Tissue-Specific Expression Phenotypes of Hawaiian Drosophila *Adh* Genes in *Drosophila melanogaster* Transformants

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ABSTRACT

Interspecific differences in the tissue-specific patterns of expression displayed by the *alcohol dehydrogenase* (*Adh*) genes within the Hawaiian picture-winged Drosophila represent a rich source of evolutionary variation in gene regulation. Study of the *cis*-acting elements responsible for regulatory differences between *Adh* genes from various species is greatly facilitated by analyzing the behavior of the different *Adh* genes in a homogeneous background. Accordingly, the *Adh* gene from *Drosophila grimshawi* was introduced into the germ line of *Drosophila melanogaster* by means of *P* element-mediated transformation, and transformants carrying this gene were compared to transformants carrying the *Adh* genes from *Drosophila affinidisjuncta* and *Drosophila hawaiiensis*. The results indicate that the *D. affinidisjuncta* and *D. grimshawi* genes have relatively higher levels of expression and broader tissue distribution of expression than the *D. hawaiiensis* gene in larvae. All three genes are expressed at similar overall levels in adults, with differences in tissue distribution of enzyme activity corresponding to the pattern in the donor species. However, certain systematic differences between *Adh* gene expression in transformants and in the Hawaiian Drosophila are noted along with tissue-specific position effects in some cases. The implications of these findings for the understanding of evolved regulatory variation are discussed.

ALCOHOL dehydrogenase (*Adh*) genes in Drosophila display complex expression patterns during development. The homologous *Adh* genes from closely related species of the Hawaiian picture-winged Drosophila show distinct tissue- and stage-specific patterns of regulation (DICKINSON 1980a). It has been proposed that such regulatory variation might provide an important source of evolutionarily significant genetic variability in natural populations (WILSON 1976; WHITT 1983). However, the mechanisms responsible for this differential regulation are not well understood. Previous studies employing interspecific hybrids of certain Hawaiian Drosophila species have demonstrated that the major differences in the developmental patterns of alcohol dehydrogenase enzyme (ADH) distribution are controlled by *cis*-acting elements linked to the corresponding structural genes (DICKINSON 1980b; DICKINSON and CARSON 1979).

In order to facilitate analysis of the expression of the Hawaiian Drosophila *Adh* genes, *P* element-mediated transformation has been utilized to introduce cloned genes from Hawaiian species in *Drosophila melanogaster* (SPRADLING and RUBIN 1982; RUBIN and SPRADLING 1982; BRENNAN and DICKINSON 1988; BRENNAN, WU and BERRY 1988). This allows *in vivo* study of the tissue-specific regulation of the introduced genes in transformants. As a host species, *D. melanogaster* has the advantages over the Hawaiian Drosophila of shorter generation time, easier stock maintenance and controllable genetic background. In previous work, *D. melanogaster* transformants carrying the *Adh* genes from *Drosophila affinidisjuncta* and *Drosophila hawaiiensis* were constructed, and the patterns of ADH production in larval transformants were compared (BRENNAN and Dickinson 1988; BRENNAN, WU and BERRY 1988). The results demonstrated that the expression patterns in larval transformants closely parallel those in the corresponding donor species.

In the present study, the *Adh* gene of *Drosophila grimshawi*, another member of the grimshawi complex of picture-winged Drosophila, was cloned and introduced into germ line chromosomes of *D. melanogaster*. The expression patterns of this and of the other two homologous *Adh* genes in both larval and adult transformants were compared by measuring ADH RNA levels and ADH activity and by determining tissue-specific expression phenotypes via gel electrophoresis and histochemical staining. The results indicate that the major tissue-specific differences in expression patterns among the Hawaiian Drosophila *Adh* genes are conserved for most tissues examined. Consistent with previously published results for the donor species (DICKINSON 1980a), in transformants the *D. grimshawi* gene was found to have a similar larval expression pattern, but a different adult expression pattern, than the *Adh* gene of *D. affinidisjuncta*. These data confirm that differences in developmental patterns of ADH production are under the control of *cis*-acting regu-

latory elements linked to the structural genes.

However, not all aspects of tissue-specific expression are conserved perfectly in transformants. Some variations from the donor pattern, particularly at the histochemical level, were noted. At least some of the differences between the donor pattern and the pattern displayed by the same gene in transformants represent systematic differences between transformants and the Hawaiian flies as a group. We also address the influence of position of transposon integration on total enzyme production and on tissue-specific expression. Position effects for larval and adult tissues were found for all three genes, with each gene being differently sensitive to tissue-specific position effects.

MATERIALS AND METHODS

Drosophila stocks: The *D. grimshawi*, stock G1, the *D. affinidisjuncta*, stock S36G1, and the *D. hawaiiensis*, stock J14B8 were used as sources of the genomic DNAs for cloning, and for tissues in histochemical preparations (DICKINSON 1980a). Stocks were maintained according to the method of WHEELER and CLAYTON (1965). All *D. melanogaster* stocks were maintained on Formula 4-24 instant Drosophila medium (Carolina Biological Supply Co.) at 25°. The *Adh* null stock, Adh^{fn6} *cn*; ry^{506}, was used as a host for *P* element-mediated transformation. This *Adh* allele produces a low level of incompletely processed *Adh* RNA and no detectable ADH protein (BENYAJATI *et al.* 1982). For all analyses transformants were harvested as feeding third instar larvae or as adults aged 4–8 days posteclosion.

Construction of plasmids and *P* element-mediated transformation: The construction of *P* element vectors carrying the *Adh* genes of *D. affinidisjuncta* and *D. hawaiiensis* has been described (BRENNAN and DICKINSON 1988; BRENNAN, WU and BERRY 1988). Two bacteriophage lambda clones (G1 and G3) carrying portions of the *D. grimshawi Adh* gene, were obtained in previous work (RABINOW and DICKINSON 1986). In order to reconstitute an intact *D. grimshawi Adh* gene, upstream sequences from the clone G3 were combined with promoter and structural gene sequences from clone G1. A 2.1-kb *Bgl*II/*Pst*I fragment carrying upstream sequence from clone G3 was combined with a 3.1-kb *Pst*I/*Xho*I fragment carrying promoter and structural gene sequence from clone G1 (Figure 1). The resulting 5.2-kb fragment was introduced into the *P* element vector, pUChs-*neo* (STELLER and PIRROTTA 1985). The *P* element plasmids carrying the *Adh* gene in either orientation were obtained and purified by CsCl density gradient centrifugation prior to use for microinjection. Restriction endonucleases and other DNA modifying enzymes were obtained from New England Biolabs (Beverly, Massachusetts) and used according to the supplier's recommendations. All of the DNA manipulations were according to standard techniques (MANIATIS, FRITSCH and SAMBROOK 1982).

