The Effect of an Intronic Polymorphism on Alcohol Dehydrogenase Expression in *Drosophila melanogaster*

Cathy C. Laurie and Lynn F. Stam

Department of Zoology, Duke University, Durham, North Carolina 27708

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ABSTRACT

Several lines of evidence indicate that natural selection controls the frequencies of an allozyme polymorphism at the alcohol dehydrogenase (*Adh*) locus in *Drosophila melanogaster*. However, because of associations among sequence polymorphisms in the *Adh* region, it is not clear whether selection acts directly (or solely) on the allozymic site. This problem has been approached by using *in vitro* mutagenesis to distinguish among the effects on *Adh* expression of individual polymorphisms. This study shows that a polymorphism within the first *Adh* intron ($\nabla 1$) has a significant effect on the level of ADH protein. Like the allozyme, $\nabla 1$ shows a geographic cline in frequency, indicating that it may also be a target of natural selection. These results suggest that multisite selection models may be required to understand the evolutionary dynamics of individual loci.

THE alcohol dehydrogenase enzyme (ADH, EC 1.1.1.1) of *Drosophila melanogaster* plays an important role in the detoxification of environmental alcohols and in energy metabolism. Flies lacking ADH activity are extremely sensitive to the toxic effects of alcohols (DAVID *et al.* 1976), and naturally occurring variation in ADH activity is frequently associated with variation in alcohol tolerance (GIBSON and OAKESHOTT 1982; VAN DELDEN 1982). ADH catalyzes a key step in the metabolism of ethanol to lipid, which occurs very efficiently in larvae (GEER *et al.* 1985).

In *D. melanogaster*, ADH is encoded by a single gene, which produces two distinct transcripts from alternative promoters (Figure 1A). These transcripts are developmentally regulated such that the distal transcript is the predominant form in adults and the proximal transcript is predominant in larvae until late third instar (BENYAJATI *et al.* 1983; SAVAKIS *et al.* 1986). 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 (CORBIN and MANIATIS 1989, 1990; POSAKONY *et al.* 1985).

Natural populations of *D. melanogaster* harbor extensive nucleotide variation throughout the *Adh* region (AQUADRO *et al.* 1986; KREITMAN 1983) and they show extensive genetic variation of ADH activity level in adults (LAURIE-AHLBERG 1985). A large part of this activity variation is associated with a worldwide allozyme polymorphism (LAURIE-AHLBERG 1985), which is due to a single amino acid replacement (Figure 1A) (KREITMAN 1983). Fast homozygotes generally have a 2.5–3-fold higher ADH activity level than Slow homozygotes, which is partly due to a difference in catalytic efficiency and partly to a difference in concentration of the ADH-protein (estimated immunologically) (LAURIE-AHLBERG 1985). Both of these differences map to a 2.3-kb *Hpa/Cla* fragment (see Figure 1A) that includes the *Adh* coding sequences and introns, but excludes the 5′-flanking region (LAURIE-AHLBERG and STAM 1987). Using *in vitro* mutagenesis and transgenic flies, we have shown that the amino acid replacement clearly causes the catalytic efficiency difference, but it has no effect on the concentration of ADH protein (CHOUDHARY and LAURIE 1991).

Population survey data have suggested that the typical 1.5-fold difference in ADH protein level between allozymes might be due to a polymorphism called $\nabla 1$ (LAURIE *et al.* 1991), which consists of a complex sequence substitution within the first intron of the distal transcriptional unit (Figure 1A). There is a strong association between the $\nabla 1$ and amino acid replacement polymorphisms such that *Slow* alleles nearly always have the 29 bp version of $\nabla 1$ (denoted $\nabla 1$-*S*) and *Fast* alleles predominantly have the 34-bp version ($\nabla 1$-*F*). This association occurs in natural populations throughout North America (BERRY and KREITMAN 1993; KREITMAN and AGUADÉ 1986; LAURIE *et al.* 1991), in Spain (AGUADÉ 1988) and in an African population (BÉNASSI *et al.* 1993), indicating that $\nabla 1$ could account for the worldwide difference in ADH protein level between the allozymes. Here we use *in vitro* mutagenesis and *P* element transformation to test directly the hypothesis that $\nabla 1$ affects ADH protein level.

