). Among the 49 chromosomes analyzed, only four have one of the two rare haplotypes (−S and +F). Two of these rare haplotype alleles show very unusual levels of ADH: one +F line has a low activity typical of a S line and one −S line has a high activity typical of a F line. These results suggested that the difference in ADH level between allozymes might not be due to the amino acid substitution itself, but rather to association with a regulatory polymorphism located 5′ of the S/F substitution site. Under this hypothesis, the two rare haplotype lines that appear to have switched activity class are interpreted as recombinants between the S/F substitution site and the putative regulatory site while the inferred recombination site for the other two rare haplotypes occurs further upstream, between the putative regulatory site and the *Bam*HI site. Here we describe a transformation experiment designed to test this hypothesis by localizing the difference in ADH level to a restriction fragment within the *Adh* region. The results clearly rule out involvement of any polymorphisms within the 5′ flanking region and narrow the range of likely possibilities to one of three substitutions within the third coding exon.

MATERIALS AND METHODS

Plasmid constructions: Construction of the transposon containing plasmids is outlined in Figure 1. The pPLΔ-1 (J. POSAKONY, personal communication) and cDm2837 (COTÉ *et al.* 1986) plasmids were obtained from J. POSAKONY and the *Adh*-λ1059 clones from M. KREITMAN. First, an 8.6-kb *Sac*I-*Cla*I fragment was subcloned from each of two *Adh*-λ1059 clones (Wa-s and Wa-f, derived from S and F isochromosomal stocks, respectively; KREITMAN 1983) into the pPLΔ-1 *P*-element vector. Recombinants between these two plasmids were generated by digestion with *Hpa*I and *Cla*I, gel purification of the two fragments generated, cross ligation and transformation of *Escherichia coli* strain DH1. Finally, the 8.1-kb *Sal*I *rosy*$^+$ fragment from cDm2837 was inserted (in each orientation) at the *Xho*I site of each of the four *Adh*/*P*-element plasmids (two parentals and the two reciprocal recombinants). The resulting plasmids are designated pΔ1Waij where i = 1, 2, 3, 4 according to the type of *Adh* fragment and j = A, B according to the *rosy* fragment orientation.

***P*-element transformation:** Microinjection of embryos was performed essentially as described by GOLDBERG, POSAKONY and MANIATIS (1983). Embryos from the host stock, *Adh*^{fn6} *cn*; *ry*^{506} (provided by J. POSAKONY) were injected with 5 mM KCl/0.1 M sodium phosphate, pH 6.8, containing the wings-clipped helper *P*-element plasmid, pπ25.7wc (KARESS and RUBIN 1984), at 150 μg/ml and one of the pΔ1Waij plasmids at 300 μg/ml.

Construction of isochromosomal stocks: Adults derived from injected embryos (the G_0 generation) were mated to *Adh*^{fn6} *cn*; *ry*^{506} partners and about 9% of fertile crosses produced one or more phenotypically *ry*$^+$ offspring (G_1s), which presumably contain one or more *P*[*Adh*$^+$, *ry*$^+$] transposon inserts. A separate stock was established for each of