D. melanogaster Adh^{fn6} *cn*; ry^{506} embryos were used as hosts for microinjection of the above plasmids. Each plasmid (300 μg/ml) was injected with the helper plasmid pUChsπ at 100 μg/ml as described (SPRADLING and RUBIN 1982; RUBIN and SPRADLING 1982; STELLER and PIRROTTA 1986). Resistance to the antibiotic, G418 (conferred by pUChs-*neo*) was used as a selectable marker for transformation (STELLER and PIRROTTA 1985).

Transformants were crossed with Adh^{fn6} *cn*; ry^{506} flies and integrations were made homozygous by employing *CyO* and *In(3LR)CXD* as balancer chromosomes (LINDSLEY and GRELL 1967). All lines analyzed were homozygous for single intact transposons carrying the *Adh* gene as indicated by genomic Southern analysis (SOUTHERN 1975). Isolation of transformants carrying the *Adh* genes of *D. affinidisjuncta* and *D. hawaiiensis* have been reported previously (BRENNAN and DICKINSON 1988; BRENNAN, WU and BERRY 1988). In no case have we observed systematic differences in tissue-specific expression patterns due to the orientation of the *Adh* gene within the *P* element vector.

Quantitative primer extension: Total nucleic acid was prepared by homogenization of whole larvae or adults in RNA extraction buffer [100 mM Tris-HCl, 50 mM EDTA, 1% SDS (pH 7.4)], containing 1 mg/ml freshly added proteinase K. Homogenates were incubated for 1–4 hr at 37° and extracted three times with an equal volume of chloroform:isoamyl alcohol:phenol 24:1:24 (v/v/v). RNAs were precipitated twice by standard methods with 95% ethanol.

Two synthetic oligonucleotides were used for primer-extension analysis. One of these is complementary to nucleotides 2056–2085 of the *D. affinidisjuncta Adh* gene (ROWAN and DICKINSON 1988). Based on DNA sequence analysis, this oligonucleotide is complementary to a portion of the protein coding sequence that is identical for mRNAs encoded by the three Hawaiian genes used in the present study (P. THORPE and W. J. DICKINSON, personal communication). The second oligonucleotide is complementary to nucleotides 1081 through 1119 in exon two of the cytoplasmic actin 5C gene (BOND and DAVIDSON 1986). Actin 5C mRNAs carrying exon two have been shown to be expressed constitutively at low levels in most (perhaps all) tissues of *D. melanogaster* (BOND and DAVIDSON 1986; BURN, VIGOREAUX and TOBIN 1989).

Oligonucleotides were labeled at their 5′ ends by incubation of 7 pmol of oligonucleotide and 35 pmol of γ-[^{32}P]-ATP (New England Nuclear, 6000 Ci/mmol) with bacteriophage T4 polynucleotide kinase. Reactions were stopped by extraction with an equal volume of water-saturated phenol. Radiolabeled oligonucleotides were freed from unincorporated nucleotide by multiple rounds of ethanol precipitation. Typical specific activities for oligonucleotides were approximately 7×10^6 cpm/pmol.

Total nucleic acids from larvae and adults (10 μg) were mixed with approximately 0.2 pmol of each oligonucleotide in a 7 μl mixture containing 180 mM KCl and 10 mM Tris-HCl (pH 7.2). Assuming that *Adh* mRNA represents a total of about 1% of the poly(A)-containing mRNA, this amount of oligonucleotide is in excess by at least 20-fold. Hybridization mixtures were heated to 65° for 5 min and transferred to 42° for 6 to 12 h. Annealing reactions were chilled on ice, and diluted with 50 μl of reverse transcriptase buffer [75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 800 μM of each dNTP, Tris-HCl (pH 8.3)] containing 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (Bethesda Research Laboratories). Reactions were incubated for 90 min at 37°, and were terminated by chilling on ice and adding 0.1 volume of 4 M NaCl followed by ethanol precipitation. Nucleic acids were recovered by centrifugation, and analyzed on 8% polyacrylamide-urea gels as described (MAXAM and GILBERT 1980). Fixed and dried gels were fluorographed using Kodak XAR-5 film and Dupont Cronex intensifying screens at −80°.

Signal strength was determined by scanning densitometry of X-ray films. Relative RNA levels were initially expressed as a ratio of *Adh*-specific to actin-specific primer-extension products. In order to control for possible variation in the relative specific activities of the two primers, ratios were normalized to the value obtained for a standard RNA in-

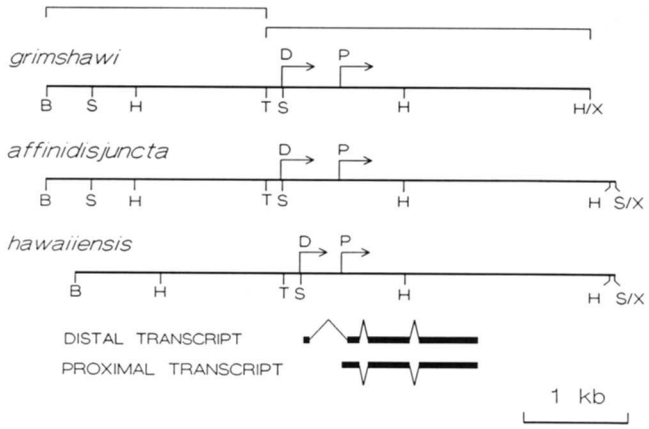


FIGURE 1.—The three *Adh* genomic regions introduced into the germ line of *D. melanogaster*. The genomic regions are aligned in order to allow comparison of the organization of the genes. The lines above the *D. grimshawi* gene map represent the two bacteriophage lambda clone fragments which were combined in order to reconstitute the intact gene. The arrows labeled D and P designate the distal and proximal promoters for each gene. Restriction endonuclease cleavage sites are shown by vertical lines and are abbreviated as follows: B, *Bgl*II; H, *Hin*dIII; T, *Pst*I; S, *Sal*I; X, *Xho*I. *Xho*I linkers were added to the 3′ end of each gene to allow insertion into a *P* element vector. The processing patterns for the distal and proximal transcripts are shown below the gene maps (ROWAN, BRENNAN and DICKINSON 1986; ROWAN and DICKINSON 1988). Dark boxes represent exons present in the mature *Adh* RNAs. The three genes differ by fewer than 20 bp in the size of the two smaller introns. The spacing between the proximal and distal promoters is similar for the *D. grimshawi* and *D. affinidisjuncta* genes but approximately 200 bp shorter for the *D. hawaiiensis* gene.