MATERIALS AND METHODS

Plasmid constructions: The plasmids used for *P* element transformation all have the same basic structure (see Figure 1 of LAURIE-AHLBERG and STAM 1987). The vector pPLΔ1 consists of a defective *P* element containing a polylinker, which was inserted into pUC9 (J. POSAKONY, personal communication). An 8.0-kb *SacI/ClaI* frament containing the *Adh* gene

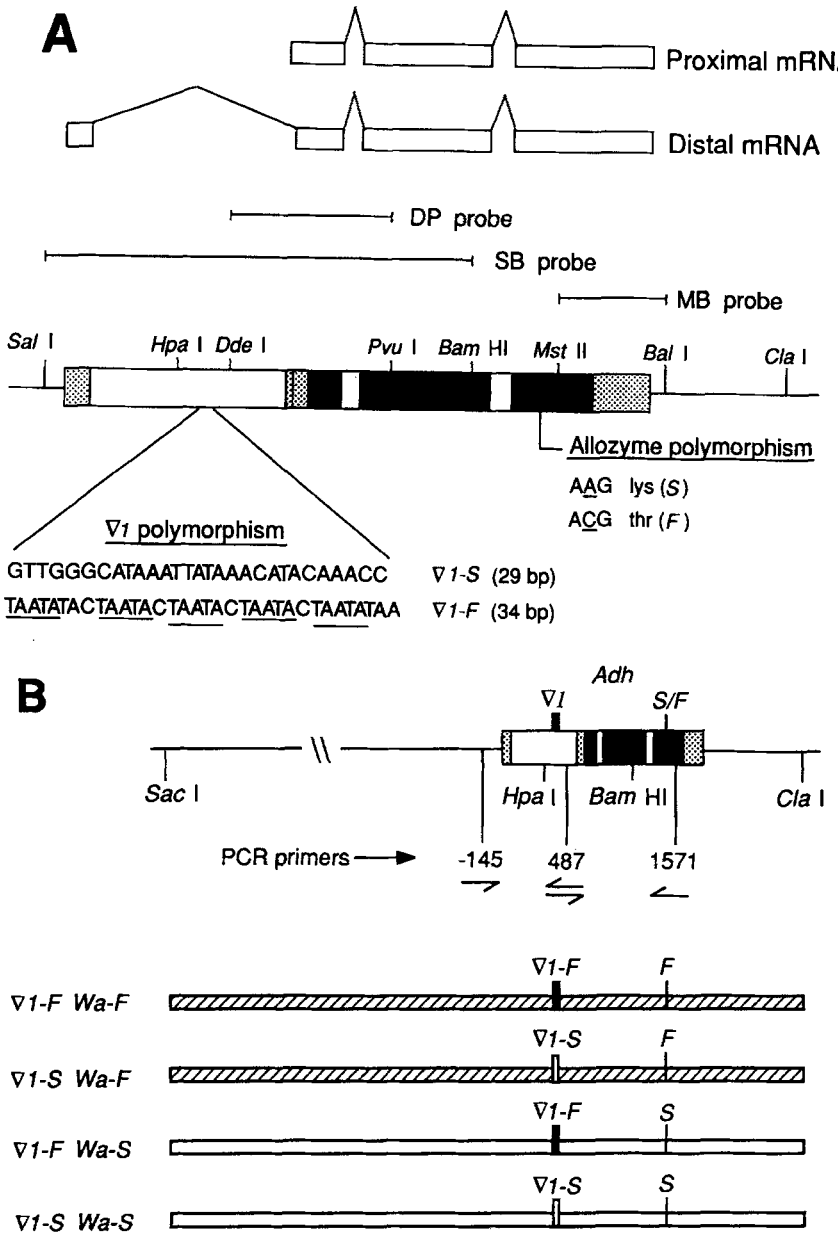

FIGURE 1.—The *Adh* transcriptional unit (1.9 kb in length) lies within an 8.0 kb *SacI/ClaI* fragment used in the transformation experiment. This fragment contains 5.3 kb of 5′-flanking DNA and 0.8 kb of 3′-flanking DNA. Within the transcriptional unit, stippled areas are untranslated regions, open areas are introns and solid areas are protein-coding. (**A**) The ∇*1* polymorphism begins at nucleotide 448 and the allozyme polymorphism is due to an amino acid replacement at 1490 (where sequence numbering begins at the distal transcript start site) (KREITMAN 1983). Locations of the probes used in RNase protection assays (Figure 3) are shown. (**B**) Four different *SacI/ClaI* fragments were used in the transformation experiment. The hatched fragment represents the *Wa-F* allele, which contains the ∇*1-F* sequence (solid black) and the F amino acid. The open fragment represents the *Wa-S* allele, which contains the ∇*1-S* sequence (open) and the S amino acid. The first and fourth fragments are completely wild-type sequences, while the second and third are chimeric fragments.

(Figure 1A) and an 8.1-kb *SalI* fragment containing a wild-type *ry* gene are inserted into the polylinker. The *Adh* fragments originate from the *Wa-S* and *Wa-F* λ1059 clones of KREITMAN (1983). Plasmids were constructed using standard methods (MANIATIS *et al.* 1982).

Recombinant polymerase chain reaction (PCR): The two chimeric constructs in Figure 1B were constructed by a recombinant PCR procedure (HIGUCHI 1990). The ∇*1-F* sequence at 448 in *Wa-F* was replaced by ∇*1-S* from *Wa-S* as follows. A chimeric *HpaI/Bam*HI fragment was produced using primers at nucleotides −145 (upstream of *Hpa*I), 487 (just downstream of ∇*1*) and 1571 (downstream of *Bam*HI). A −145 to 487 fragment from *Wa-S* (which contains no sequence differences from the corresponding *Wa-F* sequence other than ∇*1*) was amplified and annealed to a 487–1571 fragment amplified from *Wa-F*. The chimeric PCR product was digested with *Hpa*I and *Bam*HI and used to replace the corresponding *HpaI/Bam*HI fragment in *Wa-F* to produce ∇*1-S Wa-F*. ∇*1-F Wa-S* was constructed by a similar procedure. In both cases, the *HpaI/Bam*HI fragment was completely sequenced to verify that no unwanted mutations were introduced by the *Pfu* polymerase (LUNDBERG *et al.* 1991) used in the PCR.

