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Genetics

Genetic basis of the difference in alcohol dehydrogenase expression between *Drosophila melanogaster* and *Drosophila simulans*

(molecular evolution/gene regulation/interspecific variation)

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ABSTRACT **_Drosophila melanogaster_ and its sibling species, _Drosophila simulans_, differ in expression of the enzyme alcohol dehydrogenase (ADH). Adult _melanogaster_ flies that are homozygous for the Slow allozyme have approximately twice the level of ADH activity and crossreacting material as _simulans_ adults. There is no corresponding difference in ADH mRNA, however, so this difference in ADH protein level is evidently due to a difference in the rate of translation of the two RNAs and/or to a difference in protein stability. Here we report an interspecific gene-transfer experiment, using _P_-element transformation, to determine whether this expression difference is due to genetic background differences between the species (trans-acting modifiers) or to cis-acting factors within the _Adh_ gene. When the _Adh_ genes from _D. melanogaster_ and _D. simulans_ are put into the same genetic background, there is no detectable difference in their level of expression. The level is relatively high in the _melanogaster_ background and relatively low in the _simulans_ background. Therefore, the interspecific difference in _Adh_ expression is due entirely to trans-acting modifiers, in spite of the many sequence differences between the _Adh_ genes of the two species, which include two amino acid substitutions.**

Drosophila melanogaster and its sibling species, *Drosophila simulans*, are essentially cosmopolitan in distribution and live in close association with man (1). Both species utilize fermenting fruits in which ethanol concentrations range up to several percent by volume (2, 3), but *D. melanogaster* appears to be more adapted to high alcohol environments than *D. simulans*. In laboratory toxicity tests, *melanogaster* adults consistently show a higher level of tolerance to ethanol and other alcohols than *simulans* adults (4–8). This difference in tolerance may be due to a difference in alcohol dehydrogenase (ADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) expression between the species.

The ADH enzyme of *D. melanogaster* clearly plays an important role in alcohol detoxification and metabolism. Flies homozygous for a null allele of *Adh* are extremely sensitive to the toxic effects of environmental alcohols (9). More than 90% of the ethanol that is metabolized to lipid in larvae goes through a pathway that is dependent on ADH activity (10). Furthermore, genetic variation in ADH activity levels in *melanogaster*, particularly the difference between allozymes, is frequently associated with variation in tolerance to ethanol and other alcohols (for review, see refs. 11 and 12).

The *D. melanogaster Adh* gene produces two different transcripts, which are differentially expressed during development but produce identical proteins (13, 14) (see Fig. 1). We have shown that the timing of usage of the two promoters during development is very similar in *melanogaster* and *simulans* (15). We have also compared the pattern of *Adh* expression throughout development in the two species by measuring ADH activity, ADH-crossreacting material (CRM), and ADH mRNA for several strains of each species, which derive from diverse geographic locations (15). All of the *melanogaster* strains in that study were homozygous for a Slow *Adh* (Adh^S) allele, because sequence analysis clearly shows that the *simulans Adh* gene is much more similar to *melanogaster* Adh^S alleles than to Adh^F alleles (see discussion in ref. 15). The only consistent differences between the species are in pupal RNA level and in adult enzyme activity and CRM level. Adult *melanogaster* flies with the Slow ADH allozyme have approximately twice the level of ADH activity and CRM (per milligram of soluble protein) found in *simulans*. However, there is no significant difference between the species in ADH mRNA level in adults. Therefore, the interspecific difference in ADH level in adults must be due to a difference in the rate of translation of the two RNAs and/or to a difference in protein stability. Either of these mechanisms could operate through genetic background differences between the species (trans-acting modifier genes) or cis-acting factors located within the *Adh* sequences that specify the mature message.

Here we report an interspecific gene-transfer experiment, using *P*-element-mediated transformation, to determine the genetic basis of the difference in ADH levels between adults of the two species. This experiment shows that, in spite of the many sequence differences between the *Adh* genes of *melanogaster* and *simulans*, the difference in adult expression is due entirely to trans-acting modifier genes.