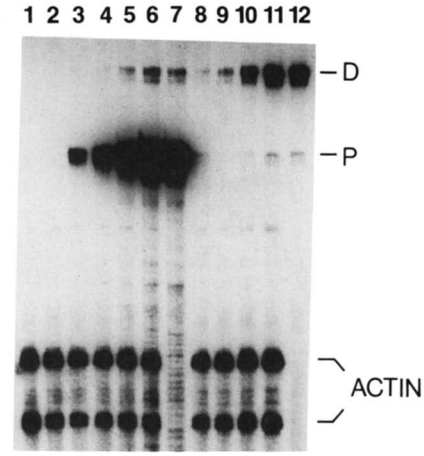


FIGURE 2.—RNA analysis by primer extension. RNAs were annealed with two oligonucleotides. One of these is complementary to a portion of the coding region held in common by all of the Hawaiian *Adh* mRNAs. The other is complementary to the *D. melanogaster* actin 5C mRNA (see MATERIALS AND METHODS). The extension products corresponding to the distal and proximal promoters of the *Adh* gene are labeled D and P, respectively. The two extension products from the actin oligonucleotide probe differ from one another by 9 nucleotides. Similar microheterogeneity was reported by BOND and DAVIDSON (1986). Lane 1, 10 μg of total RNA from the adult *D. melanogaster* host. Lanes 2 through 6 and 8 through 11 are from reactions containing 10 μg of total RNA from the larval *D. melanogaster* host. Lanes 7 and 12 contain only *D. affinidisjuncta* RNAs plus 10 μg of yeast carrier RNA. The amounts of *D. affinidisjuncta* poly(A)-containing RNAs are as follows: Lanes 1 and 2, none; lane 3, 3 ng larval; lane 4, 10 ng larval; lane 5, 33 ng larval; lanes 6 and 7, 100 ng larval; lane 8, 3 ng adult; lane 9, 10 ng adult; lane 10, 33 ng adult; lanes 11 and 12, 100 ng adult.

cluded in each set of reactions. Because the absolute levels of actin 5C transcripts in larvae and adults are not known, larval and adult RNA values obtained by this method cannot be compared directly.

ADH activity measurement, gel electrophoresis and histochemical staining: Determination of ADH specific activity was done as previously described (SOFER and URSPRUNG 1968) using 2-propanol as the substrate. Protein determination was by the micro-Biuret method using bovine serum albumin as the standard (LEGGET-BAILEY 1962).

ADH activity was quantified in pooled tissues dissected from feeding third instar larvae and from adults aged from 4 to 8 days posteclosion. The percentage of total ADH activity of selected tissues was determined by gel electrophoresis and scanning densitometry as described (DICKINSON and CARSON 1979; BRENNAN and DICKINSON 1988; BRENNAN, WU and BERRY 1988). Since fat body tissue could not be recovered quantitatively, the amount of ADH activity in the fat body was estimated by subtracting the activity in the other tissues from the total. Histochemical localization of ADH was performed on dissected tissue stained at 37° for 50 min. In all cases, control tissues were incubated in the absence of substrate. The fixing and staining procedure was described previously (BRENNAN and DICKINSON 1988).

RESULTS

Overall expression levels in transformants: The *Adh* genes of three Hawaiian species *D. affinidisjuncta, D. hawaiiensis* and *D. grimshawi* are diagrammed in Figure 1. In these Hawaiian species, the *Adh* genes contain two promoters (BRENNAN *et al.* 1984; ROWAN, BRENNAN and DICKINSON 1986; ROWAN and DICKINSON, 1986). The *Adh* transcripts in larvae derive mostly from a proximal promoter (P in Figure 1), and the adult transcripts are from a distal promoter (D in Figure 1). The introduced *Adh* gene segments are 5.1 kb (*D. hawaiiensis)* to 5.4 kb (*D. affinidisjuncta*) in size and carry sequences from about 2.5 kb upstream of the distal promoters to 1.4 kb downstream of the structural genes.

After obtaining homozygous lines carrying each of the *Adh* genes, we first determined the levels of ADH enzyme and RNA in intact organisms. RNA levels were measured by primer extension analysis in the presence of an excess of primer, with the cytoplasmic actin 5C RNA acting as an internal standard. To illustrate this method, varying amounts of adult or larval *D. affinidisjuncta* RNA were combined with a constant amount of *D. melanogaster* host RNA (Figure 2). As can be seen in Figure 2, lanes 1 and 2, neither larval nor adult nucleic acid samples from the host contain any RNA that results in detectable primer-extension products for the ADH probe used (D and P in Figure 2). As expected, however, RNA samples from the host do produce readily detectable signals for the actin primer-extension products. The addition of increasing amounts of *D. affinidisjuncta* RNA re-

TABLE 1

Relative ADH activities and RNA levels in larvae

Transformant	ADH activity[a]	RNA level[a]
D. affinidisjuncta	1.00 ± 0.04	1.00 ± 0.06
D. grimshawi	1.18 ± 0.04	1.17 ± 0.19
D. hawaiiensis	0.52 ± 0.02	0.49 ± 0.02

Extracts and RNAs were prepared from feeding third instar larvae. Means and standard errors are shown. ADH activity per milligram of protein per gene was determined as described previously (BRENNAN and DICKINSON 1988) and normalized to the value obtained for larval *D. affinidisjuncta* transformants. Four lines carrying different integrations of each gene were analyzed. ADH activity was assayed on four independent extracts for each line. RNA values were determined as the ratio of ADH-specific primer extension products (on a per gene basis) to actin 5C products (see MATERIALS AND METHODS), with two determinations for each line. These also were normalized to the value obtained for larval transformants carrying the *D. affinidisjuncta* gene.

[a] All values are significantly different ($P < 0.005$).

TABLE 2

Relative ADH activities and RNA levels in adults

Transformant	ADH activity[a]	RNA level[b]
D. affinidisjuncta	1.0 ± 0.06	1.0 ± 0.11
D. grimshawi	0.92 ± 0.06	0.33 ± 0.04
D. hawaiiensis	0.72 ± 0.10	0.17 ± 0.04

Extracts and RNAs were prepared from adults aged 4–7 days after eclosion (females and males pooled in equal numbers). The values shown and the number of independent determinations are as given for Table 1. The ADH specific activity (units per milligram of protein per gene) and RNA primer extension ratios per gene are normalized to the values obtained for adult transformants carrying the *D. affinidisjuncta* gene. These values cannot be compared directly to those in Table 1 because they are independently normalized.

[a] The *D. hawaiiensis* value is significantly different from the other two ($P < 0.05$).

[b] All three values are significantly different ($P < 0.01$).

sults in increasing extension products corresponding to RNA transcribed from the *Adh* gene (Figure 2, lanes 3–6 and 8–11). Densitometric determination of the ratio of ADH to actin extension products verifies that the ADH signals are linearly proportional to the amount of input RNA (data not presented). This method simultaneously allows relative quantitation of ADH RNA levels in various samples and a determination of RNA type (proximal or distal) in those samples.

Tables 1 and 2 summarize the RNA data and enzyme specific activity data for larval and adult stages, respectively. In larvae, transformants carrying the *D. affinidisjuncta* and *D. grimshawi* genes display comparable levels of RNA and enzyme activity, while transformants carrying the *D. hawaiiensis* gene contain about half as much of each.

