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Quantitative analysis of RNA produced by Slow and Fast alleles of *Adh* in *Drosophila melanogaster*

(gene regulation/alcohol dehydrogenase/molecular evolution/polymorphism)

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ABSTRACT **The alcohol dehydrogenase (ADH) locus (*Adh*) of *Drosophila melanogaster* is polymorphic on a worldwide basis for two allozymes, Fast and Slow. This study was undertaken to determine whether the well-established difference in ADH protein concentration between the allozymes is due to a difference in mRNA levels. RNA gel blot hybridization and an RNase protection assay were used to quantify ADH mRNA levels. Each method used an *Adh* null mutant as an internal standard. Several Slow and Fast allele pairs of different geographic origins were analyzed. The results provide strong evidence that the ADH protein concentration difference is not accounted for by RNA level.**

The alcohol dehydrogenase (ADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) of *Drosophila melanogaster* is encoded by a single gene (*Adh*) on chromosome arm 2L, which produces two developmentally regulated transcripts (distal/proximal) that share the same coding sequence but differ in their 5′ untranslated leader (1, 2). In natural populations throughout the world, the *Adh* gene is polymorphic for two allozymes, designated Slow (S) and Fast (F) on the basis of electrophoretic mobility. Amino acid sequencing has shown that ADH-F differs from ADH-S by a threonine/lysine substitution at residue 192 (3), and DNA sequencing of several alleles of each electrophoretic type indicates that this is generally the only difference in primary structure (4). The ADH allozyme polymorphism has been intensively studied at both molecular and population levels but is still poorly understood (for reviews, see refs. 5–7).

Lines homozygous for Adh^F alleles generally have 2–3 times higher ADH enzyme activity per fly than Adh^S lines. Several investigators have shown that a large part, but probably not all, of this activity difference is accounted for by a difference in the concentration of ADH protein estimated immunologically (8–13). The active-site titration experiments of Winberg *et al.* (14) indicated that ADH-F has a higher catalytic efficiency, in addition to being more concentrated in the fly. The basis for the concentration difference between allozymes was addressed by Anderson and McDonald (15) in a protein-turnover study and by analysis of ADH mRNA levels. Their results suggested that Adh^F lines have a higher ADH concentration because of a higher rate of synthesis *in vivo*, which is associated with a higher concentration of ADH mRNA.

A study of restriction fragment length polymorphism in the *Adh* region revealed a pattern of strong nonrandom association among ADH activity level, ADH allozyme, and several restriction-site polymorphisms (16). These results, along with the difference in ADH mRNA concentration reported by Anderson and McDonald (15), suggested that the difference in *Adh* expression between allozymes might be due in part to linkage disequilibrium with a regulatory-site polymorphism. We have begun to test this hypothesis through the use of *P*-element transformation to identify the nucleotide substitution(s) responsible for the quantitative differences between allozymes. The first transformation experiment clearly localized the differences in ADH activity and ADH protein level to a 2.3-kilobase restriction fragment that includes all of the *Adh* coding sequence and some intron and 3′ flanking sequence but excludes all of the 5′ flanking sequence of the distal (adult) transcriptional unit (17). Analysis of DNA sequences for this fragment indicated that the effect is very likely due to one of three substitutions. One of these is the amino acid replacement and the other two are nearby third-position silent substitutions. Although any of these substitutions could theoretically cause differences in ADH mRNA levels through differential transcript processing or stability, these results suggest that additional quantitative analysis of RNA levels produced by *Adh* alleles from diverse sources is necessary for a thorough understanding of the allozyme polymorphism. Here we present the results of such a study, which, unlike the results of Anderson and McDonald (15), provide strong evidence that F lines do not have a higher concentration of ADH mRNA than S lines.