MATERIALS AND METHODS

Fly Stocks. The *D. melanogaster* stock KA27 (Ash^S) was constructed by extracting a second chromosome into an isogenic genetic background (16). The *melanogaster* transformation host strain, Adh^{fn6}; ry^{506}, was constructed by removing the *cn* mutant from the strain Adh^{fn6} *cn*; ry^{506}, which was provided by J. Posakony (University of California at San Diego). The *D. simulans* stock Rall-2 was made isoallelic for the *Adh* region as described by Thomson *et al.* (15). The *simulans* null mutant, $Adh^{nA5\text{-}1}$, was induced by diepoxybutane mutagenesis; it has no detectable ADH-CRM or ADH mRNA. The *simulans* transformation host strain, $Adh^{nA5\text{-}1}$; ry^{i83}, was constructed by combining the $Adh^{nA5\text{-}1}$ mutant with the ry^{i83} mutant provided by D. Hartl (Washington Univer-

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Abbreviations: ADH, alcohol dehydrogenase; CRM, crossreacting material; ANOVA, analysis of variance.

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sity). The stock Wa-s is isogenic for the second chromosome and was obtained from M. Kreitman (Princeton University).

Cloning and Sequencing. Complete *Bgl* II digests of genomic DNA from lines KA27 and Ral1-2 were used to make λEMBL-4 and λEMBL-3 libraries, respectively (17). *Adh*-containing clones were identified by plaque hybridization. The λKA27 clone was provided by C. F. Aquadro (Cornell University). For each of the two types of phage clones, a 2.7-kilobase (kb) *Sal* I–*Cla* I fragment containing the *Adh* transcriptional unit (Fig. 1) was subcloned into a plasmid vector for sequencing. Both strands of the 2.7-kb fragment were sequenced completely by the dideoxy chain-termination method using oligonucleotide primers located every 200 bases along the sequence. The average overlap between sequenced fragments was 60 base pairs (bp). These sequences are stored under GenBank accession numbers M36581 (Ral1-2) and M36580 (KA27). These sequences, as well as the *simulans* sequences of Bodmer and Ashburner (18) and Cohn and Moore (19), were aligned with Kreitman's *melanogaster* consensus sequence (20) by using the GAP program of the University of Wisconsin Genetics Computer Group software package (21).

Plasmid Constructions. The *P*-element plasmids used for transformation contained the following insertions within the defective *P*-element sequence of the plasmid pPLΔ-1 (obtained from J. Posakony): (*i*) an 8.7-kb *Bam*HI–*Cla* I fragment containing the *Adh* transcriptional unit along with 6 kb of 5′ flanking DNA and 0.8 kb of 3′ flanking DNA; (*ii*) an 8.1-kb *Sal* I fragment containing a wild-type *ry* gene from cDM2837 (22). Four types of *P*-element plasmids were constructed that differed in the constitution of the *Adh* fragment. As shown in Fig. 1, two of the plasmids (MM and SS) contained the original *Bam*HI–*Cla* I fragment from either *melanogaster* (KA27) or *simulans* (Ral1-2). The other two plasmids (SM and MS) contained chimeric *Adh* fragments in which the 5′ flanking region and the coding regions from the two species were switched at a *Hpa* I restriction site located in the first intron of the distal transcript.

***P*-Element Transformation.** Microinjection of embryos was performed essentially as described by Goldberg *et al.* (23). Embryos from the host stock were injected with 5 mM KCl/0.1 M sodium phosphate, pH 6.8, containing the wings-clipped helper *P*-element plasmid pπ25.7wc (24) at 150 μg/ml and one of the plasmids MM, MS, SM or SS at 600 μg/ml.