Consistent with previous data (BRENNAN and DICKINSON 1988), for all three types of transformants (as well as the Hawaiian flies themselves) the total activity levels are five to ten fold lower in adults than in larvae (data not presented). Table 2 summarizes the ADH activity and RNA data for adult transformants. Because the absolute levels of actin 5C transcripts in larvae and adults are not known the values in Table 2 have been normalized independently of those in Table 1. Surprisingly, Table 2 indicates that the level of activity corresponding to a particular RNA level in adults is different for each type of transformant. This appears to be donor gene specific in that the relationship between RNA level and enzyme activity is relatively constant when one compares different integrations of the same transposon (not shown). However, both enzyme and RNA levels for multiple samples from the same stock were more variable in adults than in larvae.

Tissue-specific expression patterns in transformants: In order to determine if there is a correlation between total expression level and tissue-specific expression, ADH activity was measured by gel electrophoresis in selected tissues (Table 3). Included in this analysis are the larval and adult fat bodies which account for the majority of ADH synthesis in all cases. As can be seen, midgut and Malpighian tubules are relatively active tissues in larvae for transformants carrying the *Adh* genes of *D. affinidisjuncta* and *D. grimshawi*. However, the amount of ADH activity in the larval midgut and Malpighian tubules is significantly lower for the *D. grimshawi* gene than for the *D. affinidisjuncta* gene. In contrast, the *D. hawaiiensis* gene produces quite low levels of ADH activity in the larval midgut and undetectable activity in the larval Malpighian tubules. In earlier studies on the donor species, *D. grimshawi* and *D. affinidisjuncta* were scored as positive for larval midgut and Malpighian tubules, while *D. hawaiiensis* was scored as negative (DICKINSON 1980a). All three donor species were scored as positive for the carcass (hypodermis and body wall musculature).

The adult midgut expression addresses the major difference between the *D. affinidisjuncta* and *D. grimshawi* donor species. Of these two closely related species, only *D. affinidisjuncta* produces significant levels of ADH in the adult midgut (DICKINSON 1980a). Consistently, as shown in Table 3, the *D. affinidisjuncta* gene produces about twenty times more ADH activity in the adult midgut than does the *D. grimshawi* gene in transformants. By contrast, the adult Malpighian tubule activity is only about twofold higher in transformants carrying the *D. affinidisjuncta* gene. Also consistent with results for the donor species, there is no detectable activity found in either midgut or Malpighian tubules for adult transformants with the *D. hawaiiensis* gene. Thus in both larval and adult transformants, all three *Adh* genes maintain the major tissue-specific regulatory differences displayed in the donor species. Moreover, these tissue-specific regulatory differences do not relate in a simple way to overall

TABLE 3

Relative amounts of ADH activity per individual in transformants

Source of ADH gene	Larval				Adult		
	Fat body	Midgut	Carcass	Malpighian tubules	Fat body	Midgut	Malpighian tubules
D. affinidisjuncta	181 ± 7.2	49.4 ± 1.5	12.1 ± 1.2	3.3 ± 0.3	33.3 ± 3.7	1.00 ± 0.07	0.20 ± 0.03
D. grimshawi	249 ± 8.4	32.9 ± 1.7	11.1 ± 1.2	1.5 ± 0.3	31.6 ± 3.8	0.05 ± 0.003	0.10 ± 0.01
D. hawaiiensis	123 ± 4.7	2.1 ± 0.3	3.8 ± 0.4	ND[a]	25.0 ± 5.9	ND[b]	ND[b]

The level of ADH activity in different tissues was determined by gel electrophoresis and densitometric scanning of activity gels. The results are normalized to the amount found in the adult midgut of *D. affinidisjuncta* transformants. Means and standard errors are shown. In each line, four independent determinations were made for all tissues. For a given tissue, only values with nonoverlapping error ranges are significantly different ($P < 0.005$).

[a] ADH was not detectable in three lines. In the fourth, ADH activity was too little to measure by densitometry but was estimated by comparison with serial dilutions to be less than 0.06.

[b] There was no detectable activity in adult midgut and Malpighian tubules in three of the four lines analyzed. The relative amount of activity in adult midgut and Malpighian tubules for the exceptional stock were 0.17 ± 0.04 and 0.06 ± 0.01, respectively. This is the same transformed line that had barely detectable activity in the larval Malpighian tubules.

levels of gene expression in transformants.

It is interesting to note that there are some effects of integration position on tissue-specificity of expression. Figure 3 shows the relative level of total enzyme production and tissue-specific enzyme production in larval and adult tissues for all three genes, each with four independent lines representing different transposon integrations. The influence of position effect is different for each gene and each tissue. Considering larval activity first, there is a correlation for all three genes between total activity (representing primarily larval fat body activity) and activity in the carcass. In larvae, the *D. grimshawi* gene appears more sensitive to position effect than do the other two genes. Different integrations of only this gene show significant differences in total activity and midgut values ($P < 0.05$). Interestingly, both the *D. grimshawi* gene and the *D. affinidisjuncta* gene are noticeably sensitive to position effects for the carcass and Malpighian tubules.

For all three genes, total ADH activity in adults (representing primarily adult fat body ADH synthesis) does not correlate with total larval activity (Figure 3). This is consistent with the accumulation of primarily the proximal transcript in larvae and the distal transcript in adults as confirmed by primer-extension analysis of transformants. In adults, all three genes display tissue-specific position effects. Notably, one integration of the *D. hawaiiensis* gene (*HAW.*, stock 2) has expressed detectable levels of ADH in both the adult midgut and Malpighian tubules. Similarly, one integration of the *D. affinidisjuncta* gene (*AFF.*, stock 3) shows higher levels of expression in all adult tissues. Other than this, no consistent correlations between activity levels in various adult tissues for given integrations are observed.

Since the ADH isozyme produced by the *D. hawaiiensis* gene is electrophoretically distinguishable from those encoded by the *D. grimshawi* and *D. affinidisjuncta* genes, electrophoretic analysis may be used as a tool to demonstrate *cis*-acting regulation in a single individual carrying two different *Adh* genes. Taking advantage of this, flies homozygous for each donor gene were crossed and the tissue expression patterns of the progeny were examined. Previous experiments of this sort analyzing the larval tissue expression patterns from the offspring of the crosses between *D. melanogaster* transformants carrying the *D. affinidisjuncta* gene and those carrying the *D. hawaiiensis* gene demonstrated that tissue-specific differences between these transformants in larvae are *cis*-dominant (BRENNAN, WU and BERRY 1988). Figure 4 shows ADH activity gel electrophoresis on larval and adult tissues from transformants carrying either the *D. grimshawi* and *D. hawaiiensis* genes (Figure 4A) or the *D. affinidisjuncta* and *D. hawaiiensis* genes (Figure 4B). Scanning densitometry of such gels consistently gives activity ratios that are in agreement with the hypothesis that tissue-specific differences are determined by *cis*-acting sequences. Due to position effects, the particular ratios obtained depend upon the transformants analyzed.