Production and analysis 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. Southern blot analysis was used to select isochromosomal stocks with only a single insert. The 54 single-insert stocks were sampled in a randomized block design with two blocks and two replicates per block. ADH activity was measured spectrophotometrically, ADH-protein was measured by radial immunodiffusion and total protein was measured by the bicinchoninic acid procedure (SMITH *et al.* 1985).

RNase protection assays: Total nucleic acids were extracted from sets of 100 adult flies (7–8 days old) by grinding in 2 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.35 M NaCl,

7 M urea, 2% NaDodSO$_4$), centrifugation for 5 min at 15,000 × *g*, extraction with phenol-chloroform, precipitation twice in ethanol and resuspension in 50 µl 10 mM Tris-HCl, pH 8.0/1 mM EDTA. RNA transcription, hybridization, digestion and acrylamide gel analysis were performed as described by MELTON *et al.* (1984) with minor modifications. Each transcription reaction (25 µl) contained 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl, 10 mM dithiothreitol, 0.6 mM GTP, 0.3 mM ATP and UTP, 12 µM CTP, 50 µCi [α-^{32}P]CTP (3000 Ci/mmol), 40 units RNasin, 1 µg DNA template and 50 units of either T3 or T7 RNA polymerase. RNA hybrids were digested at 37° for 30 min with 400 units of RNase T1 for all probes and, in addition, with 1.2 µg RNase A for the SB probe.

RESULTS

To test for the effect of the ▽*1* polymorphism on ADH protein level, four alleles were constructed *in vitro* and introduced into flies by *P* element-mediated germline transformation (Figure 1B). Two of the alleles consist of the wild-type *Wa-S* and *Wa-F* sequences, which were cloned from isochromosomal fly stocks showing the typical Slow/Fast difference in ADH level (KREITMAN 1983; LAURIE-AHLBERG and STAM 1987). There are many sequence differences between these alleles within the 8.0-kb *Sac*I/*Cla*I fragment used for *P* element transformation (KREITMAN 1983; KREITMAN and HUDSON 1991). The wild-type alleles are designated ▽*1-F Wa-F* and ▽*1-S Wa-S*. The other two alleles are reciprocal chimeric constructs. One chimera consists of the *Wa-F* allele in which the ▽*1-F* sequence is replaced by the Slow-typical form of ▽*1* (▽*1-S Wa-F*) and the other is the *Wa-S* allele in which ▽*1-S* is replaced by the Fast-typical form (▽*1-F Wa-S*). The replacements were made precisely (with no other sequence alterations) by using a recombinant PCR technique. Multiple independent transformants of each allelic type (in an Adh^{fn6} null background) were analyzed for variation in ADH level.