MATERIALS AND METHODS

Stocks. Three sets of wild-type stocks were used. (*i*) The isochromosomal "Kreitman lines" (4): Wa-s and Wa-f from Washington; Fl-2s and Fl-f from Florida; Fr-s and Fr-f from Bully, France; Ja-s and Ja-f from Ishigaki, Japan. (*ii*) Isogenic second-chromosome substitution lines (18): KA13 (F) and KA16 (S) from Kansas; WI08 (F) and WI09 (S) from Wisconsin; RI42 (S) from Rhode Island. In a survey of 50 such lines, the KA and WI lines showed modal ADH activity values within their respective allozymic classes (18). (*iii*) The "CA" lines are John McDonald's S1 and F1 isogenic lines from California (19). Two mutant strains were used: Adh^{fn23} *pr cn* (from W. Sofer, Waksman Institute, Piscataway, NJ) and *b* Adh^{nLA248} *cn bw* (from M. Ashburner, Cambridge University, Cambridge, UK). Transformant stocks, each homozygous for a single insert (in an Adh^{fn6} *cn*; ry^{506} background) were also used in one experiment. Each insert consists of a *P* element containing a ry^+ fragment and the *Sac* I–*Cla* I *Adh* fragment from either the Wa-s or Wa-f clone (17).

ADH Activity and Protein Level. For assaying ADH activity, the spectrophotometric method described by Maroni (20) was used with isopropanol as substrate. ADH units are nmol of NAD^+ reduced per min. ADH protein was estimated as crossreacting material (CRM) by radial immunodiffusion (21). This procedure was tested with purified ADH-F and ADH-S (22, 23) to verify that there is no difference between

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Abbreviations: ADH, alcohol dehydrogenase; F, Fast; S, Slow; CRM, crossreacting material; ANOVA, analysis of variance.

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allozymes in the extent of antibody–antigen reaction (i.e., equal quantities of ADH protein gave equal immunodiffusion diameters). A dilution series of a standard fly extract was included on each immunodiffusion plate to ensure linearity over the range of sample values. ADH CRM units are given in terms of mg of fly wet weight per ml of this standard extract. ADH activity and CRM levels are given as units per mg of wet weight.

RNA Preparation. For experiments 1 and 4 (see below), total RNA was prepared from sets of 120 flies (7- to 8-day adult males) by urea lysis and pelleting through a CsCl cushion, as described by Goldberg *et al.* (24). For experiments 2 and 3, total nucleic acids were prepared by a modification of the method of Fischer and Maniatis (25). Sets of 100 flies (7- to 8-day adult males) were ground in liquid N_2, suspended in 2 ml of homogenization buffer (50 mM Tris·HCl, pH 7.5/10 mM EDTA/50 mM NaCl/0.5% $NaDodSO_4$ containing proteinase K at 0.25 mg/ml), and then ground in a Dounce glass homogenizer. The brei was incubated at 37°C for 30 min, mixed with 0.3 ml of 8 M potassium acetate (pH 5.5) and put on ice for 30–60 min. After centrifugation at 12,000 × *g* for 5 min, the supernatant was ethanol-precipitated and resuspended in 70 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA.

RNA Gel Blot Assays. Nick-translation, formaldehyde/agarose gel electrophoresis, transfer to nitrocellulose, and hybridization were essentially as described by Maniatis *et al.* (26). The *Adh* probe used was plasmid p13E3, which contains the central *Eco*RI fragment inserted in pUC13. The RNA was quantified by computing the peak areas of bands on autoradiograms with scanning laser densitometry. Pairs of F and S alleles to be compared were always run together in the same gel.

Plasmid Construction. The plasmid pBSBD was constructed by isolating the 423-base-pair fragment that extends from the *Bam*HI site at nucleotide 1257 to the *Dde* I site at nucleotide 1680 of Kreitman's Wa-f *Adh* clone (4), blunting the ends with Klenow DNA polymerase, and inserting the blunt-ended fragment into the *Sma* I site of Stratagene's pBSM13+ vector. The plasmid was linearized with *Eco*RI and used as a template for RNA transcription.

