Post-Translational Control of Alcohol Dehydrogenase Levels in Drosophila melanogaster

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ABSTRACT

A trans-acting regulatory gene that alters in vivo protein levels of alcohol dehydrogenase (ADH) has been mapped to a region of the third chromosome of Drosophila melanogaster. The gene has been found to affect the in vivo stability of ADH protein. It was not found to alter levels of total protein of two other enzymes assayed. The action of the gene over development and its possible mode of control are discussed.

O NE of the central concerns of developmental genetics is how the cell regulates gene expression. In recent years there has been a considerable advance toward understanding the regulatory processes occurring in higher eukaryotes. Several genetic elements that influence the timing and/or level of expression of various structural genes have been localized and characterized (Pajen 1979). While in most instances these are cis-acting elements (Schibler et al. 1983; Palmer et al. 1983; Fodler, Burnett and Kaplan 1983; Lusis et al. 1983; Maroni and Laurie-Ahlberg 1983; Bewley 1981; Chovnick 1976), there have been reports of trans-acting regulatory elements in mice (Kozak 1985), maize (Sandalias et al. 1980) and Drosophila (Abraham and Doane 1978; King and McDonald 1983).

Due to difficulties in isolating suitable genetic material for the study of gene regulation in eukaryotes by classical mutagenesis (Sherman et al. 1981; Donahue, Farabaugh and Fink 1982), naturally occurring variants have been utilized in recent studies (e.g., Estelle and Hodgetts 1984). Natural populations of Drosophila melanogaster have been shown to contain high degrees of regulatory gene variation for a number of enzyme loci including alcohol dehydrogenase (Adh) (McDonald and Ayala 1978a), α-glycerophosphate dehydrogenase (Gpdh) (Wilson and McDonald 1980), and seven other enzyme loci (Laurie-Ahlberg et al. 1980). In the aforementioned studies, detection of regulatory variation relies on quantitative enzyme assay comparisons between genetic lines constructed from natural populations. Quantitative differences between lines are attributed to the genetic difference constructed between lines (most often a chromosome). Quantitative effects attributed to genetic elements unlinked to the structural loci they influence are referred to as trans-acting. In a few instances it is known that the quantity of enzyme is altered (King and McDonald 1983; Maroni and Laurie-Ahlberg 1983; McDonald and Ayala 1978a) but in most cases the genetics and biochemistry of these trans-acting effects are unknown.

The Adh locus (2-50.1) codes for a soluble Drosophila alcohol dehydrogenase (DADH), an enzyme believed to play a key role in the detoxification of ethanol and other alcohols (David et al. 1976). The structural gene has been cloned (Goldberg 1980) and the DNA sequence of several variants is known (Kreitman 1983). In addition to molecular and biochemical aspects, Adh has been the subject of many developmental (e.g., Anderson and McDonald 1981) and population (e.g., McDonald et al. 1977; Cavener and Clegg 1978; Oakeshott et al. 1982) genetic studies as well. We report here on the genetic localization and biochemical characterization of a trans-acting regulatory element which alters the expression of Adh in D. melanogaster.

MATERIALS AND METHODS

Drosophila strains: A wild-type strain (S2) made completely homozygous for the first, second and third chromosomes was derived from a single male collected from a Napa County, California, population (McDonald, Anderson and Santos 1980). A substituted strain (S2, MM3) was constructed to have the same second (and thus the same Adh genotype) and X chromosome constitution as the wild-type strain but homozygous for the multiply marked third chromosome carrying the markers roughoid (ru, 3-0.0), hairy (h, 3-26.5), thread (th, 3-43.2), scarlet (st, 3-44.0), curled (cu, 3-50.0), stripe (sr, 3-62.0), ebony-soozy (e′, 3-70.7) and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7). Our method of substituted strain construction utilizes the balancer stock Cy/Bl², Sb Ser/e¹ and claret (ca, 3-100.7).