Construction and Analysis of Transformant Lines. For *melanogaster* transformants, isochromosomal lines were established using balancer chromosomes (25). For *simulans* transformants, single G_1 ry^+ males were backcrossed to the host strain to establish a line. The transformant lines of both species were analyzed by Southern blotting (as in ref. 25) to determine the number and location of insertions. Each line analyzed for *Adh* expression contains a single insertion at a unique location. The *melanogaster* Slow ADH and *simulans* ADH proteins differ in electrophoretic mobility and this difference was used to verify that each of the transformant lines expressed an ADH of the appropriate mobility.

Experimental Design. *Adh* expression was analyzed in two separate experiments. One experiment analyzed adult males from KA27, RAl1-2, and 49 *melanogaster* transformant lines with autosomal insertions. The other experiment analyzed both males and females from KA27, Ral1-2, and 7 *simulans* transformant lines (4 autosomal and 3 X chromosome-linked). In both experiments, each line was crossed to either the *melanogaster* host strain (Adh^{fn6}; ry^{506}) or the *simulans* host strain ($Adh^{nA5\text{-}1}$; ry^{i83}) and, in the case of transformant lines, ry^+ progeny were selected for analysis. Progeny were aged 7–9 days posteclosion and homogenized in sets of 10. The crosses were set up in a randomized block design with two blocks and two replicates per block per line.

Protein Assays. ADH activity, ADH-CRM, and total protein were assayed as described (15). ADH activity units are nanomoles of NAD^+ reduced per minute per milligram of total protein. ADH-CRM was estimated by radial immunodiffusion. Each diffusion plate contained a dilution series of a standard extract of Hochi-R flies to ensure linearity and to provide a standard for comparison among plates. ADH-CRM units are given in terms of Hochi-R fly equivalents per milligram of total protein.

RNase Protection Assay. RNA was prepared essentially as described by Fischer and Maniatis (26) except that no proteinase K was used. RNA transcription, hybridization, digestion, and acrylamide gel analysis were performed by the method of Melton *et al.* (27) with minor modifications.

Polymerase Chain Reaction. Amplification of genomic DNA from KA27 for sequencing was performed as described by Higuchi and Ochman (28).

RESULTS

Sequence and Expression Analysis of the KA27 and Ral1-2 Alleles. One *Adh* allele from each species was cloned and used in a *P*-element-mediated gene-transfer experiment. Previous analyses of the isoallelic lines from which these clones were derived (KA27 ad Ral1-2) established that each allele has a level of *Adh* expression typical of its species. The DNA sequence of each allele was obtained to verify that KA27 codes for a typical *melanogaster* Slow ADH protein and that Ral1-2 codes for a typical *simulans* ADH, which differs from Slow by two amino acid substitutions.

The KA27 allele was completely sequenced from a *Sal* I site at −63 from the distal transcript start site to a *Cla* I site located about 0.8 kb downstream of the polyadenylylation site (Fig. 1). It codes for an ADH protein that has an amino acid sequence identical to those encoded by the six Slow *Adh*

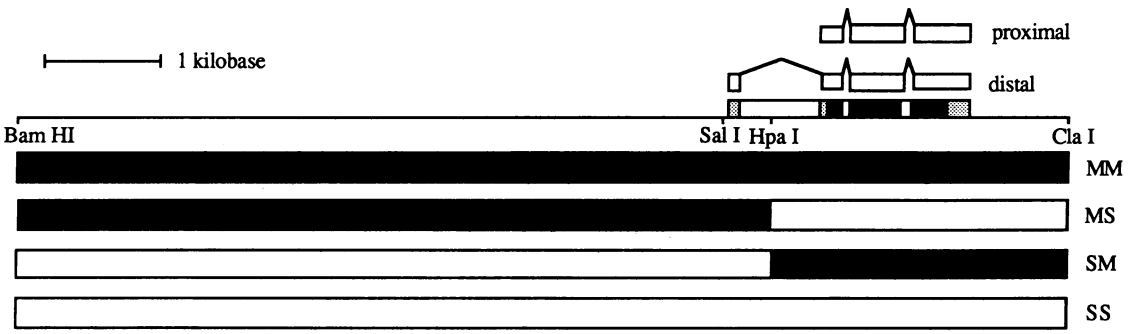

FIG. 1. The 8.7-kb *Adh*-containing DNA fragment used in the transformation experiments. The distal and proximal transcripts are mapped above the *Adh* gene, which has coding regions solid, introns open, and untranslated regions stippled. The constitution of the four types of fragments (MM, MS, SM, and SS) is shown. The first letter refers to the source of the upstream region and the second letter refers to the source of the coding and downstream region (M for *melanogaster* and S for *simulans*).