The transformation results (Figure 2 and Table 1) show that ▽*1-F* confers a higher level of ADH-protein (and consequently ADH activity) than ▽*1-S*, as expected. A two-way factorial analysis of variance was performed to test for the effects of the ▽*1* sequence and *Adh* allelic background (*Wa-F vs. Wa-S*, which includes the allozymic difference). Both sources of variation have highly significant effects on both ADH-protein and activity levels ($P < 0.0001$ without and $P < 0.001$ with an outlier line).

The transformants with wild-type alleles have F/S ratios of 1.6 for ADH-protein and 3.0 for activity, which are typical of wild-type flies (Table 1) (LAURIE and STAM 1988). The 1.6-fold difference in ADH-protein translates into a 3.0-fold difference in activity because of an approximately twofold difference in catalytic efficiency, which is clear from the allozymic difference in slope of the regression of activity on protein (Figure 2). Although ▽*1* clearly affects ADH-protein, the magnitude of the effect is insufficient to account for the 1.6-fold ADH-protein level difference between the *Wa-S* and *Wa-F* wild-type alleles. The ratios of ▽*1-F*/▽*1-S* for ADH-protein are just 1.25 on the *Wa-F Adh* background and 1.16 on the *Wa-S* background.

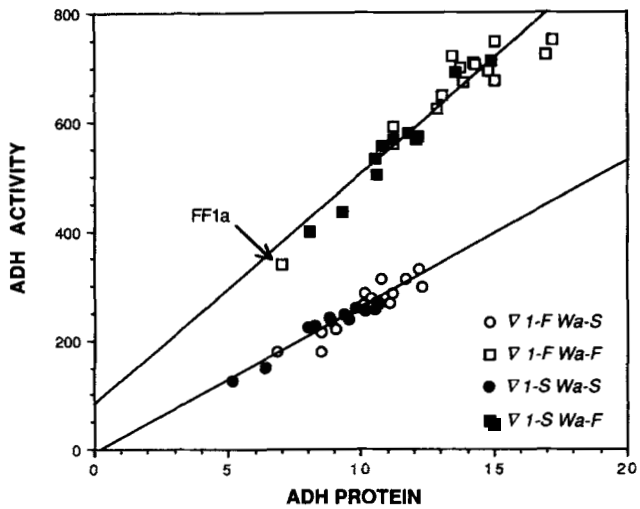

FIGURE 2.—A plot of ADH activity *vs.* ADH-protein level in 6–8-day-old adult males, where each point represents the average of four values from an independent, single-insert transformant stock. The slope of the regression of activity on protein is significantly different ($P > 0.0001$) for lines having the Fast (slope = 42.3) *vs.* Slow (slope = 26.7) form of the enzyme. An outlier line (FF1a) is marked by the arrow. Units are nanomoles NAD^+ reduced per min per mg total protein for activity and the number of standard (Hochi-R inbred) fly-equivalents per mg total protein for ADH-protein level.

TABLE 1

Average values of ADH activity and ADH protein for each of four types of Adh alleles in transgenic flies

Allele	ADH activity[a]	ADH protein level[b]	N[c]
▽1-F Wa-F	680[d]	14.1[d]	14
▽1-S Wa-F	556	11.3	11
▽1-F Wa-S	263	10.2	15
▽1-S Wa-S	228	8.8	13

[a] Units are nanomoles NAD^+ reduced per minute per milligram total protein.

[b] Units are number of standard (Hochi-R inbred) fly equivalents per milligram total protein.

[c] *N* = number of single-insert, independent transformant lines analyzed.