Histochemical analysis of gene expression in transformants: Previous results comparing the histochemical distribution of ADH in *D. melanogaster* larvae carrying the *Adh* genes of *D. affinidisjuncta* and *D. hawaiiensis* indicated that the larval midgut and Malpighian tubule patterns closely resemble those in the corresponding donor species (BRENNAN and DICKINSON 1988; BRENNAN, WU and BERRY 1988). Specifically, staining in larvae for both *D. hawaiiensis* and transformants carrying the *D. hawaiiensis* gene is limited to fat body, and light staining in the gastric caeca and in a few cells in the middle midgut, while *D. affinidisjuncta* and transformants carrying the *D. affinidisjuncta* gene show strong staining throughout the midgut and Malpighian tubules.

As can be seen in Figure 5, the *D. grimshawi* gene

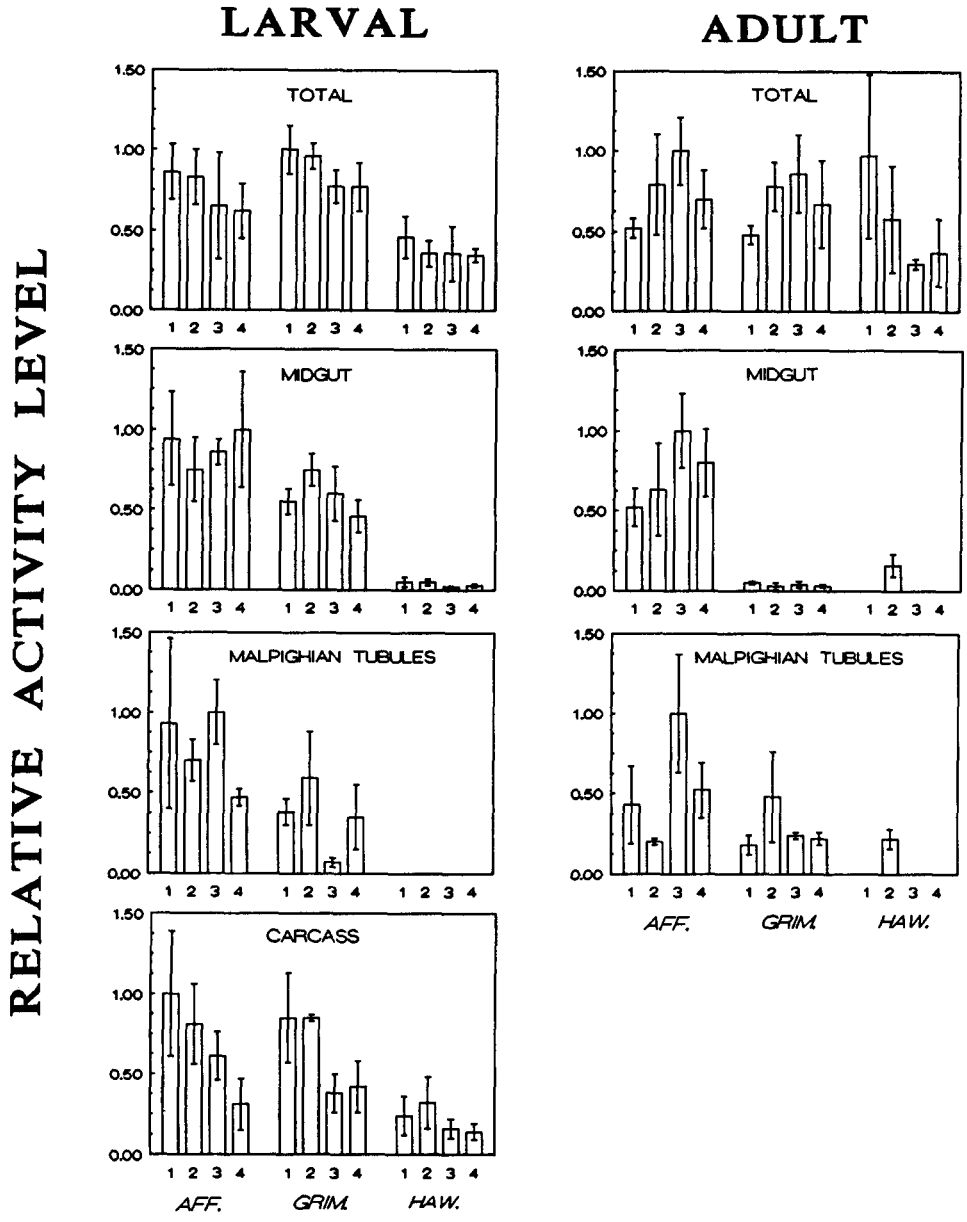


FIGURE 3.—The influence of transposon integration site on total and tissue-specific ADH activity. Particular transformed stocks are specified by an abbreviation of the donor species name (bottom) and a numerical designation (*AFF.*, *D. affinidisjuncta*; GRIM., *D. grimshawi*; *HAW.*, *D. hawaiiensis*). Total activity represents ADH specific activity per gene. To obtain tissue-specific activity levels, the percent of total activity, as determined by gel electrophoresis, was multiplied by the mean total specific activity level. Values are normalized to the highest one for the specific panel, with a value of 1.0 representing maximal ADH activity for the particular tissue or stage. Four determinations were made in all cases. Bars and error lines represent means and standard deviations. Results were compared pairwise for the different integrations of the same gene in each tissue or developmental stage. In all cases where neither mean falls within the error range for the other sample group, the results differ significantly ($P < 0.05$).

in transformants represents an intermediate case with detectable but weak staining in the posterior region of the midgut and Malpighian tubules. Representative examples for *D. grimshawi* (Figure 5D) and for transformants carrying the corresponding gene (Figure 5C) are shown. The specimens shown illustrate the differences between transformants and the donor species. While histochemical analyses of larvae consistently reveal readily detectable midgut and Malpighian tubule staining in both *D. grimshawi* and transformants, there are certain differences. Most often, larval transformants have lighter staining on the gastric caeca, hindgut and Malpighian tubules and heavier staining on the body of the anterior midgut than is displayed by *D. grimshawi* individuals. These same relative differences are seen also when comparing transformants carrying the *D. affinidisjuncta* and *D. hawaiiensis* genes to the corresponding donor species (BRENNAN, WU and BERRY 1988; see also Table 4).