Enzyme activity assay: DADH, α-glycerophosphate dehydrogenase (GPDH), and phosphoglucone isomerase (PGI) activities were measured spectrophotometrically according to a modification of the procedures of McDonald and

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AVISE (1976). Whole fly extracts of ten adults (4–8 days posteclosion) were homogenized in 0.5 ml of 100 mM Tris-HCl (pH 8.6) with 8 mM EDTA added for GPDH assays. All homogenates were centrifuged at 5000 x g at 12,000 x g for 20 min, and the supernate was recovered for enzymatic analysis. Activity was measured on Beckman Du-8 spectrophotometer using 10 μl of crude extract in 990 μl of the following reaction mixture; 5% isopropanol (DADH) or 90 mM DL-GPDH in 100 mM Tris-HCl (pH 8.6) and 1 mM NAD+. PGI activity was measured according to the previously published techniques of AVISE and MCDONALD (1976). All flies examined in this study were determined to be insignificantly different in weight. Enzyme activities are expressed as Δ OD/min x 10² ± standard error.

Electrophoresis: Polyacrylamide gel electrophoresis was carried out according to SMITH (1968). Gels were stained specifically for GPDH or DADH as described by AYALA et al. (1972).

Gel sieving: Polyacrylamide gel sieving was performed as described by JOHNSON (1975) using bovine hemoglobin as a standard.

DADH antiserum: DADH antiserum was prepared in New Zealand white rabbits against DADH purified to greater than 95% homogeneity (MCDONALD et al. 1977). Specificity of the antiserum was verified radioimmunologically (ANDERSON and MCDONALD 1983). Goat anti-rabbit serum was purchased from Research Products International (Elk Grove Village, Illinois).

DADH cross-reacting material determinations: Immunodiffusion gels were run according to MANCINI, CARBONARA and HEREMANS (1965), placing 10 μl of sample in 4-mm wells formed in 1% agarose containing 1% DADH antiserum gels. After 2 days, the gels were stained for DADH activity and the ring diameters measured. Serial dilutions of one sample were used as standards for comparison (FAHEY and MCKELVEY 1965).

In vivo estimates of relative DADH synthesis rates (k): The rate of incorporation of [3H]-labeled amino acids into DADH was used as a measure of the relative rate of the protein’s synthesis. Adult male Drosophila were fasted for 4 hr at 25° under conditions of low humidity (25%) and then placed in shell vials containing filter paper tabs (Whatman No. 1) that were saturated with [3H]-labeled amino acids (25 μCi/mmol, New England Nuclear) and 3% sucrose in distilled H2O (250 μl). At 1–2 hr intervals, flies were removed from the label and homogenized in 100 mM Tris-HCl (pH 8.6) (45 mg of net weight adult males/ml buffer). The homogenate was centrifuged (10 min in Eppendorf microfuge) and the supernate was recovered and divided into four 20-μl replicate aliquots to which 20 μl of DADH antiserum was added. After 10–20 hr of incubation (5°), 45 μl of goat anti-rabbit IgG were added and incubated for an additional 1.5 hr (5°) to aid in the precipitation of the antigen-antibody complex. After centrifugation, the pellet was resuspended in buffer and repelleted three times in order to wash away trapped, nonspecific counts. The final pellet was dissolved in scintillation fluid (BRAY 1960) and radioactivity was measured in a scintillation spectrophotometer. Relative synthesis rate was determined by fitting the data points (counts per minute x 10⁻¹⁴ vs. time in hours) to a line by least squares regression analysis (correlation coefficient >0.95) and calculating the slope (k). Eight replicates were taken at each of six time points over a period of 8 hr. Since the half-life of DADH has been previously measured to be approximately 50 hr (ANDERSON and MCDONALD 1981), enzyme degradation should not interfere with this measurement.

Rate of incorporation into total protein was determined analogously on the same homogenates by trapping trichlo-roacetic acid (TCA) precipitable counts (5% TCA at 4° for 30 min) on Millipore filters (pore size 0.45 μm). Filters were placed in scintillation fluid of 5 ml of a toluene fluor [16 g (New England Nuclear) Omni-fluor per 3.8 liters of toluene] before counting. In this study eight replicate homogenates at each of six time points were taken.

RESULTS

A gene that alters DADH activity maps to a specific region (26.5–43.2) on chromosome III: To identify and localize naturally occurring Adh regulatory variation, a series (three) of third chromosomes were extracted from a natural population (McDonald Ranch, Napa County, California) and combined with a single wild second chromosome (designated S2; see McDO-NALD, ANDERSON and SANTOS (1980)). The relative DADH activities of these strains were determined and contrasted to a strain having the same second (S2) and X chromosome as the previously described stocks but homozygous for the recessively marked third chromosome MM3 (see MATERIALS AND METHODS). DADH activity measurements of the wild third chromosomes, revealed that one designated +3 was associated with significantly lower (P < 0.001, Student’s t-test) DADH activity than MM3. Moreover, the higher activity associated with MM3 was found to be dominant to the +3-associated activity (S2, +3 = 1.43; S2, MM3/+3 = 1.48; S2, MM3 = 1.40 relative units). The genetic element(s) residing on the third chromosome which influence DADH activity are not located within the known Adh structural gene, since the gene coding for DADH is located on the second chromosome. This effect on Adh expression will be referred to as regulatory by virtue of the fact that its effect on Adh expression is due to element(s) residing outside the structural gene coding for DADH. Use of the term regulatory in this context does not imply any knowledge of the mechanism by which these element(s) may influence DADH activity.

