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.

ONE 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 (PAIGEN 1979). While in most instances these are *cis*-acting elements (SCHIBLER *et al.* 1983; PALMER *et al.* 1983; FELDER, BURNETT AND KAPLAN 1983; LUSIS *et al.* 1983; MARONI and LAURIE-AHLBERG 1983; BEWLEY 1981; CHOVIK 1976), there have been reports of *trans*-acting regulatory elements in mice (KOZAK 1985), maize (SCANDALIOS *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-sooty* (*e'*, 3-70.7) and *claret* (*ca*, 3-100.7). Our method of substituted strain construction utilizes the balancer stock *Cy/Bl*², *Sb Ser/e*¹¹ and has been described earlier (McDONALD and AYALA 1978b).

Enzyme activity assay: DADH, α -glycerophosphate dehydrogenase (GPDH), and phosphoglucose 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 5° at 12,000 × *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 D,L-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 $\Delta OD/min \times 10^2 \pm$ 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 a mixture of [³H]-amino acids (25 µl of 1.0 mCi/mol, 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 × 10⁻⁴ 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 trichloroacetic 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 McDONALD, 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 = 1.00; S2, *MM3*/+3 = 1.43; 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.

FIGURE 1.—Genetic construction of S2, MM3 and production of recombinant types (see text for details).

FIGURE 2.—Genetic isolation of individual recombinants.

Localization of the regulatory effect could then proceed in a stepwise fashion. DADH activity assays of the recombinant line S2, *ru h* were found to be insignificantly different from the S2, +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,

FIGURE 3.—Genetic map of MM3 chromosome with R^{26-43} indicated.

TABLE 1

Relative DADH activities of recombinant and paternal phenotypes

Visible genotypes	Relative DADH activity	Significance
+ . . + . . +	1.00	
<i>ru</i> . . <i>cu</i> . . <i>ca</i>	1.40	$P < 0.001$
<i>ru</i> . . <i>h</i>	0.88	NS
<i>h</i> . . <i>th</i>	1.48	$P < 0.001$
<i>th</i> . . <i>st</i>	1.40	$P < 0.001$
<i>st</i> . . <i>cu</i>	1.12	NS
<i>cu</i> . . <i>sr</i>	0.94	NS
<i>sr</i> . . <i>e^s</i>	0.89	NS
<i>e^s</i> . . <i>ca</i>	1.17	NS

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 S2, +3 line (Table 1). One recombinant line (not shown) containing only the *h* marker contained an DADH activity significantly higher than the S2, +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 regulatory region 26–43. The upper case R^{26-43} will designate the dominant, high DADH activity allele originally carried on the MM3 marker chromosome; the lower case r^{26-43} will designate the recessive, low activity allele originally carried on the +3 wild extracted chromosome. We conclude that a gene or genes located within the R^{26-43} region is responsible for the increased DADH activity of the S2, MM3 strain.

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 S2, +3 line by this small region of the third chromosome (approximately 3 map units). For convenience this strain will be referred to as S2, *R* and the S2, +3 line will analogously be designated S2, *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 S2, *R* line

TABLE 2

DADH activity and cross-reactive material (CRM) levels associated with the mapped region (R)

Strain	Adult activity	CRM	Relative activity	Relative CRM
S2, R	19.3 ± 0.43	1.40 ± 0.08	1.41**	1.41**
S2, r	13.7 ± 0.36	0.99 ± 0.03	1.00	1.00

** $P < 0.001$ level of significance.

TABLE 3

DADH activity of larval extracts associated with the localized region (R)

Strain	Larval activity	Relative activity	Significance ^a
S2, R	46.65 ± 2.68	1.37	$P < 0.001$
S2, r	34.15 ± 0.88	1.00	

^a Statistical significance based on Student's *t*-test comparison (d.f. = 18).

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 R²⁶⁻⁴³ 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 R²⁶⁻⁴³ 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 R²⁶⁻⁴³ 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 4

DADH activity distribution of DADH-5 and DADH-1 forms compared between strains

Strain	Percent Activity ± SE	
	DADH-5	DADH-3
S2, R	70.41 ± 5.39	29.27 ± 5.55
S2, r	67.12 ± 4.29	32.88 ± 4.29

TABLE 5

In vivo K_i, K_d, and relative CRM levels of the two strains (S2, R and S2, r)

Strain	K _i ^a	K _d ^b	Relative CRM
S2, R	0.020	0.014	1.41
S2, r	0.019	0.019	1.00

^a K_i is the rate of ¹⁴C incorporation into DADH protein.^b K_d is calculated according to the steady state rate equation, $[E] = K_i/K_d$. Relative CRM was used as an estimate of the enzyme concentration $[E]$.

FIGURE 4.—Ferguson plot of sequential gel electrophoresis, comparing the two strains (S2, R and S2, r).

ANDERSON 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 R²⁶⁻⁴³ 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.*, JOHNSON, FINNERTY and HARTL, 1981). Although we were unable to detect evidence of such R²⁶⁻⁴³ 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 JOHNSON (1975). The results presented in Figure 4 indicate no significant effect of the regulatory region (R²⁶⁻⁴³) on DADH net charge (M₀) or conformation (K_r); hence we detect no qualitative alterations of DADH enzyme.

R²⁶⁻⁴³ regulatory effect is not due to differences in DADH synthesis rates: The results demonstrate

FIGURE 5.—Incorporation of ^3H -labeled amino acids into DADH protein vs. time. Points represent average of eight determinations.

that the R^{26-43} 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 HODGETTS 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 (R^{26-43}) 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); R^{26-43} , therefore, codes for a gene or genes responsible for a *trans*-acting regulatory effect. DADH synthesis measurement indicated that R^{26-43} alters the stability of DADH protein post-translationally. The regulatory effect is not ubiquitous; that is, we could detect no effects of the R^{26-43} region on the activities of other *Drosophila* enzymes. We were unable to detect post-translational modification of DADH, which suggests that R^{26-43} 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 SCHIMKE (1969)] maize [*Adr1*; LAI and SCANDALIOS (1980)] and *Drosophila* [$r_{3-55.4}$; KING and MCDONALD (1983)]. It is equally likely that R^{26-43} 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 R^{26-43} 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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