[d] These averages exclude transformant line FF1a (Figure 2), which is an outlier that satisfies a statistical rejection rule ($P < 0.025$) (SNEDECOR and COCHRAN 1967). Including the outlier gives values of 657 for activity and 13.6 for ADH-protein.

The analysis of variance also revealed a significant allelic background-by-▽*1* interaction effect on activity ($P < 0.05$), but not on ADH protein. This significant interaction is probably due to the catalytic efficiency difference between the allozymes, which causes nonadditivity in the effect on activity of changing ADH protein levels. For example, the same increase in ADH protein level would cause a proportionally greater increase in the ADH activity of Fast than Slow strains because of the

greater catalytic efficiency of ADH-F (*i.e.*, greater activity per ADH molecule).

The ▽*1* polymorphism is located within a region that serves both as the first intron of the distal transcriptional unit and as the 5′ flanking region of the proximal transcript (Figure 1A). Previous work indicates that proximal RNA is predominant in larvae, while adults produce almost entirely distal RNA (Benyajati *et al.* 1983). We used an RNase protection assay to show that adults of all four of the transformant types produce abundant distal mRNA but no trace of proximal mRNA (Figure 3A). These results (and those in Figure 3B) also show that there is no evidence for aberrantly initiated mRNAs, which might result from the multiple TAATA elements in the ▽*1-F* sequence (Figure 1A). Therefore, the observed effect of ▽*1* on ADH level in adults is due to its role as an intronic sequence of the distal transcript.

The ADH-RNA level in wild-type *F* and *S* strains (which differ in their ▽*1* sequences) is not significantly different, even though they show a 1.5-fold difference in ADH-protein level (Laurie and Stam 1988). This suggests that ▽*1* does not affect the level of transcription or mRNA stability, but may affect translational efficiency. An effect on translation is possible if ▽*1* alters pre-mRNA processing (perhaps capping, splicing or polyadenylation), leading to mRNAs of different structure.

Another RNase protection experiment (Figure 3B) shows that all four transformant types produce RNA in which the first two introns are spliced exactly as predicted for the distal transcript (Benyajati *et al.* 1983). The 90-base fragment found in all genotypes indicates that the distal RNA is initiated at the same site. In addition, there is no evidence for intron inclusion or that the GT within ▽*1-S* serves as a cryptic 5′ donor splicing site. Another RNase protection assay with the MB probe (Figure 1A), shows a 352-base fragment for all genotypes, as expected if they all have the same polyadenylation site (data not shown). Finally, we have previously shown that wild-type fly strains that differ in ▽*1* sequence show the same splicing sites for the third intron (Laurie and Stam 1988).

We conclude that the distal mRNAs from both ▽*1* alleles have the same initiation, polyadenylation and splicing sites, within a level of resolution on the order of 10 bases. Exon skipping (which could not be detected in our RNase assays), capping and polyadenylation have not been examined. Since intron splicing is mechanistically coupled with polyadenylation (Buchman and Berg 1988; Huang and Gorman 1990), and the presence of a poly(A) tail stimulates translation (Munroe and Jacobson 1990), it is conceivable that ▽*1* exerts an indirect effect on translational efficiency through differential polyadenylation. Further work is needed to establish the mechanism of the ▽*1* effect on *Adh* expression.

DISCUSSION

Several types of observations suggest a role for natural selection in controlling the frequencies of the *Adh* allozymes in natural populations. These include geographic frequency clines, increases in *Fast* frequency under ethanol selection in the laboratory (Gibson and Oakeshott 1982; Van Delden 1982) and an excess of silent polymorphism closely linked to the allozymic site (Kreitman and Hudson 1991). Perhaps the most convincing evidence is that an increase in *Fast* frequency with latitude occurs in several geographically distant parts of the world (David *et al.* 1989; Jiang *et al.* 1989; Oakeshott *et al.* 1982; Parkash *et al.* 1992).While historical artifacts of colonization can account for any one cline (Simmons *et al.* 1989), it is very difficult to devise a neutralist scenario to account for repeatable clines that occur in different continents.