The adult patterns of the three Hawaiian flies and *D. melanogaster* transformants carrying the corresponding *Adh* genes are shown in Figure 6. Again, in general, the distribution of histochemical staining for the donor species and the corresponding transformants reveal remarkably similar patterns of ADH production. For *D. affinidisjuncta*, the staining of the midgut is fairly strong and similarly distributed in donor and transformants. Similarly, both donor and host show light and patchy staining in the Malpighian tubules. The major difference between the ADH activity distribution in adults of *D. affinidisjuncta* and *D. grimshawi* is in the adult midgut. The adult midgut of *D. affinidisjuncta* has readily detectable activity, while that of *D. grimshawi* does not (Figure 6, F and H). In this regard, histochemical observation of transform-

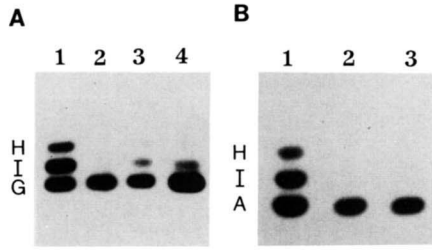


FIGURE 4.—ADH activity gel electrophoresis on larval and adult tissues. Extracts were prepared from whole organisms or hand-dissected tissues, electrophoresed on 0.8% Noble agar gels and stained for ADH activity. The ADH isozymes are labeled as follows: H, *D. hawaiiensis* ADH; I, interspecific heterodimer; A, *D. affinidisjuncta* ADH; G, *D. grimshawi* ADH. Tissues were pooled from the same group of larval or adult flies but they were homogenized in various volumes so that the amount of ADH activity would be comparable for each lane. Panel A: Larval tissues from the F_1 offspring of a cross between transformants carrying the *D. grimshawi* and *D. hawaiiensis* genes. Lanes: 1, Total larval extract representing the material from one half larva; 2, Malpighian tubules from 24 individuals; 3, carcasses from 12 individuals; 4, midguts from six individuals. Panel B: Adult tissues from a line homozygous for an integration of the *D. affinidisjuncta* gene on chromosome *2* and for an integration of the *D. hawaiiensis* gene on chromosome *3*. Lanes: 1, Total adult extract representing the activity from one-half of one individual; 2, midguts from 12 individuals; 3, Malpighian tubules from 24 individuals.

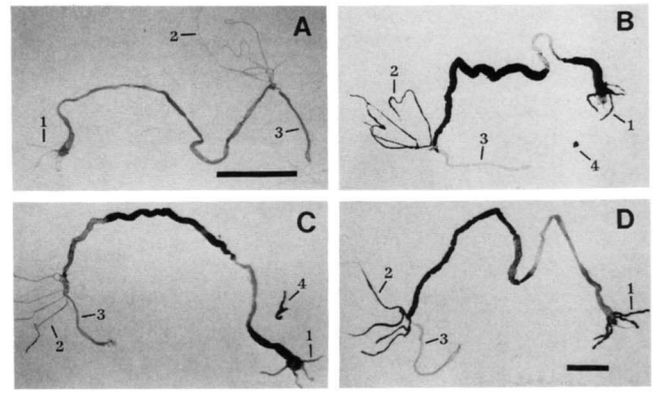


FIGURE 5.—Histochemical localization of ADH in larval tissues. Third instar larvae were dissected and the tissues were stained histochemically for ADH activity. Structures are indicated by numbers as follows: 1, gastric caeca; 2, Malpighian tubules; 3, hindgut; 4, fat body. The midgut extends anteriorly from the sperical proventriculus and posteriorly to where the Malpighian tubules and the hindgut meet. Tissues are from the following: (A) *D. melanogaster, Adh*^{fn6} *cn*; *ry*^{506} host; (B) *D. melanogaster, Adh*^{F}; *ry*^{506} homozygous for the wild-type *Adh*^{F}-allele; (C) *D. melanogaster* transformant carrying the *D. grimshawi Adh* gene; (D) *D. grimshawi*. [A, bar = 2 mm (A–C); D, bar = 2 mm.]

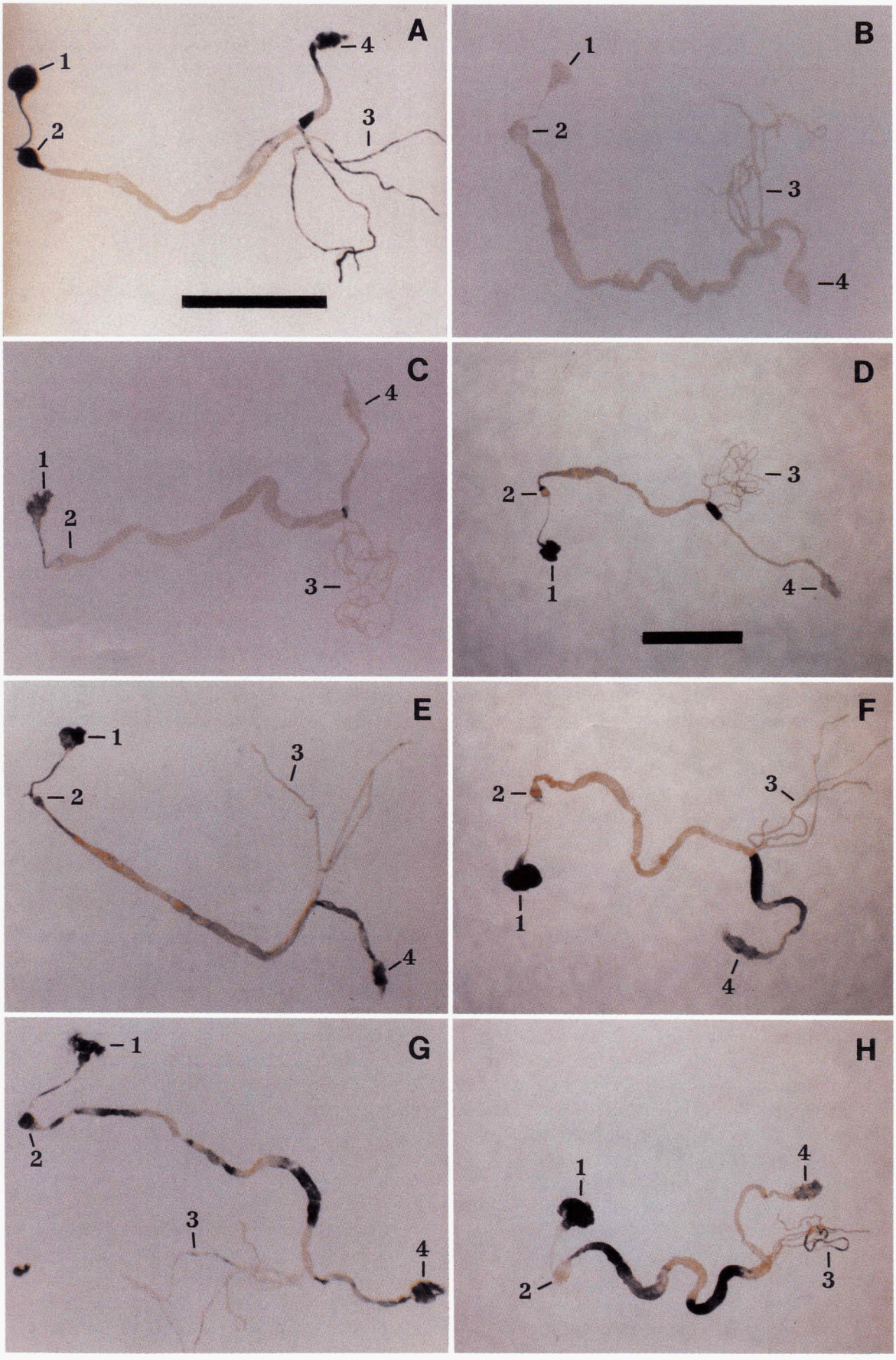
ants carrying the *Adh* genes from those two species agree completely (Figure 6, E and G, and Table 4). The *D. hawaiiensis* gene in adults of the donor and host species, consistent with the results in Table 3 and Figure 4, displays no detectable activity on the body of the adult midgut or on the Malpighian tubules. As in the case of larval histochemistry, certain systematic differences between transformants and the Hawaiian flies were noted for adults. For example, for the *D. grimshawi* and *D. affinidisjuncta* genes, the posterior region of the adult hindgut and the rectum stain more reproducibly in transformants than in the Hawaiian flies (Table 4).