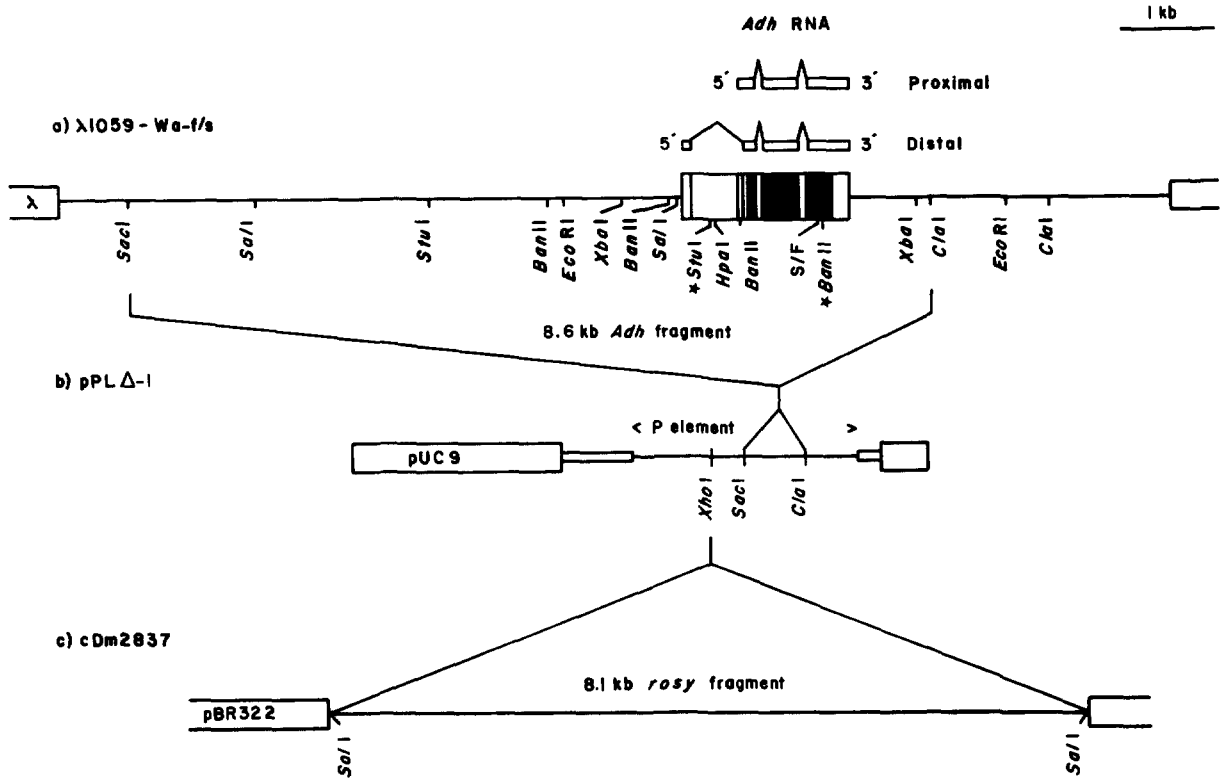

FIGURE 1.—Outline of plasmid constructions. Vector DNA is represented by labeled open blocks. The full length of vector sequence is shown only for pPLΔ-1. The open blocks of intermediate size in pPLΔ-1 represent *white* locus DNA. All *Sal*I, *Stu*I, *Hpa*I, *Ban*II, and *Xba*I sites between the leftmost *Sal*I and *Cla*I sites are shown (from sequences of KREITMAN 1983; KREITMAN and AGUADÉ 1986). The polymorphic *Stu*I and *Ban*II sites are marked by stars. Of the six *Eco*RI sites within the *Adh* fragment, only the two that define the p13E3 plasmid insert are shown.

the *P*[*Adh*$^+$, *ry*$^+$] G_1 offspring (up to five per G_0) by individually crossing them to *Adh*^{fn6} *cn*; *ry*^{506} and inbreeding their ry+ offspring for several generations. From each inbred stock, a single chromosome containing one or more *P*[*Adh*$^+$, *ry*$^+$] inserts was extracted into the genetic background of the host strain with the use of balancer stocks. These were constructed by substituting either *In(1)FM7* or *CyO*, *Adh*^{nB}/*In(2LR)bw*^{V1}, *Sp* or *In(3LR)TM2*, *Ubx ry e*^{s}/*MKRS* into the host strain genome as in LAURIE-AHLBERG *et al.* (1980). Inserts associated with recessive lethal or sterile effects were maintained over the appropriate balancer. See LINDSLEY and GRELL (1968) for balancer and mutant descriptions.

Nucleic acid methods: Minor modifications of published procedures were used for DNA extraction from flies (BENDER, SPIERER and HOGNESS 1983), Southern blotting (SMITH and SUMMERS 1980) and *in situ* hybridization to polytene chromosomes (BINGHAM, LEVIS and RUBIN 1981). The *Adh* probe used in both Southern and *in situ* hybridizations is p13E3 (constructed by J. POSAKONY), which contains the *Eco*RI fragment shown in Figure 1 inserted in pUC13.

Enzyme assays: For assaying ADH activity, the spectrophotometric method described by MARONI (1978) was used with isopropanol as substrate. ADH units are nanomoles NAD^+ reduced per min. For xanthine dehydrogenase (XDH) activity, the fluorimetric procedure described by MCCARRON, O'DONNELL and CHOVNICK (1979) was used. XDH activity is expressed in arbitrary fluorescence units per min. Unless indicated otherwise, ADH and XDH refer to specific activities (units per μg total protein). Total protein was determined by the Folin phenol procedure (LOWRY *et al.* 1951).

Kreitman line activity survey: Nine of the original isochromosomal lines that provided the *Adh* region clones for KREITMAN's (1983) sequencing study were analyzed for ADH activity variation. Each line was reared in six vials and one sample of five 6–8-day adult males per vial was assayed.

Transformant line activity survey: In one experiment, adults were sampled from each of the 121 single insert stocks during each of two time blocks. Within each block, males (autosomal lines) or females (*X* chromosome lines) from each transformant stock were crossed with flies from the host stock *Adh*^{fn6} *cn*; *ry*^{506} and two sets of 6–8-day adults of each sex were collected from the offspring (five flies per ADH set and 20 per XDH set). In the case of lethal or sterile inserts being maintained over a balancer, ry$^+$ progeny were selected. In another experiment, third instar wandering larvae were collected from each of 24 homozygous transformant stocks (each from a different G_0). During each of two time blocks, two sets of larvae of each sex were collected from each line (five larvae per set for ADH and ten for XDH). KREITMAN's Wa-s and Wa-f isochromosomal stocks were also included in each of these two experiments.

ADH-protein level: The Wa-s, Wa-f and 8 homozygous transformant lines (two of each of the 4 *Adh* sequence types) were analyzed. In each of four time blocks, two samples of five 6–8-day adult males from each transformant line were collected. The Wa-s and Wa-f lines were included in two of the four blocks. ADH-protein level was estimated by radial immunodiffusion (MANCINI, CARBONARA and HEREMANS 1965). This procedure was tested with purified ADH-F and ADH-S (LEE 1982; CHAMBERS, FLETCHER and AYALA 1984) to verify that there is no difference between allozymes in the extent of antibody-antigen reaction (*i.e.*, equal quantities

of ADH-protein gave equal immunodiffusion diameters). A dilution series of a standard fly extract was included on each immunodiffusion plate to insure linearity over the range of sample values. The same frozen extract was used on every plate. ADH-protein levels are reported in terms of mg of fly wet weight per ml of this standard extract.

RESULTS

Experimental plan: In order to localize the DNA sequence alteration responsible for the difference in *Adh* expression between S and F alleles, two *Adh* clones were selected that derive from flies showing the typical two- to threefold difference in ADH activity level. Figure 2 shows the ADH activity levels of nine of the isochromosomal lines that provided the *Adh* region clones for KREITMAN's (1983) sequencing study. The Wa-s and Wa-f clones were chosen for this study because they show roughly modal activities within their respective allozymic classes and they also show a pair of convenient restriction site differences (see below).