Further evidence of the selective basis of the eastern North American cline in allozyme frequency is provided by Berry and Kreitman (1993) who found that only a few of the more than 20 *Adh* polymorphisms with intermediate frequencies show a significant regression of frequency on latitude. Moreover, ▽*1* shows a significant cline that is even steeper than the allozyme cline and therefore cannot be explained solely by linkage disequilibrium with the allozyme. The lack of regional differentiation for most other polymorphisms in *Adh* indicates a high level of gene flow, which argues against historical explanations of the clines at these two sites. Berry and Kreitman suggested that clinal selection may be acting epistatically on the allozyme and ▽*1* polymorphisms, in part because of a marked deficiency of ▽*1-F* in Slow alleles.

Here we show that the ▽*1* polymorphism has a highly significant effect on ADH-protein level, which lends credence to the suggestion that it is subject to clinal selection. Although the demonstration of an effect at the protein level does not necessarily mean there is an effect on fitness, the failure to find a protein level effect would have provided a very strong argument against the selection hypothesis. On the other hand, the protein-level analysis does not illuminate the suggestion of epistatic selection of a type that could account for the deficiency of ▽*1-F* in Slow alleles. While there is nonadditivity in the effects of ▽*1* on ADH activity, the ▽*1-F*, Slow combination has intermediate levels of ADH expression, so it is not clear why it might be disadvantageous relative to the ▽*1-S*, Slow combination. Of course, any firm conclusions regarding epistatic selection requires a knowledge of how ADH-protein level and catalytic efficiency translate into fitness differences.

Although some introns have been shown to contain regulatory sequences such as enhancers (*e.g.*, Aronow *et al.* 1989; Gasch *et al.* 1989; Huang *et al.* 1993; Pogulis and Freytag 1993), the relative scarcity of such instances