Table 4 gives the number of transformed lines and the frequency of individuals of the donor and host species that show histochemically detectable ADH in a variety of specific tissues. Three points may be made with reference to these data. First, in qualitative detail, the staining in transformants follows the donor pattern closely. Secondly, it is difficult to arrive at a consensus phenotype based on histochemical observations alone. All tissues examined showed threshold effects, with two-fold to three-fold differences in activity levels having dramatic consequences in terms of staining intensity. Also, many "exceptions" were noted even for the endogenous genes in the host and donor species. Though this may reflect some variability in genetic background, particularly for the Hawaiian flies, it is noteworthy that we observed comparable variability from individual to individual within a tranformed line. Most of the exceptions for the Hawaiian flies involved light staining of one to six cells as in the case of the larval salivary gland and adult Malpighian tubules in *D. hawaiiensis* (Table 4). In these cases, electrophoresis on pooled tissues provides no evidence of ADH production. Last, there is one tissue, the larval salivary gland, for which staining is seen (for some integrations) in transformants but, with the possible exception of *D. hawaiiensis*, not in the donor or host species.

DISCUSSION

The introduction of *Adh* genes from different Hawaiian picture-winged Drosophila species into the germ line of *D. melanogaster* allows the analysis of the expression of these genes in a single host species. This provides a method of functional comparison of genes that would otherwise be impossible. The *cis*-dominant regulation of certain Hawaiian Drosophila *Adh* genes was inferred from previous studies employing interspecific hybrids of only two species pairs (DICKINSON and CARSON 1979; DICKINSON 1980b). Only one of these studies involved members of the *D. grimshawi* complex, and in this case, adult tissues were not analyzed. In order for such studies to be informative, the species under study must be readily hybridizable and their *Adh* genes must encode electrophoretically distinguishable enzymes.

The results presented here indicate that major differences in tissue-specific phenotypes for the Hawaiian Drosophila *Adh* genes are conserved when the genes are introduced into a common host species. Previous work had suggested that this was the case for larval transformants carrying the *D. hawaiiensis* and *D. affinidisjuncta* genes (BRENNAN, WU and BERRY 1988).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The present study further extends this analysis by considering both larval and adult transformants carrying three genes each with different tissue-specific phenotypes in the donor species. Indeed, with the exception of the larval carcass, the expression patterns of these three genes are representative of the entire range of variation described for the *Adh* genes in 27 picture-winged species (DICKINSON 1980a).

Interestingly, the larval expression phenotypes for the endogenous *D. grimshawi* and *D. affinidisjuncta* genes are similar, but their adult phenotypes differ primarily in that only the *D. affinidisjuncta* gene is expressed at a high level in the cells making up the body of the adult midgut (DICKINSON 1980a; Figure 6; Table 4). The endogenous *D. hawaiiensis* gene differs from both in having low larval and adult midgut and Malpighian tubule expression (DICKINSON 1980a; BRENNAN, WU and BERRY 1988; Table 4; Figures 5 and 6). These aspects and other details of the developmental regulation of the three genes are conserved in transformants (Tables 3 and 4; Figures 4–6). Consistently, intraspecific transformation experiments involving the *Adh* gene of *D. melanogaster*, as well as other genes from this species, indicate that the *cis*-acting sequences necessary for correct tissue-specific expression may be carried on relatively small segments of cloned DNA (GOLDBERG, POSAKONY and MANIATIS 1983; SCHOLNICK, MORGAN and HIRSH 1983; WAKIMOTO, KALFAYAN and SPRADLING 1986).

Though the production of interspecific hybrids in the Hawaiian flies had indicated that regulatory differences between the *Adh* genes were *cis*-dominant, it did not follow that major differences in the tissue distribution of relevant *trans*-acting factors would be relatively invariable over evolutionary time. Our findings indicate that the latter is indeed the case. A model reconciling complex tissue-specific expression with the other available data would be one in which the genes in question possess (or are influenced by) multiple, *cis*-acting sequences that interact with a small number of *trans*-acting components each of which has been strongly conserved in evolution. Such a model is consistent with a growing body of evidence indicating that a single eukaryotic DNA binding protein may interact with a variety of genes, sometimes with alternative consequences (ARNDT, STYLES and FINK 1987; PFEIFER, PREZANT and GUARENTE 1987; DAVIDSON *et al.* 1988). Also in agreement are experiments indicating interspecific conservation of functional and structural features of eukaryotic transcription factors (WIEDERRECHT *et al.* 1987; KAKIDANI and PTASHNE 1988; FISCHER *et al.* 1988; WEBSTER *et al.* 1988).

Two lines of evidence indicate that the above model may be appropriate for the Hawaiian Drosophila *Adh* genes. First, these genes are measurably sensitive to tissue-specific position effects in transformants (Figure 3; Table 4). This indicates, by analogy to enhancer-sensitive genes with weak promoters (O'KANE and GEHRING 1987), that the tissue-specificity of expression for these genes is responsive to surrounding chromosomal context. When a given gene is expressed at a high level in the particular tissue the position effects are negligible. An example of the latter is the larval midgut in transformants carrying the *D. affinidisjuncta* gene (Figure 3). The second line of evidence involves recent analyses of chimeric *Adh* genes (our unpublished results). Briefly, these experiments indicate that multiple, separable *cis*-acting sequences are needed for the fully donor-specific expression patterns.

The present study has emphasized the measurement of ADH enzyme levels because original observations of regulatory variation for the *Adh* genes in the Hawaiian flies were made at this level (DICKINSON 1980a). However, additional insight is gained by analysis of RNA. We found without exception that the proximal transcript makes up greater than 99% of the total in larval transformants and that the distal transcript makes up 75–99% of the total in adult transformants. Again, this indicates strong conservation of *trans*-acting components involved in the regulation of Drosophila *Adh* genes.