RNase Protection Assays. RNA transcription, hybridization, digestion, and acrylamide gel analysis were performed as described by Melton *et al.* (27) with some modification. The transcription reaction mixtures (20 μl) contained 40 mM Tris·HCl (pH 7.5); 8 mM $MgCl_2$; 2 mM spermidine; 25 mM dithiothreitol; 50 mM NaCl; 0.4 mM ATP, CTP, and UTP; 15 μM nonradioactive GTP; 50 μCi of [α-^{32}P]GTP (800 Ci/mmol; 1 Ci = 37 GBq); 40 units of RNase inhibitor (RNasin; Promega Biotec, Madison, WI); 20 units of phage T3 RNA polymerase; and 1 μg of DNA template (pBSBD). Digestion was carried out at 37°C for 30 min with RNase T1 at 2 μg/ml (no RNase A). The desired fragments (located by autoradiography) were cut out of the 6% acrylamide gel and quantified by scintillation counting.

***Adh* Mutants as Controls.** Two procedures were used for quantitative analysis of ADH mRNA levels, each of which makes use of an internal control provided by an *Adh* null mutant allele. The first procedure uses the null allele Adh^{nLA248}, a duplication mutant that makes an *Adh* message about 200 bases longer than wild type (28). Heterozygotes for the *nLA248* allele and a wild-type allele produce two distinct bands of ADH mRNA on an RNA gel blot. Fig. 1 shows a dilution series of two RNA samples, each prepared from a heterozygote for *nLA248* and either Ja-f or Ja-s. The amount of message produced by the wild-type allele relative to the mutant control is estimated as the slope of the regression of wild-type band density on mutant band density over the dilution series. A dilution series was used for each sample analyzed to ensure linearity of the densitometric response of the autoradiogram.

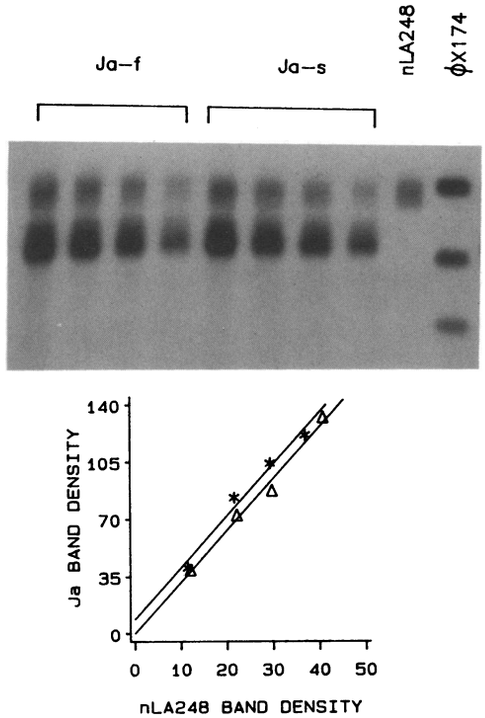


FIG. 1. (*Upper*) Autoradiogram of an RNA gel blot showing a dilution series for RNA from flies heterozygous for *nLA248* and a wild-type allele (Ja-f or Ja-s). Lane nLA248, RNA sample from flies homozygous for *nLA248*; lane φX174, an *Hae* III digest of bacteriophage φX174 DNA for size markers (1353, 1078, and 872 bases; these appear because a small amount of φX174 DNA was added to the probe). (*Lower*) Linear regression of wild-type band density on *nLA248* band density for the F (asterisks) and S (triangles) dilution series.