Figure 1 shows the 8.6-kb *Sac*I/*Cla*I *Adh* fragment contained within each transposon in the transformation experiment. This fragment contains about 0.8 kb of 3′ flanking DNA and about 5.4 kb of 5′ flanking DNA (but does not include the polymorphic *Bam*HI site referred to in the introduction, which is located about 0.5 kb upstream of the *Sac*I site). GOLDBERG, POSAKONY and MANIATIS (1983) previously demonstrated that this amount of 5′ flanking DNA is sufficient to provide a normal tissue and developmental

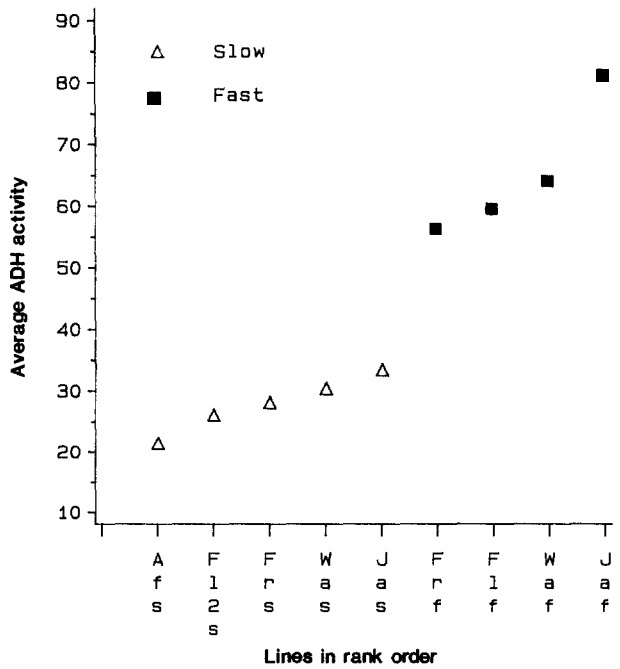

FIGURE 2.—Distribution of average ADH activity per fly ($n = 6$) for the KREITMAN (1983) isochromosomal lines. The minimum significant difference between any two Fast lines is 11.6 and between Slow lines is 8.0 (95% confidence, Tukey's multiple comparison procedure).

specificity and approximately normal levels of *Adh* expression in transformed adults and larvae. The first objective was to determine whether any of the ADH activity level difference maps to the 5′ flanking region, so recombinants between the Wa-s and Wa-f fragments were constructed *in vitro* by exchanging the *Sac*I/*Hpa*I and the *Hpa*I/*Cla*I fragments as described above. The *Hpa*I site lies 328 bp downstream from the start site of the distal transcript and 380 bp upstream of the proximal start site (Figure 1). The distal transcript occurs primarily in adults and the proximal transcript primarily in larvae (BENYAJATI *et al.* 1983; SAVAKIS, ASHBURNER and WILLIS 1986). Therefore, the recombination site separates all of the 5′ flanking sequence of the distal transcript from all of the coding sequences. Thus, the *Sac*I/*Hpa*I fragment will be referred to as the flanking region fragment and the *Hpa*I/*Cla*I fragment as the coding region fragment. A *rosy*$^+$ fragment was also included in each transposon so that transformants could be detected by eye color. Transformants were obtained from injection of each of eight different plasmid types, the four *Adh* sequence types (two parentals and the reciprocal recombinants) combined with each of the two possible *rosy* orientations.

Characterization of transformant stocks: From each transformed G_1 offspring (up to five per G_0), an isochromosomal stock was established in which one or more inserts on a single chromosome only are fixed (or maintained over a balancer in the case of lethal and sterile stocks). The number and location of the insert(s) in each stock were determined by Southern blotting and *in situ* hybridization to the polytene chromosomes. Southern blots of genomic DNA from each stock digested with *Bam*HI/*Eco*RI/*Xho*I were hybridized to an *Adh* probe (p13E3 containing the *Eco*RI fragment shown in Figure 1). This procedure visualizes one fragment of constant size (2.45 kb) that derives from the resident *Adh* gene and the homologous sequence internal to each transposon, a second fragment of constant size (1.97 kb) that derives from the resident gene only and one unique-sized junction fragment for each insertion (data not shown). In a few cases additional digestions with either *Sac*I/*Sal*I/*Hpa*I or *Kpn*I/*Pvu*II were analyzed to verify the number of unique junction fragments. Table 1 summarizes the number of single insert stocks of each type established. It shows that, on the average, two independent isolations of each insertion were made. The legend of Figure 4 gives the cytogenetic locations determined by *in situ* hybridization. Among the 62 different locations, ten occur on the *X*, 21 on the second, and 31 on the third chromosome. Among the 52 different autosomal insertions, 11 are associated with a recessive lethal or sterile effect. Just one *X*-linked lethal insert was recovered from a single G_1 female.

TABLE 1

Number of single insert transformant stocks

Transposon type					
Adh					
Flank	Code	*rosy* orientation	No. of G_0s	No. of locations	No. of stocks
S	S	A	2	2	3
S	S	B	8	8	22
S	F	A	7	9	17
S	F	B	5	5	9
F	S	A	2	2	7
F	S	B	5	5	12
F	F	A	3	3	3
F	F	B	14	28	48
Total			46	62	121

In defining a transposon type, S and F refer to sources of the *Adh* flanking and coding region fragments (the Wa-s and Wa-f clones, respectively) and A and B refer to the two possible *rosy* fragment orientations (the same or opposite transcriptional orientations, respectively).

One stock of each *Adh* sequence type was analyzed to verify that the correct type of transposon was integrated at a novel chromosomal location. Both the *Sac*I/*Hpa*I and the *Hpa*I/*Cla*I fragments that were recombined contain a restriction site difference between Wa-s and Wa-f (*Stu*I and *Ban*II, respectively; Figure 1). Wa-s lacks the *Stu*I site at +287 and Wa-f lacks the *Ban*II site at +1517 (KREITMAN 1983), which results in the fragment length variation marked by arrows in Figure 3. Note that the two transformant types that should have received the *Sac*I/*Hpa*I fragment from the Wa-s clone show the Wa-s-like pattern in the *Stu*I digest, as expected, and the two transformant types that should have received the *Hpa*I/*Cla*I fragment from Wa-s show the Wa-s-like pattern in the *Ban*II digest, as expected. The unique-sized bands in each of the *Ban*II digests of the transformants represent junction fragments and provide the evidence for novel chromosomal location (in addition to *in situ* hybridization results).