alleles sequenced by Kreitman (20). It differs from all six of Kreitman's Slow alleles by four base substitutions within the sequences that encode the two mature messages, by a 41-base deletion in the 5′ distal transcript leader, and by three base substitutions and a 2-base insertion within the first intron of the distal transcript.

The Rall-2 allele was also sequenced from the −63 *Sal* I site to the 3′ *Cla* I site. It codes for an ADH protein identical to those encoded by two previously sequenced *simulans* alleles (18, 19). The Rall-2 allele differs from both of the other *simulans* sequences by six intronic base substitutions and four substitutions within the sequences that encode the mature messages.

The *simulans* ADH protein differs from the *melanogaster* Slow ADH by two amino acids. The *simulans* sequence has alanine rather than serine at amino acid residue 1 and lysine rather than glutamine at residue 82. In addition to the two amino acid replacement differences between the KA27 and Rall-2 alleles, the sequences that encode the mature messages contain 19 substitutions and 2 insertion/deletion differences. The introns contain 28 substitutions and 8 insertion/deletion differences. When the flanking regions are included, there are 82 substitutions and 16 insertion/deletion differences over the 2658 aligned bases. The sequence alignments are available upon request.

Thomson *et al.* (15) measured ADH activity and CRM in adult males of KA27, Rall-2, and four isofemale lines of each species that derive from four different geographic continents. All of the *melanogaster* lines were homozygous for the Slow allozyme. Both KA27 and Rall-2 have ADH activity and CRM levels that are similar to the average and fall within the range of the four isofemale lines of their respective species. In addition, Aquadro *et al.* (29) analyzed the ADH activity levels of 32 isogenic *melanogaster* lines with the Slow allozyme, one of which was KA27. In that study, the activity of KA27 (3.13 units per fly) was also similar to the mean (3.48) and fell well within the range of activities of the other lines (2.14–5.25; exclusive of one high-activity outlier). Therefore, the KA27 and Rall-2 alleles can be considered representative of their respective species in terms of *Adh* expression.

It is notable that the 41-bp deletion (bases 32–72) within the 5′ distal leader sequence of the KA27 allele has no apparent effect on *Adh* expression. Two procedures were employed to verify that the KA27 flies used in the experiments reported here and by Thomson *et al.* (15) actually had the leader deletion in their *Adh* gene and transcript. First, the polymerase chain reaction was used to amplify *Adh* DNA from KA27 flies. When this DNA was sequenced through the distal leader, the predicted 41-bp deletion was found. Second, an RNase protection assay was used to verify that the *Adh* transcript in adults actually has the predicted deletion. An RNA probe extending from the *Sal* I site at −63 to a *Bam*HI site at +1257 was used to protect RNA isolated from flies from the KA27 line, from an MM transformant line (see below), and from Wa-s (one of Kreitman's Slow lines, which does not have the deletion). As expected, the KA27 and MM samples showed the presence of a 31-base fragment corresponding to the beginning of the distal leader region (5′ of the deletion), which was absent from the Wa-s sample (data not shown). Also, KA27 showed the absence of an 87-base fragment corresponding to the entire distal leader region, which was present in the Wa-s sample. Therefore, it is clear that the 41-bp distal leader deletion has no major effects on *Adh* expression.

***P*-Element Transformation.** Four different types of *P*-element transposons were constructed that differed in the constitution of the *Adh*-containing fragment (Fig. 1). The MM and SS transposons contained a *Bam*HI–*Cla* I fragment from the original KA27 and Rall-2 clones, respectively. The MS and SM transposons contained chimeric fragments in which the 5′ flanking and coding regions of the MM and SS fragments were switched. Multiple independent insertions of each of these four transposon types were obtained by injection of the *melanogaster* host strain. The numbers of independent insertions analyzed were 12 each for MM, MS, and SS and 13 for SM.