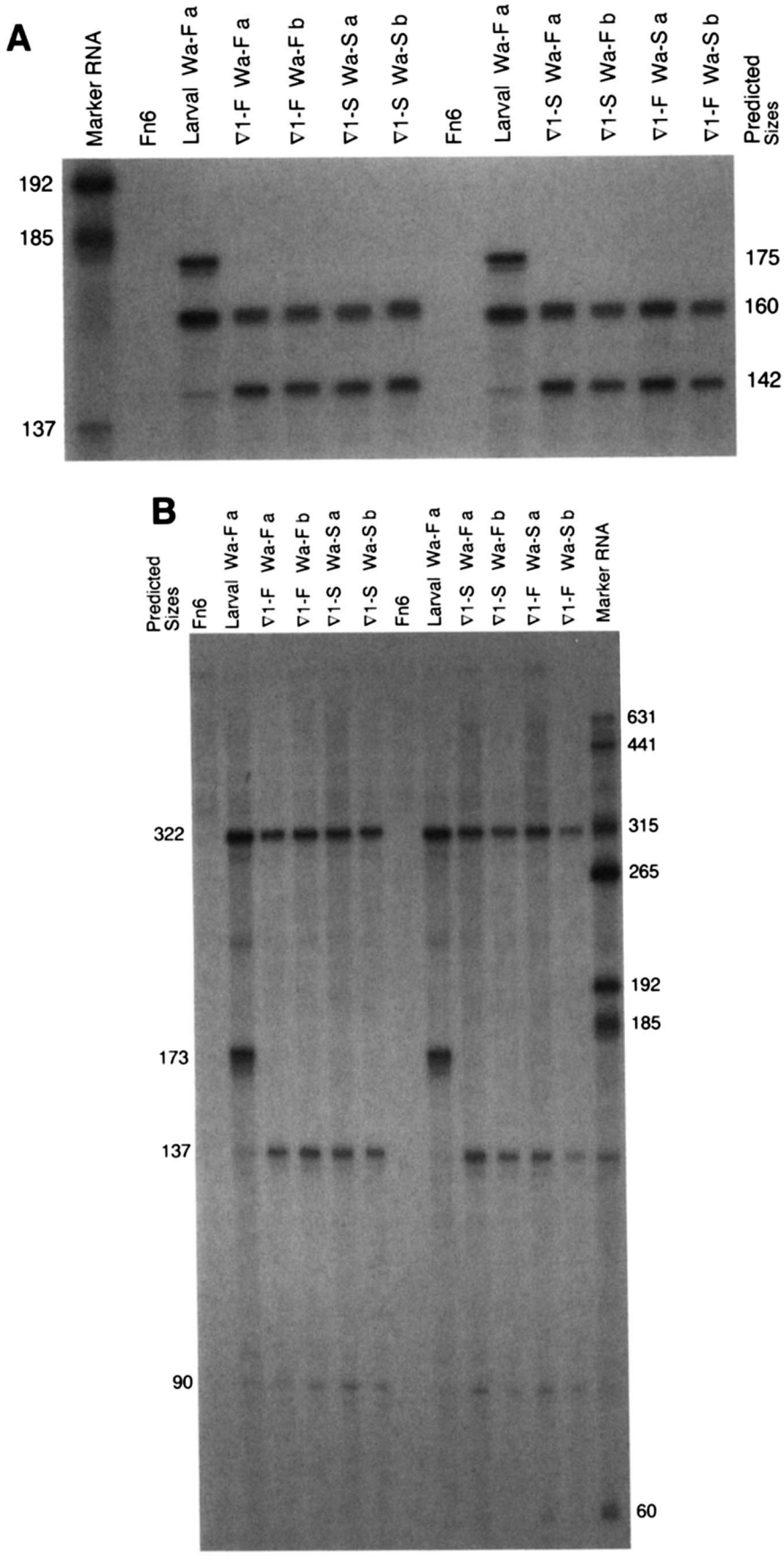

FIGURE 3.—RNase protection analyses of *Adh* RNA. Adult flies from two independent transformant lines of each type (a and b) and from the host strain (Adh^{fn6} *cn*; ry^{506}-labeled "Fn6") were analyzed. Lanes labeled "larval Wa-F a" contain RNA from a mixture of larval stages from the transformant line ∇1-FWa-F a. Predicted sizes of protected fragments are based on the RNA splicing pattern for distal and proximal mRNAs (Figure 1A) as determined by BENYAJATI *et al.* (1983) and take into account whether only RNase T1 or both RNases T1 and A were used. The RNA probes are shown in Figure 1A. **A**, DP probe; **B**, SB probe. In **B**, marker RNAs were mixed with a sample from the ∇1-FWa-F a line and only the sizes of markers are shown on the right.

suggests that functional sequences within introns are uncommon. Moreover, because introns evolve at a high rate similar to pseudogenes (Li and Graur 1991), it is frequently assumed that intronic substitutions and polymorphisms are neutral. The effect of $\nabla 1$ on ADH protein and its clinal variation suggest that these generalizations do not apply to the *Adh* intron containing $\nabla 1$. To our knowledge, this is the first analysis of an intronic polymorphism that is capable of detecting small quantitative effects. Additional analyses of this type are needed to determine whether specific intronic sequence variations frequently have subtle effects on gene expression.

We have shown that multiple polymorphisms within the *Adh* region of *D. melanogaster* affect ADH activity levels. Previous analyses of the *Wa-S* and *Wa-F* alleles have detected an 8% difference in activity that maps to the 5′-flanking region (Laurie-Ahlberg and Stam 1987) and a twofold difference in catalytic efficiency due to the amino acid replacement that generates the allozyme polymorphism (Choudhary and Laurie 1991). Here we show that the intronic $\nabla 1$ polymorphism has a 15–20% effect on ADH-protein level. Moreover, there must be at least one additional polymorphism affecting ADH-protein level within the *HpaI/ClaI* fragment (Figure 1A), since $\nabla 1$ accounts for only about one-third of the difference that maps to this fragment (Laurie-Ahlberg and Stam 1987). The association between functional effects and geographic variation in allelic frequencies indicates that selection may be operating on at least two of these sites. These results suggest that multiple sites within a single gene may be subject to selection simultaneously in a population and, therefore, that multisite selection models may be required to understand the evolutionary dynamics of individual loci.

These results also have some implications for human genetic epidemiology. For example, many studies have shown linkage disequilibrium among DNA polymorphisms within the genes that code for apolipoproteins and, in several cases, specific polymorphisms have shown effects on apolipoprotein level and other serum traits associated with cardiovascular disease (Dunning *et al.* 1992; Sing and Moll 1990). Our studies of Drosophila *Adh* suggest that several polymorphisms within a single gene may affect apolipoprotein level and that direct experimental approaches are required to discriminate among the effects of the many candidate polymorphisms that show linkage disequilibrium with each other.

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