To localize the third chromosome gene or genes responsible for the third chromosome regulatory effect, a backcross was made between the S2, MM3/+3 heterozygote and the S2, MM3 marker stock, and recombinant progeny isolated and scored for visible phenotype (Figure 1). Individual recombinant males were then crossed to a strain designated S2, Sb Ser/+3 which is identical to the S2, +3 stock except it contains the balancer chromosome Sb Ser (see MATERIALS AND METHODS). Individual male progeny were subsequently backcrossed to the S2, Sb Ser/+3 stock and their progeny were inter-crossed to isolate the recombinant chromosome as illustrated in Figure 2. The isolation of each individual chromosome is necessary because the regulatory effect associated with the multiply marked (MM3) chromosome is dominant to the wild-type (+3). In all, over 40 recombination events were recovered in this manner.
Post-Translational Control

Localization of the regulatory effect could then proceed in a stepwise fashion. DADH activity assays of the recombinant line $S_2$, $ru\ h$ were found to be insignificantly different from the $S_2$, $+3$ line. Based on this result, we concluded that the regulatory effect is not associated with the left end of the third chromosome ($0$–$26.5$, cf. Figure 3). In a similar fashion, the region from marker $st$ to $ca$ was also eliminated ($44.0$–$100.0$). These results localize the regulatory effect between $h$ and $st$. In addition to eliminating the regions above, the assay of recombinant lines containing the $h$ to $th$ region always contained activity levels significantly higher than the $S_2$, $+3$ line (Table 1). One recombinant line (not shown) containing only the $h$ marker contained an DADH activity significantly higher than the $S_2$, $+3$ strain, which indicates that the regulatory effect is contained between the $h$ and $th$ markers ($26.5$–$43.2$). Henceforth, the region $26.5$–$43.2$ on the third chromosome shall be referred to as the regulatory region $26-43$.

DADH activity differences associated with the $R^{26-43}$ region is accounted for by differences in in vivo levels of DADH cross-reacting material: To explore the biochemical/molecular basis of the localized regulatory effect, we utilized a recombinant line which contained only the $h$ and $th$ markers and as such only differs from the $S_2$, $+3$ line by this small region of the third chromosome (approximately $3$ map units). For convenience this strain will be referred to as $S_2$, $R$ and the $S_2$, $+3$ line will analogously be designated $S_2$, $r$ in order to call attention to the fact that the strains differ only in the $R^{26-43}$ region.

The DADH activity associated with the $S_2$, $R$ line

<table>
<thead>
<tr>
<th>Visible genotypes</th>
<th>Relative DADH activity</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+.+.+.+$</td>
<td>1.00</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>$ru\ .\ cu\ .\ ca$</td>
<td>1.40</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>$ru\ .\ h$</td>
<td>0.88</td>
<td>NS</td>
</tr>
<tr>
<td>$h\ .\ th$</td>
<td>1.48</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>$th\ .\ st$</td>
<td>1.40</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>$st\ .\ cu$</td>
<td>1.12</td>
<td>NS</td>
</tr>
<tr>
<td>$cu\ .\ sr$</td>
<td>0.94</td>
<td>NS</td>
</tr>
<tr>
<td>$sr\ .\ e'$</td>
<td>0.89</td>
<td>NS</td>
</tr>
<tr>
<td>$e'\ .\ ca$</td>
<td>1.17</td>
<td>NS</td>
</tr>
</tbody>
</table>

The DADH activity associated with the $S_2$, $R$ line...
and the gels stained for DADH activity. The activity of polyacrylamide gel electrophoresis of crude extracts of the DADH forms were then spectrophoto-

noassayed cross-reactive material number of DADH molecules as estimated by immu-

metrically scanned. The distribution of DADH activity among the forms agrees with that reported by

accounts for the increased DADH activity of the original S2, MM3 line and is due to an increase in the number of DADH molecules as estimated by immunoassayed cross-reactive material (CRM) (Table 2).