Transformation of the *simulans* host by microinjection was very difficult. The problem was due partly to low fertility and partly to a low transformation efficiency. Injected individuals were so infertile that surviving adults were backcrossed to the host strain in groups of five per vial so that larval densities would be sufficiently high to give a good culture. The number of such vials that gave rise to one or more transformed offspring (ry^+) was 7 out of a total of 980 potential G_0 parents (0.71%). Nearly all vials produced some progeny, so the minimum number of fertile G_0 parents is 196. Thus, a maximum estimate of the transformation efficiency is 3.6% (7/196). Both of these transformation-efficiency estimates for *simulans* are much lower than the estimate for *melanogaster*, which is 17.6% (45 out of 255 fertile G_0 adults). The low transformation efficiency of *simulans* might be simply a strain-specific effect or it might be due to an interspecific difference in host factors required for *P*-element transposition.

Because of the low transformation efficiency of the *simulans* host, we injected only the MM and SS transposons. For MM, we obtained two X-linked and two autosomal insertions, and for SS, we obtained one X-linked and two autosomal insertions. Analyses of variance (ANOVAs) were performed on ADH activity and CRM level (for each sex separately) to test for a difference between the X-linked and autosomal insertions. None of the *F* tests were significant, so chromosomal location was ignored in subsequent analyses.

***Adh* Expression in Transformant Lines.** Fig. 2 shows the average ADH activity and CRM levels (per milligram of soluble protein) in males for 49 *melanogaster* transformant lines with autosomal insertions. There is clearly a considerable amount of overlap among the four transposon classes for both variables. The average ADH activities for the four classes are shown in Fig. 3, along with the values for the original *melanogaster* and *simulans* lines from which the *Adh* clones were derived. The original lines show the typical 2-fold difference in activity that is characteristic of these species (15), and this difference is highly significant ($P < 0.005$). However, the four transformant classes have very similar means and the ANOVA of these data does not show a significant effect of transposon type. The mean values of the

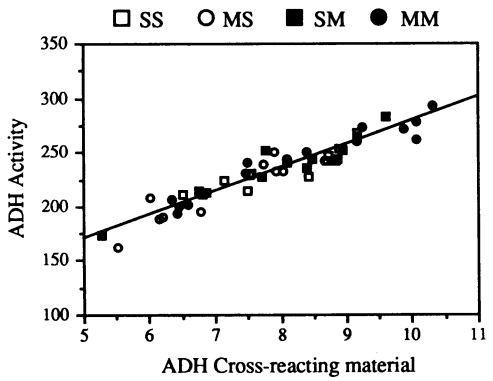

FIG. 2. Average ADH activity versus average CRM level for 49 *melanogaster* transformants. Units are nanomoles of NAD^+ reduced per minute per milligram of total protein for activity and Hochi-R fly equivalents per milligram of total protein for CRM (see *Materials and Methods*). Each point is the average of four observations. The regression of activity on CRM has a coefficient of determination (r^2) of 0.89.