Sources of variation in activity level among transformants: Of course the source of primary interest is the fixed variation in transposon structure, which must be distinguished from other genetic sources of variation among transformant stocks by obtaining a number of independent insertions of each type. Other genetic sources include alterations in transposon structure that occur during the experiment (mutations), position effects due to the chromosomal site of insertion, sex (and *X vs.* autosome) effects and possibly the effects of modifier genes segregating in the host stock that become differentially distributed among transformant lines. Because of the large number of insertions obtained in this experiment, the importance of some of these additional genetic effects could be investigated (see below). First, the fixed differences among transposon types, which were investigated by analyses

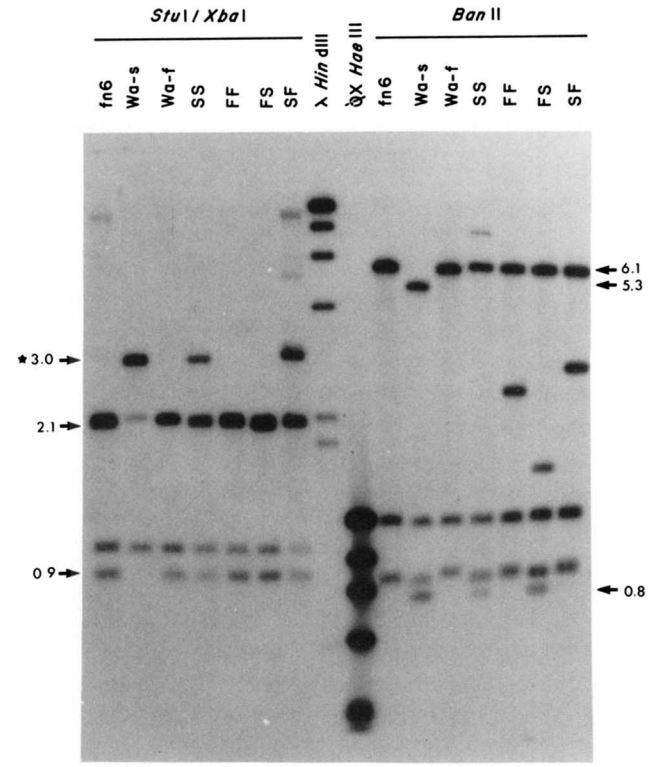

FIGURE 3.—Southern blot of genomic DNA digested with *Stu*I/*Xba*I or *Ban*II and probed with a mixture of *Adh* (p13E3), λ and ϕX174 DNA. The two center lanes contain λ*Hin*dIII or ϕX174 *Hae*III fragments as size markers. Wa-s and Wa-f lanes contain DNA from the original isochromosomal stocks from which the corresponding clones were derived. The lane marked *fn6* contains DNA from the host strain Adh^{fn6} *cn*; ry^{506}. The other lanes contain DNA from transformant stocks; the first letter in each designation refers to the source of the flanking region fragment (*Sac*I/*Hpa*I) and the second letter to the source of the coding region fragment (*Hpa*I/*Cla*I). S refers to the Wa-s clone and F to the Wa-f clone. Arrows mark the fragments that vary among stocks because of the *Stu*I and *Ban*II polymorphisms (the second arrow at the left marks the lower band of a doublet). The 3.0-kb fragment is the internal *Xba*I fragment (Figure 1), which is cut by *Stu*I into the 2.1- and 0.9-kb fragments in all stocks except those in which the flanking region derives from Wa-s. This 3.0 kb *Xba*I fragment (marked with a star) is diagnostic for the source of the flanking region fragment. The 6.1-kb fragment extends from the *Ban*II site within the proximal 5′ leader to a *Ban*II site beyond the right boundary of the fragment shown in Figure 1 (*i.e.*, outside of the *Sac*I/*Cla*I transposon fragment). In Wa-s this fragment is cut within the third coding exon, producing the 5.3- and 0.8-kb fragments. The 0.8-kb *Ban*II fragment (marked with a star) is diagnostic for the source of the coding region fragment.

of variance of the means of single insert lines, will be discussed.

Effects of transposon type on ADH: A transposon type is defined by the combination of source of *Adh* flanking fragment, *Adh* coding fragment and the *rosy* orientation. Factorial analysis of variance of the line means for adult ADH revealed no significant effect due to *rosy* orientation (neither the main effect nor any interaction) and no significant difference between second and third chromosome inserts. Therefore *rosy* orientation is ignored and the autosomes are pooled

TABLE 2

Analyses of variance of ADH specific activity from single insert transformant stocks

Source	Adult females			Adult males		
	d.f.	*F*-test	*P*	d.f.	*F*-test	*P*
Flank	1	2.8	0.10	1	4.2	0.05
Code	1	152.9	<0.0001	1	120.6	<0.0001
F × C	1	0.7	0.40	1	4.0	0.05
Chr	1	0.8	0.37	1	21.9	<0.0001
F × Chr	1	0.7	0.40	1	1.0	0.31
C × Chr	1	0.4	0.55	1	3.1	0.08
F × C × Chr	1	0.4	0.52	1	0.4	0.54
Loc (F × C × Chr)	53	6.4	<0.0001	52	8.6	<0.0001
Error	58			58		
Total	118			117		

The data points for these analyses are the activities averaged over the four observations for each single insert line. The two lines representing location 54, which have no ADH activity, are excluded from both male and female analyses. The recessive lethal *X* chromosome insert (location 09) is absent from the male analysis. "Flank" and "Code" (or "F" and "C") refer to sources of the *Adh* flanking and coding region fragments, "Chr" refers to chromosome (*X vs.* autosome) and "Loc (F × C × Chr)" refers to insert location nested within a transposon type by chromosome combination. In constructing *F*-tests, it was assumed that Flank, Code and Chr are fixed effects and Loc is random.