**Regulatory effect is not ubiquitous**: It is possible that the R<sup>26-43</sup> region either directly or indirectly influences gene products other than DADH. For this reason activity assays were conducted on two other abundant Drosophila enzymes, GPDH and PGI. Like DADH, both of these enzymes are encoded by structural genes located on the second chromosome (Gpdh, 17.8; Pgi, 58.6) (O'BRIEN 1980). Comparison of S2, R and S2, r with regard to the activities of these enzymes indicates that neither GPDH or PGI activity is affected by the R<sup>26-43</sup> region. The regulatory effect is thus not ubiquitous.

**Regulatory effect is consistent over development**: The DADH activity of third instar larvae was compared between S2, R and S2, r and the difference was similar to that observed in adults (Table 3). This indicates that the action of the regulatory effect is consistent over development. There is no evidence to indicate that this region has temporal effects on Adh or the expression of any other gene.

**Regulatory effect does not alter the distribution of products of known post-translational modification of DADH**: Electrophoretically, the DADH enzyme appears in three forms designated DADH-5, DADH-3, and DADH-1 which are the result of differential NAD-carbonyl complex formations (EVERSE et al. 1971). To test the hypothesis that R<sup>26-43</sup> alters the distribution of these forms, they were separated by polyacrylamide gel electrophoresis of crude extracts and the gels stained for DADH activity. The activity bands of the DADH forms were then spectrophotometrically scanned. The distribution of DADH activity among the forms agrees with that reported by

<table>
<thead>
<tr>
<th>Strain</th>
<th>Adult activity</th>
<th>CRM</th>
<th>Relative activity</th>
<th>Relative CRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2, R</td>
<td>19.3 ± 0.43</td>
<td>1.40 ± 0.08</td>
<td>1.41**</td>
<td>1.41**</td>
</tr>
<tr>
<td>S2, r</td>
<td>13.7 ± 0.36</td>
<td>0.99 ± 0.05</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**P < 0.001 level of significance.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Larval activity</th>
<th>Relative activity</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2, R</td>
<td>46.65 ± 2.68</td>
<td>1.37</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>S2, r</td>
<td>54.15 ± 0.88</td>
<td>1.00</td>
<td></td>
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* Statistical significance based on Student's t-test comparison (d.f. = 18).

**Regulatory effect is not due to differences in DADH synthesis rates**: The results demonstrate

<table>
<thead>
<tr>
<th>Strain</th>
<th>K&lt;sup&gt;+&lt;/sup&gt;</th>
<th>K&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Relative CRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2, R</td>
<td>0.020</td>
<td>0.014</td>
<td>1.41</td>
</tr>
<tr>
<td>S2, r</td>
<td>0.019</td>
<td>0.019</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*<sup>+</sup>K<sub>p</sub> is the rate of 14C incorporation into DADH protein.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percent Activity ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2, R</td>
<td>DADH-5 70.41 ± 5.39</td>
</tr>
<tr>
<td></td>
<td>DADH-3 29.27 ± 5.55</td>
</tr>
<tr>
<td>S2, r</td>
<td>DADH-5 67.12 ± 4.29</td>
</tr>
<tr>
<td></td>
<td>DADH-3 32.88 ± 4.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>% T</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2, R</td>
<td>-1.505 ± 0.035</td>
<td></td>
</tr>
<tr>
<td>S2, r</td>
<td>-1.505 ± 0.035</td>
<td></td>
</tr>
</tbody>
</table>

**In vivo K<sub>p</sub>, K<sub>-</sub>, and relative CRM levels of the two strains (S2, R and S2, r) **

**Figure 4**: Ferguson plot of sequential gel electrophoresis, comparing the two strains (S2, R and S2, r). ANGELERSON and MCDONALD (1981) with DADH-5 being the predominant form and DADH-1 being negligible. This distribution did not vary significantly between the strains (Table 4).**

**Action of the R<sup>26-43</sup> region on DADH activity does not involve detectable changes in protein conformation**: Evidence has been presented that naturally occurring gene variants exist that can alter the conformation and/or charge of specific Drosophila proteins (e.g., JOHNSTON, FINNERTY and HARTL, 1981). Although we were unable to detect evidence of such R<sup>26-43</sup> mediated-DADH modification via standard 7% polyacrylamide gel electrophoresis, we explored the question by subjecting S2, R and S2, r extracts to the gel sieving techniques of JOHNSTON (1975). The results presented in Figure 4 indicate no significant effect of the regulatory region (R<sup>26-43</sup>) on DADH net charge (M<sub>0</sub>) or conformation (K<sub>p</sub>); hence we detect no qualitative alterations of DADH enzyme.