In larvae, there is a constant relationship between ADH activity and RNA levels (Table 2). However, in adults a particular activity level corresponds to a different transcript level for each of the three foreign genes introduced (Table 3). This points to the complexity inherent in the analysis of multiple genes, each encoding a distinct polypeptide. Many factors contribute to this complexity including the possibilities of differential translatability of three mRNAs in adults, differential stability of the polypeptides in adults, or different catalytic properties of the enzymes in adult extracts. Although much remains to be done, initial analysis of chimeric *Adh* genes indicates that the differences in RNA to activity ratios in adults are not

FIGURE 6.—Histochemical localization of ADH in adult tissues. *D. melanogaster* adults were aged 4–8 days and Hawaiian Drosophila adults were aged 10–17 days posteclosion. Tissues were hand dissected and stained for ADH activity. The structures are indicated by number as follows: 1, crop; 2, cardia; 3, Malpighian tubules; 4, rectum. The midgut extends anteriorly from the cardia and posteriorly to where the hindgut and Malpighian tubules meet. The hindgut extends between the rectum and the Malpighian tubules. Tissues are from the following: (A) *D. melanogaster* Adh^F; ry^{506} carrying the wild-type Adh^F allele; (B) *D. melanogaster*, Adh^{fn6} *cn*; ry^{506} host; (C) *D. melanogaster* transformant carrying the *D. hawaiiensis Adh* gene; (D) *D. hawaiiensis*; (E) *D. melanogaster* transformant carrying the *D. grimshawi Adh* gene; (F) *D. grimshawi*; (G) *D. melanogaster* transformant carrying the *D. affinidisjuncta Adh* gene; (H) *D. affinidisjuncta*. [A, bar = 2 mm (A, B, C, E, G); D bar = 2 mm (D, F, H).]

TABLE 4

Histochemical localization of ADH in larval and adult tissues of species and transformants

Stock		Larval									Adult							
		Gc	PV	AMG	MMG	PMG	HG	MT	CAR	SG	Crop	Cardia	AMG	PMG	AHG	PHG	MT	R
D. melanogaster[a]	(+)	0.22	0	1.00	1.00	1.00	0	0.17	0	0	1.00	1.00	0	0.71	0.93	1.00	0.93	1.00
Adh^{f}; *ry*^{506}	(+/−)	0.78	0.06	0	0	0	0.22	0.83	1.00	0	0	0	0.14	0.29	0.07	0	0.07	0
	(−)	0	0.94	0	0	0	0.78	0	0	1.00	0	0	0.86	0	0	0	0	0
D. affinidisjuncta[a]	(+)	1.00	0.93	1.00	0.86	1.00	0.71	1.00	1.00	0	0.92	0	0.58	0.58	0.17	0.08	0.17	0
	(+/−)	0	0.07	0	0.14	0	0.29	0	0	0	0.08	1.00	0.42	0.42	0.25	0.50	0.75	1.00
	(−)	0	0	0	0	0	0	0	0	1.00	0	0	0	0	0.58	0.42	0.08	0
D. affinidisjuncta[b]	(+)	2	3	4	3	0	0	1	2	2	4	3	3	2	1	1	0	3
transformant	(+/−)	2	1	0	1	4	3	3	2	2	0	1	1	2	3	3	4	1
	(−)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
D. grimshawi[a]	(+)	1.00	0.33	0	0.33	1.00	0	1.00	0	0	1.00	0.135	0	0	0.635	0	0	0
	(+/−)	0	0.67	0.83	0.67	0	0.92	0	1.00	0	0	0.74	0.44	0	0.37	1.00	0.19	1.00
	(−)	0	0	0.17	0	0	0.08	0	0	1.00	0	0.135	0.56	1.00	0	0	0.81	0
D. grimshawi[b]	(+)	1	2	2	2	0	0	0	3	0	4	0	0	0	1	1	0	4
transformant	(+/−)	3	2	2	2	4	1	3	1	1	0	4	1	0	3	3	0	0
	(−)	0	0	0	0	0	3	1	0	3	0	0	3	4	0	0	4	0
D. hawaiiensis[a]	(+)	0	0	0	0	0	0	0	0.90	0	0.42	0.25	0	0	1.00	0	0	0
	(+/−)	1.00	0	0	1.00	0.10	0	0.08	0.10	0.10	0.58	0.67	0	0	0	0.09	0.3	0.45
	(−)	0	1.00	1.00	0	0.90	1.00	0.92	0	0.90	0	0.08	1.00	1.00	0	0.91	0.7	0.55
D. hawaiiensis[b]	(+)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
transformant	(+/−)	0	0	2[c]	3	0	0	0	4	1	4	1	0	0	4	0	0	0
	(−)	4	4	2	1	4	4	4	0	3	0	3	4	4	0	4	4	4

Histochemically detectable activity was rated as (+) for strongly staining, (+/−) for weakly staining and (−) for not detectable. Abbreviations are as follows: GC, gastric caeca; PV, proventriculus; AMG, anterior midgut; MMG, middle midgut; PMG, posterior midgut; HG, hindgut; MT, Malpighian tubules; CAR, carcass; SG, salivary gland; AHG, anterior hindgut; PHG, posterior hindgut; R, rectum.

[a] For *D. melanogaster* and the three donor species, the numbers represent the fraction of individuals showing staining of each intensity. Ten to 20 individuals were analyzed for each species.

[b] For transformants, the figures are the number of independent transformed lines showing the corresponding staining. Four lines were rated for each donor gene.

[c] Staining is limited to a few cells at the junction of the body of the anterior midgut and the gastric caeca.

due solely to the different polypeptides produced (our unpublished observations).

Comparison of the total standing RNA levels and tissue-specific expression pattern for a given gene also sheds light on the underlying circuitry involved in evoluntary alteration of tissue-specific expression patterns. The *D. hawaiiensis* gene produces about a two-fold lower standing level of RNA than do the other two genes in larvae as well the lowest level in adults (Tables 1 and 2). Since 70–95% of the total larval ADH synthesis and at least 90% of the adult ADH synthesis represent gene expression in the corresponding fat bodies, the RNA levels determined for intact organisms primarily reflect fat body synthesis. Thus, lower expression in the fat bodies for the *D. hawaiiensis* gene correlates with limited tissue distribution of ADH production by this gene (Figures 3–6; Tables 2–4). Similarly lower RNA levels in adults carrying the *D. grimshawi* gene correlate with limited tissue distribution of expression for the *D. grimshawi* gene relative to the *D. affinidisjuncta* gene. Superficially, it appears that models invoking one or more *cis*-acting regulatory elements that function to similar extents in the various tissues might account for these observations. Such a model has been proposed for the regulation of the *D. mulleri* Adh-1 gene in larval transformants of *D. melanogaster* (FISCHER and MANIATIS 1988). However, the present case appears to be more complex. For example, Table 3 and Figure 3 clearly demonstrate (despite position effects) that the magnitude of ADH activity differences between the three genes is different for various tissues. Further, total RNA levels for adults reflect almost exclusively the distal transcript, while the major regulatory difference between the *D. grimshawi* gene and *D. affinidisjuncta* gene (the adult midgut) involves only the proximal transcript (ROWAN and DICKINSON 1986; BRENNAN and DICKINSON 1988). For a given gene, then, one or a few sequences that influence expression to similar extents in all tissues may exist, but when one compares homologous genes from related species, differences in the underlying regulatory components for the particular genes are revealed. In order to understand the origins of these regulatory components, we are presently analyzing the behavior of chimeric *Adh* genes introduced into *D. melanogaster*.

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