TABLE 3

ADH specific activity means for females

Adh type		Adults		Larvae	
		No. of locations	Average ADH	No. of locations	Average ADH
Transformants					
Flank	*Code*				
F	F	31	0.205	11	0.420
S	F	13	0.182	6	0.399
F	S	7	0.077	4	0.167
S	S	10	0.077[a]	3	0.163
Marginal means					
Flank = F		38	0.141	15	0.294
Flank = S		23	0.130	9	0.281
Code = F		44	0.193	17	0.410
Code = S		17	0.077	7	0.165
Original Wa lines					
Wa-f			0.181		0.281
Wa-s			0.072		0.118

ADH activities are averaged over the location means within each class. "Flank" and "Code" refer to the sources of *Adh* flanking and coding sequences (Wa-s or Wa-f). The flank and code means are unweighted. In comparing adult and larval values, note that adults have one dose and larvae two doses of the insert.

[a] Without the high activity outlier (location 13), this value is 0.069.

into one class in the analyses shown in Table 2. This table shows that the *Adh* coding fragment provides a highly significant source of variation for both males and females but the flanking region fragment is not significant in females and just marginally significant in males. However, if a high activity outlier (location 13, Figure 4a) is excluded from the analysis, the flanking region fragment is significant for both sexes ($P < 0.04$ for females and $P < 0.03$ for males). These effects are shown graphically in Figure 4a. There is almost no overlap between the two coding fragment classes while the differences between the flanking fragment classes are subtle at best.

Analysis of larval data gave essentially the same results. The effect of the coding region fragment is highly significant but the flanking region effect is not, perhaps because many fewer transformant stocks were used in the larval experiment. The transposon type means in Table 3 show that the direction of the observed difference in flanking fragment is the same in larvae as in adults.

These results show that the major difference between Wa-s and Wa-f clearly maps to the coding region fragment, which contains none of the 5′ flanking DNA of the distal (adult) transcriptional unit. Table 3 compares the transposon type means for both larvae and adults with means for the original Wa-s and Wa-f isochromosomal lines. Comparison of absolute values is not entirely valid here because of differences in genetic background between the transformant and Wa lines. Nevertheless, the absolute values for the coding fragment means are very similar to the corresponding Wa line values and certainly the S/F differences are almost identical. This similarity means that essentially the entire original difference in ADH activity maps to the coding region fragment. Table 4 shows that the ADH-protein level difference between Wa-s and Wa-f also maps to this fragment.

Effects of transposon type on XDH: Analyses of the line means for XDH activity shows no effect of *Adh* sequence type or of *rosy* fragment orientation (see Figure 4b). *A priori* it seemed possible that *rosy* orientation might affect the degree of variation in XDH among different inserts. In orientation A *rosy* and *Adh* are transcribed in the same direction, with *rosy* on the 5′ side of *Adh* while in orientation B, *rosy* and *Adh* are transcribed divergently (COTÉ *et al.* 1986; GOLDBERG 1980). Since the 5′ end of the *rosy* gene is located