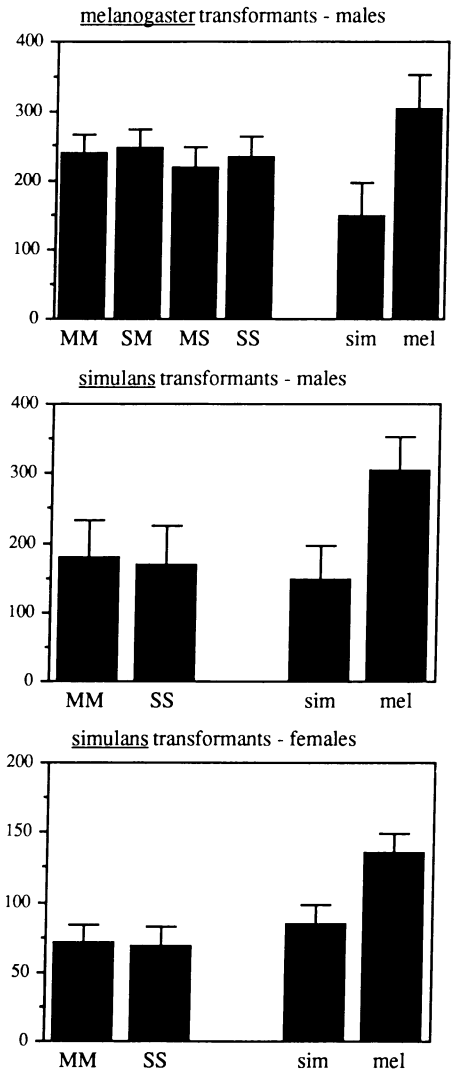

FIG. 3. Average ADH activity levels for each class of transformants. Units are nanomoles of NAD^+ reduced per minute per milligram of total protein. The averages are based on the following numbers of lines: *melanogaster* transformants, 12 each for MM, MS, and SS and 13 for SM; *simulans* transformants, 4 for MM and 3 for SS. The error bars for the *melanogaster* transformants represent Tukey's minimum significant difference (MSD) at the 5% level. All other error bars are the least significant difference (LSD) at the 5% level. These MSD and LSD values were calculated using mean squares from the ANOVAs, which were performed separately for the *melanogaster* transformants, the *simulans* transformants, and the original lines (KA27 and Ral1-2). The lines KA27 and Ral1-2 are designated mel and sim, respectively.

transformants are relatively high, but not as high as the value of the KA27 *melanogaster* line. This difference is probably due to the fact that the *melanogaster* host strain has a different genetic background than the KA27 line. The results for CRM level are virtually identical to the activity results (data not shown).

Because some of the *simulans* transformants have X-linked insertions, both males and females were analyzed. Fig. 3 shows the average ADH activity values for the transformants in comparison with the original lines. Again, the original lines show a highly significant difference for both sexes ($P < 0.005$), but the two classes of *simulans* transformants show very similar means and the ANOVAs of these two data sets show no significant effect of transposon type. The mean values of the transformants are relatively low and very similar to that of the *simulans* Ral1-2 line. The results for CRM level are virtually identical to the activity results (data not shown).

The transformation experiments for both species gave complementary and clearcut results. When the *Adh* genes from *melanogaster* and *simulans* are put into the same genetic background, there is no detectable difference in their levels of expression. Expression is relatively high when both genes are in the *melanogaster* genetic background, and it is relatively low when they are in the *simulans* genetic background. These results show that the differences in ADH activity and CRM levels between the species are due to trans-acting modifier genes that map outside of the DNA fragment used in the transformation experiment.

It is notable that the two amino acid differences between the *melanogaster* Slow and the *simulans* ADH proteins have no apparent effect on ADH activity, as measured under saturating substrate conditions. Of course, these substitutions may affect enzymatic function in some way that does not alter activity at saturating substrate concentration. In fact, the work of Heinstra *et al.* (30) suggests that certain catalytic properties of the *melanogaster* Slow ADH and the *simulans* ADH differ, and these properties may affect alcohol metabolism *in vivo* (31).

The relationship between ADH activity and CRM levels in *melanogaster* transformants is shown in Fig. 2. The regression of activity on CRM is homogeneous among the four classes, so the figure shows a single regression line calculated from the pooled data. If the *melanogaster* Slow ADH and the *simulans* ADH had different catalytic efficiencies, the regressions for transformant lines differing by these two structural forms would have different slopes. These results support our earlier conclusion that the activity difference between the species is due mostly, if not entirely, to a difference in concentration of the ADH protein (15).