**R<sup>26-43</sup> regulatory effect is not due to differences**: In DADH net charge (M<sub>0</sub>) or conformation (K<sub>p</sub>); hence we detect no qualitative alterations of DADH enzyme.
that the R26-43 region is responsible for an approximately 40% difference in the steady-state level of DADH protein on a whole fly basis. These differences may be the result of differential synthesis and/or degradation of DADH. Radioimmunological comparisons of rate of 3H-labeled amino acid incorporation into DADH was used to estimate the relative DADH synthesis rates (k_s) for S2, R and S2, r (see MATERIALS AND METHODS). The results in Figure 5 indicate that there is no difference in rate of incorporation for the two strains. We conclude that the steady state level differences in DADH are not due to differential rates of protein synthesis.

DISCUSSION

It has long been known that a great deal of regulatory variation exists in natural populations of D. melanogaster (McDonald and Ayala 1978a; Laurie-Ahlberg et al. 1980; Wilson and McDonald 1981). Only recently have studies been conducted to determine the nature of this regulatory variation. This regulatory variation can be divided into cis- and trans-acting elements. There are several examples of cis-acting regulatory effects isolated from natural populations of Drosophila. For example, DNA sequences located 5' of the dopa decarboxylase locus have been shown to regulate levels of this enzyme by altering mRNA levels (Estelle and Hodgeotts 1984a,b). Similarly a gene controlling temporal expression of GPDH levels (Bewley 1982) likely controls the synthesis of this enzyme (Bewley and Laurie-Ahlberg 1984). In addition, it has been shown that differential levels of DADH protein commonly associated with the naturally occurring DADH-F and DADH-S allozymes are, at least in part, the result of differential mRNA levels (Anderson and McDonald 1983).

Examples of trans-acting regulatory elements in natural populations of Drosophila include control of both enzyme synthesis and enzyme stability. For example, there is evidence that catalase levels are modulated by a distal trans-acting element(s) which controls the synthesis of this enzyme (Bewley and Laurie-Ahlberg 1984). While another distal trans-acting gene has been identified that post-translationally alters the stability of GPDH (King and McDonald 1983).

We have localized a region (R26-43) of the third chromosome (26.5-43.2) which controls DADH levels in Drosophila. The structural locus for Adh is located on the second chromosome (2-50.1); R26-43, therefore, codes for a gene or genes responsible for a trans-acting regulatory effect. DADH synthesis measurement indicated that R26-43 alters the stability of DADH protein post-translationally. The regulatory effect is not ubiquitous; that is, we could detect no effects of the R26-43 region on the activities of other Drosophila enzymes. We were unable to detect post-translational modification of DADH, which suggests that R26-43 may code for or regulate an DADH specific protease. This mechanism of control has been proposed before for other regulatory genes in mice [Ce; Ganschow and Schmike (1969)] maize [Adrl; Lai and Scandalios (1980)] and Drosophila [7; 55; 4; King and McDonald (1983)]. It is equally likely that R26-43 may regulate the stability of DADH by controlling the concentration of a ligand molecule that either binds loosely to DADH or results in the rapid degradation of the enzyme (Katunuma et al. 1975). As progress is made in the understanding of protein stability in vivo, the mechanism(s) of action of these genes and the R26-43 region may be determined.

Post-translational regulation may have as much to do with cellular differentiation and development as pre-translational controls. Post-translational mechanisms control numerous cellular and physiological activities, such as DNA relaxation (Ackerman, Glover and Osheroff 1985), hormone activity (Low et al. 1985) and cell proliferation (Nelson and Lasarides 1985). It is not surprising that many post-translational events are temporally (Lai and Scandalios 1980) and spatially specific (King and McDonald 1983). The existence of a high degree of regulatory variation in natural populations leads to speculation as to the role this variation might play in adaptation and speciation. It is hoped that continued analysis of regulatory variation in natural populations will contribute to an understanding of eukaryotic gene regulation and its role in evolution.

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