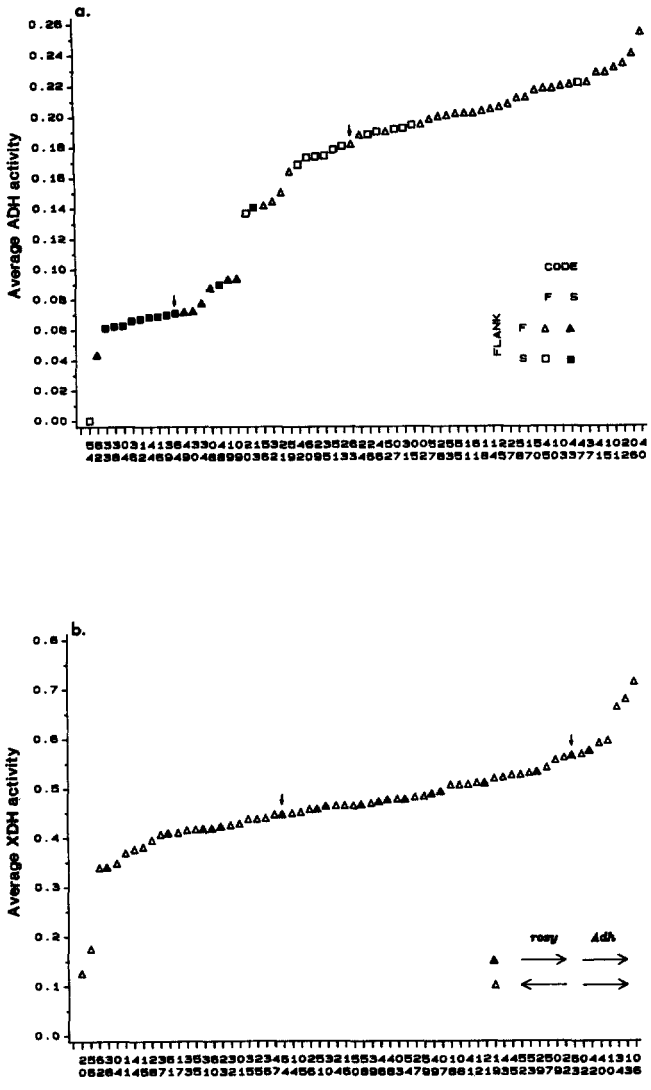

FIGURE 4.—Distributions of ADH and XDH specific activities in adult female transformants. Each point represents the average of the activities of each of the stocks having an insertion at a particular location. The following key relates location numbers on the abcissa (read vertically) to the corresponding polytene bands: 01 = 1CD, 02 = 1EF, 03 = 3F, 04 = 6A, 05 = 13D, 06 = 14B, 07 = 14D, 08 = 17D, 09 = 18D, 10 = 19BC, 11 = 22B, 12 = 26B, 13 = 26CD, 14 = 28D, 15 = 30D, 16 = 30D, 17 = 36A, 18 = 36E, 19 = 38B, 20 = 42A, 21 = 42B, 22 = 43C, 23 = 44A, 24 = 44A, 25 = 46DE, 26 = 49A, 27 = 53A, 28 = 54D, 29 = 55D, 30 = 55F, 31 = 58F, 32 = 62A, 33 = 64C, 34 = 64D, 35 = 65B, 36 = 66A, 37 = 67A, 38 = 68A, 39 = 70C, 40 = 73A, 41 = 75A, 42 = 75C, 43 = 78D, 44 = 78E, 45 = 79CD, 46 = 79CD, 47 = 82C, 48 = 84B, 49 = 85B, 50 = 86D, 51 = 87AB, 53 = 89A, 54 = 89B, 55 = 89E, 56 = 91D, 57 = 92A, 58 = 93B, 59 = 94F, 60 = 97A, 61 = 98C, 62 = 100EF. Three pairs of location numbers have the same polytene band designation, but the members of a pair derive from different injected individuals and have different insert locations at the level of Southern blot analysis. The location numbers 63 and 64 refer to the original Wa-f and Wa-s isochromosomal stocks, respectively, and those values are marked by arrows. In (a) the symbols distinguish between the sources of the *Adh* flanking and coding region fragments (S = Wa-s and F = Wa-f). In (b) the symbols distinguish between the two possible *rosy* orientations within the transposon (the solid and open triangles represent orientations A and B, respectively).

TABLE 4

ADH activity and ADH-protein levels in adult males

			ADH activity	ADH protein
Transformant line means (n = 8)				
Flank	*Code*	*Loc.*		
F	F	27	31.9	21.7
F	F	58	33.3	21.5
S	F	26	32.4	20.0
S	F	51	27.0	18.4
F	S	34	12.8	14.3
F	S	30	12.2	13.4
S	S	33	10.7	11.9
S	S	36	14.6	15.4
Transformant class means				
Flank	*Code*			
F	F		32.6	21.6
S	F		29.7	19.2
F	S		12.5	13.9
S	S		12.6	13.6
Wa line means (n = 4)				
Wa-f			36.2	20.1
Wa-s			14.0	13.9

Activities and ADH-protein levels are given on a per fly basis. ADH-protein was estimated by radial immunodiffusion. "Loc." refers to the line labels given in Figure 4.

much closer to the end of the transposon in orientation A than B, one might expect orientation A to show greater position effect variation. However, comparison of the magnitudes of the variance components for insert locations showed that, if anything, the variation is greater for B than A. In the analysis of all lines the variance component for B is significantly larger than for A but when two low activity outliers (locations 20 and 56) are excluded, the difference is not significant. The *rosy* transcriptional unit has not been localized precisely, so the extent of 5′ flanking DNA within the 8.1 kb *Sal*I fragment is unknown. Evidently, it is long enough to provide a rather effective buffer against position effect variation.

Detection of modifier variation: Variation in gene expression among independent insertions of the same transposon type is generally attributed either to mutation or position effect. However, some of the variation among single insert stocks may be due to differences at modifier genes, which are commonly polymorphic in natural populations (LAURIE-AHLBERG *et al.* 1980) and perhaps also in laboratory stocks. Although the experimental design used here does not allow a clear partitioning of modifier *vs.* position/mutational effects, it does provide two possibilities for detecting the presence of modifier variation: (1) If individual G_0 flies vary due to modifier genes, then sets of transformant stocks derived from different G_0s may show average differences in activity level. In particular, we can compare sets of transformant stocks where the members of one set derive from the same injected individual (G_0) and have insertions at differ-

ent locations within one chromosome (other chromosomes are replaced during construction of isochromosomal lines). This collection of transformant stocks contains six such sets (all of one transposon type), with two locations within each set and two or three sublines representing each location. Analysis of variance reveals no significant variation among sets for either ADH or XDH activity, thus providing no evidence for modifier variation. (2) Significant variation among the independent isolations of a particular insertion (*i.e.*, among the G_1s from one G_0 that have the same insert location) would also provide evidence for modifier variation. Analyses of variance show that this G_1 within location source is not significant for ADH for either sex or for XDH in females but is significant at $P < 0.01$ for XDH in males. In this case the variance component for G_1 within location is only about one-sixth as large as that for locations, but the "locations" source here may be confounded with additional modifier variation. These results indicate that modifier variation may contribute to variation among stocks with different insertions, but the effect is likely to be small in magnitude so in the following we assume that variation among locations represents mainly position effect or mutational variation.

Position effect/mutational variation: Table 2 shows that location within a transposon and chromosome type represents a highly significant source of variation in ADH for both sexes and the same is true for XDH. The magnitude and nature of this variation are shown in Figure 4. The distributions for each transposon type are approximately continuous, with a few notable exceptions. One insert (54) has no detectable ADH activity, whereas it lies approximately in the middle of the XDH distribution. Southern blot analysis of genomic DNA from this line reveals no detectable change in sizes of restriction fragments derived from the transposon (data not shown). Whether or not this null ADH effect is due to a mutation within the transposon or to a highly specific position effect can be determined by inducing secondary transpositions (as in LEVIS, HAZELRIGG and RUBIN 1985; DANIELS *et al.* 1986). In another case (13), the ADH activity is unusually high for the transposon type and this line is second highest in XDH activity (and digestions with three different sets of restriction enzymes revealed no evidence for more than one insert). The two lines with lowest XDH activity (20 and 56) also have moderately low ADH activities.