DISCUSSION

The results presented here clearly show that the difference in *Adh* expression between *melanogaster* and *simulans* adults is due to one or more trans-acting modifier genes that map outside of the 8.7-kb DNA fragment used in the transformation experiment. These modifier genes evidently affect either the rate of translation of the ADH mRNA and/or the rate of degradation of the ADH protein, since the interspecific difference in ADH activity is due to a difference in concentration of the ADH protein (CRM), but there is no corresponding difference in ADH mRNA level. There is no evidence that cis-acting factors play a role in this expression difference, in spite of the many sequence differences between the species in the *Adh* gene and its flanking DNA.

Dickinson *et al.* (32) analyzed *Adh* expression in hybrids between *melanogaster* and *simulans* to determine the relative role of cis- and trans-acting factors. In their experiment, hybrids were made between a *melanogaster* Adh^F strain and a *simulans* strain, which have different ADH electrophoretic mobilities. Gel scanning was used to compare activities in the parental stocks with the activities of the two homodimers in the hybrid (using ethanol as substrate). The *melanogaster*/*simulans* parental ratio was about 4 and the hybrid ratio was about 2. They concluded that both cis- and trans-acting factors were involved in the interspecific activity difference. Actually, the cis-acting component they observed is associated with the allozyme polymorphism within *melanogaster*. Fast alleles produce a 2-fold higher ADH activity on ethanol than Slow alleles, and this difference is due to a cis-acting factor very closely linked to or identical with the amino acid substitution that causes the electrophoretic mobility difference (25, 29). If all of the activity difference between the *melanogaster* Slow ADH and the *simulans* ADH is due to trans-acting modifiers, then one would expect the ratio of

Fast to *simulans* homodimers in hybrids to be about 2, just as the Fast/Slow ratio is about 2. Therefore, the results of Dickinson *et al.* (32) are consistent with our conclusion that trans-acting factors cause the difference in ADH levels between *melanogaster* Slow homozygotes and *simulans* strains.

It is not surprising that trans-acting factors play an important role in causing an interspecific difference in ADH activity, since such factors are commonly segregating in natural populations of *melanogaster* and contribute significantly to the total activity variation. We analyzed ADH activity variation among 50 second- and 50 third-chromosome substitution lines that have isogenic genetic backgrounds (16). Except for one high-activity outlier, the range of variation among the 31 second-chromosome lines having the Slow allozyme was 2.5-fold, while the range of variation among the third-chromosome lines was 2.0-fold. Thus, third-chromosome modifers contribute nearly as much variation as second-chromosome genes, which include both modifiers and the *Adh* gene itself.

The specificity of the third-chromosome modifiers segregating within *melanogaster* populations was investigated by estimating genetic correlations between ADH and 22 other enzymes (33). The correlations were significant in 12 of the 22 cases, which indicates that the effects are not highly specific, although they are clearly not simply due to variations in body weight or the total amount of soluble protein per fly. The specificity of the modifier-gene effects that differ between *melanogaster* and *simulans* is unknown, since we have not analyzed other enzymes in *simulans*. However, the interspecific difference in ADH level is also clearly not due to a difference in total soluble protein, since ANOVAs of activity and CRM per milligram of soluble protein show a highly significant species effect (15).

Other interspecific differences in *Adh* expression have been analyzed by *P*-element transformation in *Drosophila*. Brennan *et al.* (34, 35) introduced the *Adh* genes from two Hawaiian *Drosophila* species into the germ line of *melanogaster* and found that most of the differences in tissue specificity were due to cis-acting factors, although some minor differences in tissue staining seemed to be influenced by trans-acting factors. Similarly, Fischer and Maniatis (26) introduced the duplicate *Adh* genes from *Drosophila mulleri* into the *melanogaster* germ line and found that most of the species differences in tissue and temporal specificity were due to cis-acting factors, although some quantitative differences in the relative usage of the *Adh-1* and *Adh-2* promoters appeared to be controlled by trans-acting factors. These results suggest that interspecific differences involving qualitative switches in tissue or temporal specificity may generally be controlled by cis-acting regulatory elements, whereas quantitative modulation of expression may be more often influenced by trans-acting factors.

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