These associations between XDH and ADH suggest that chromosomal position may have correlated (general) effects on adjacent genes within a transposon. This possibility was investigated by estimating the correlation between XDH and ADH over the location means within each sex, chromosome and *Adh* coding fragment class. These were tested for homogeneity and then averaged by Fisher's *Z*-transform method. Excluding the sex = males and chromosome = *X* class, the correlation between XDH and ADH for adults is 0.53 [with a 95% confidence interval (CI) of 0.37–0.66] and for larvae is 0.46 (with 95% CI of 0.28–0.60). These moderately high correlations suggest that some of the position effect is general, although the magnitude of such correlations may vary according to similarity in the tissue and developmental specificity of the genes. The *Adh* and *rosy* genes have rather similar patterns of expression (URSPRUNG, SOFER and BURROUGHS 1970; MARONI and STAMEY 1983; MUNZ 1964).

Similar correlation estimates were obtained for each enzyme between adults and larvae: 0.70 (95% CI of 0.43–0.85) for ADH and 0.72 (95% CI of 0.51–0.85) for XDH. These rather high correlations suggest that most of the position effect variation is systemic rather than developmental-stage specific.

Dosage compensation: Previous studies have shown that a *ry* gene within a transposon inserted in the *X* chromosome shows partial or complete dosage compensation (SPRADLING and RUBIN 1983), while the evidence for two other autosomal genes, *Adh* and *Ddc*, is ambiguous (GOLDBERG, POSAKONY and MANIATIS 1983; SCHOLNICK, MORGAN and HIRSCH 1983; MARSH, GIBBS and TIMMONS 1985). Figure 5 shows the relationship between male and female activities per larva for eight *X* chromosome and 16 autosomal lines. In this experiment male larvae have one dose and females two doses of the insert in an *X* chromosome line, while in autosomal lines each sex has two doses of the insert. If there were full compensation, the linear relationship between male and female activity would be indistinguishable for *X* chromosome and autosomal inserts. If there were no compensation, the slope of the regression of male on female activity for *X* inserts would be one half of that for autosomal inserts. The *X* chromosome points actually appear to fall on a line of intermediate slope, but closer to full compensation for *rosy* than for *Adh*. For both enzymes, the female data show no signficant difference between *X* and autosomal lines whereas the difference for males is highly significant (as shown for adult ADH in Table 2). The average male/female ratio in larvae is, for ADH, 0.81 for autosomal inserts and 0.54 for *X* chromsome inserts and, for XDH, 0.74 for autosomal inserts and 0.61 for *X* inserts. These data confirm the previous result for *rosy* expression and show that *X* chromosome inserts of *Adh* also show partial dosage compensation, although apparently not to the same extent as *rosy*. Analysis of adult activities gives essentially the same result (data not shown).

Sample size requirements: The large number of independent insertions obtained in this experiment provide an opportunity to assess the sensitivity of

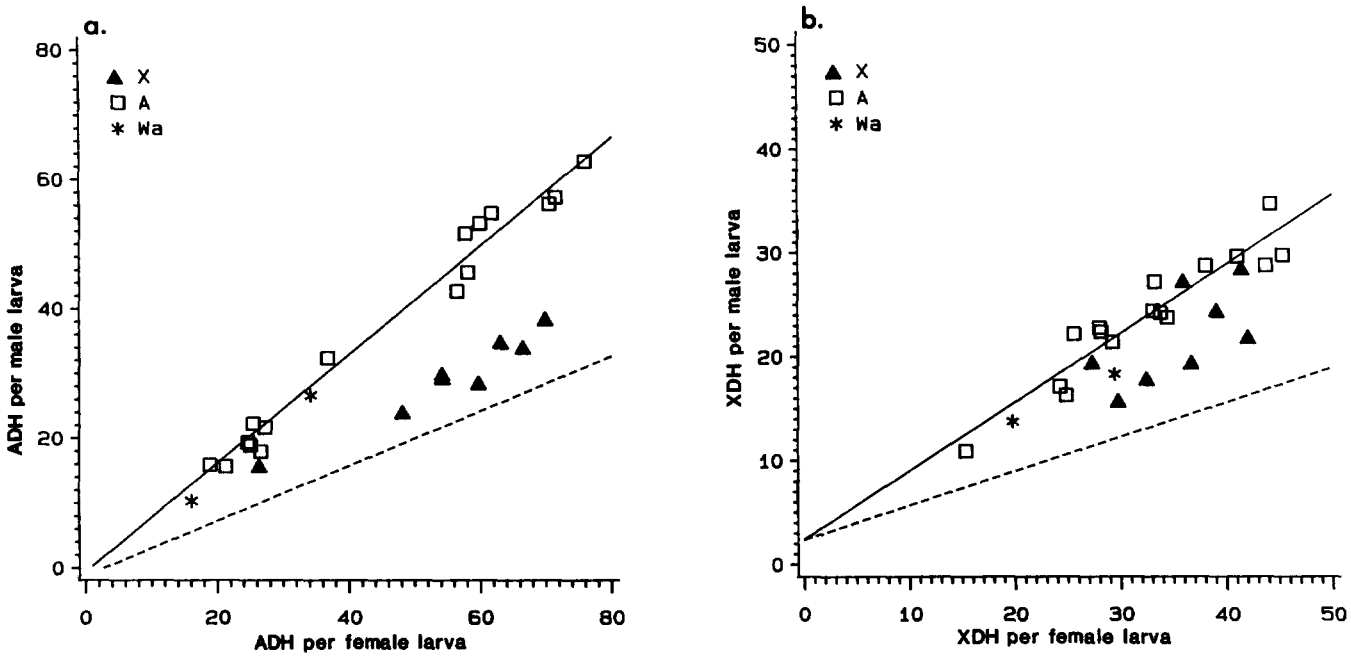

FIGURE 5.—The relationship between male and female activity per larva. Each point represents the average activity (n = 4) for a transformant stock (each with a different insert location) or for the original Wa-s or Wa-f isochromosomal stock. In the symbol key, X refers to *X* chromosome inserts, A to autosomal inserts and Wa to Wa-s or Wa-f. The solid line is the regression of male on female activity for the autosomal insert lines. The dashed line has the same Y intercept but one-half the slope of the solid line.

transformation experiments for detecting quantitative differences in gene expression. In particular, we can estimate the number of independent insertions required to detect transposon type differences of a specified magnitude. These calculations are based on the variance in enzyme activity among stocks with the same transposon inserted at different locations (*i.e.*, the mean square for locations from ANOVA as in Table 2), which is used for testing differences among transposon types. Table 5 shows estimated sample sizes required to detect differences between 10 and 100% of the mean for ADH-S, ADH-F and XDH. Differences of 50% are easily detected for all three and even differences as small as 20% can be detected with a large but feasible effort. In fact, in this experiment we detected a 17% difference between *Adh* flanking regions as significant at about the 5% level. Of course, these sample size predictions are based on a particular variance among insertion sites, which is likely to vary according to the amount of flanking DNA included in the transposon.

TABLE 5

Sample size requirements for detecting transposon type differences

	ADH-Slow	ADH-Fast	XDH
Mean	0.077	0.193	0.462
SD[a]	0.033	0.028	0.133
C.V.[b] (%)	42.9	14.5	28.8
Percent difference[c]		Sample size[d]	
10	231	28	105
15	104	13	48
20	59	8	27
35	20	4	10
50	11	3	6
100	4	—	3

[a] SD = the square root of the mean square for chromosomal locations within a transposon type (used for testing differences between transposon types).

[b] C.V. = coefficient of variation.

[c] The difference to be detected as a percentage of the mean value.

[d] The number of independent insertions per transposon type needed to be 80% confident of detecting the specified difference at the 95% confidence level (*i.e.*, a one-tailed *t*-test with $\alpha = 0.05$ and $\beta = 0.20$; STEELE and TORRIE 1980).

DISCUSSION

The results of the transformation experiment clearly show that the major difference in ADH activity and ADH-protein level between a pair of Slow and Fast alleles (Wa-s and Wa-f) maps to the *Hpa*I/*Cla*I restriction fragment (Figure 1), which includes all of the *Adh* coding sequence and some intron and 3′ flanking sequence, but excludes all of the 5′ flanking sequence of the distal (adult) transcriptional unit. Only a small, marginally significant effect could be detected for the *Sac*I/*Hpa*I fragment, which contains all of the 5′ flanking sequence for the distal transcriptional unit. Small effects of this type may contribute to the continuous variation in *Adh* expression among alleles within an allozymic class (AQUADRO *et al.* 1986).

Fortunately, the entire *Hpa*I/*Cla*I fragment to

which the major effect maps has been sequenced for Wa-s, Wa-f and nine other *Adh* alleles (KREITMAN 1983). The Wa-s/Wa-f comparison for this fragment reveals nine silent nucleotide substitutions, the one amino acid replacement substitution and four small insertion/deletion differences. However, the field can be narrowed by considering the other KREITMAN sequences. Figure 2 shows that nine of the sequenced alleles derive from isochromosomal stocks showing the typical Slow/Fast difference in ADH activity (the other two stocks were unavailable). Analysis of all nine sequences for the *Hpa*I/*Cla*I fragment shows that only three substitutions distinguish all of the F alleles from all of the S alleles. Thus, it is likely that one or more of these three substitutions causes the major difference in *Adh* expression between allozymic classes. One of these substitutions is, of course, the S/F amino acid replacement substitution (AAG to ACG at 1490) and the others are third position silent substitutions (ACC to ACG at 1443 and GCT to GCC at 1527) on either side of the S/F site.

These results indicate that the amino acid replacement substitution could be responsible for the entire difference in ADH activity, but they do not rule out involvement of silent substitutions within the *Hpa*I/*Cla*I fragment and, in fact, direct attention to the silent substitutions at 1443 and 1527. With such a small number of possibilities at this point, the use of site-directed mutagenesis to test for the effects of each substitution individually is clearly feasible. It is also important to continue investigation of the mechanism by which Fast lines have higher levels of ADH-protein than S lines, so studies of ADH-mRNA levels and *in vivo* rates of protein synthesis and degradation are in progress. If the difference in ADH-protein concentration is, for example, due to differential protein degradation *in vivo* then only the amino acid replacement could be involved, but if it is due to a difference in template availability or to differential translational efficiency than any of the three substitutions could be responsible. Both amino acid replacement and silent substitutions can affect template availability through RNA processing or stability and both could also affect translation rate through differential abundance or efficiency of tRNAs. Eventually, we hope to identify not only the nucleotide substitution(s) responsible for the effect on ADH activity but also the mechanism(s) involved.

If the ADH-protein level effect is in fact due to one of the three substitutions identified above, this result is difficult to reconcile with our original interpretation of the haplotype-ADH activity associations described in the Introduction (and detailed by AQUADRO *et al.* 1986). In order to interpret the two rare haplotype lines that switched activity class as recombinants between the S/F rate and a putative regulatory site, one would have to assume that the upstream third position silent substitution at 1443 is the regulatory site. However, it seems unlikely that among four apparent recombinants within the 7-kb region between the S/F site and the *Bam*HI site, two would have occurred within the 47 base pairs (bp) that separates the S/F site at 1490 from the silent substitution at 1443. This remains a formal possibility, but the results suggest that perhaps the activity class switches in the two rare haplotype lines are caused by other substitutions not related to the typical S/F difference. DNA sequencing and/or transformation experiments will be used to resolve this point.

The data presented here clearly demonstrate the feasibility of applying the *P*-element transformation approach to identify the molecular basis of variants with the relatively small quantitative effects typical of natural polymorphisms. This approach will be useful not only for analysis of intraspecific polymorphisms, but can also be used to identify the molecular basis of differences in enzyme expression between different species of Drosophila, since interspecific gene transfer is clearly feasible (SCAVARDA and HARTL 1985). Ultimately, this type of experiment will provide direct evidence concerning the relative importance of structural *vs.* regulatory variants in protein evolution (BRITTEN and DAVIDSON 1969; WILSON 1976).

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