The second procedure, suggested by J. Posakony, makes use of the null mutant Adh^{fn23}, which has a 34-base-pair deletion in exon 3 (29). An RNase protection assay distinguishes between RNAs produced by the deletion mutant and wild-type alleles. The pBSBD probe described above extends from the *Bam*HI site in exon 2 to a *Dde* I site in exon 3 that lies just 4 base pairs beyond the 3′ end of the *fn23* deletion. RNA from wild-type flies protects two regions of this probe: 89 bases of exon 2 and 263 bases of exon 3. When RNA from *fn23* homozygotes is used, the protected region from exon 3 is only 225 bases. Fig. 2 shows that heterozygotes for *fn23* and a wild-type allele protect both the 263- and 225-base fragments. Thus, the amount of RNA produced by the wild-type allele is estimated as the slope of the regression of number of cpm in the wild-type 263-base band on the number of cpm in the mutant 225-base band. Although some of the fly RNA·probe RNA hybrids are known to contain mismatches with a guanine in the probe strand, fragments predicted from mismatch cleavage by RNase T1 were not observed. This observation concurs with the results of Myers *et al.* (30), who reported no mismatch cleavage of RNA·DNA hybrids by RNase T1.

The *nLA248* mutant is CRM-negative (28, 31), whereas the *fn23* mutant was reported to be CRM-positive (32). However, neither mutant contributes a significant increase in immunodiffusion diameter when extracts are mixed with those from a wild-type homozygote, so the level of *fn23* CRM must be very low. Furthermore, neither mutant forms an active heterodimer when heterozygous with a *F* allele (by histochemical staining of starch gels). Therefore, we assume that all of the CRM measured in homogenates of heterozygotes with *fn23* or *nLA248* derives from the wild-type allele.

Sample Collection. In each of the four experiments, flies were reared on cornmeal/molasses medium during each of

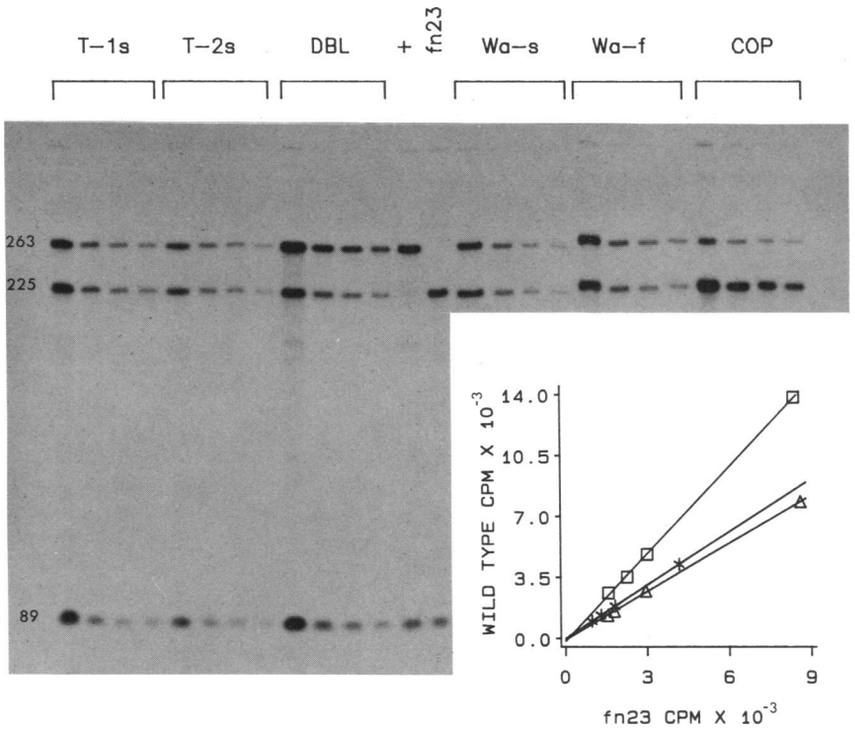


FIG. 2. Autoradiogram of an RNase protection gel showing a dilution series for each of six samples of fly RNA protected by the pBSBD probe and digested with RNase T1. The flies were produced by crossing *fn23* homozygotes to homozygous stocks of the following type: single-insert transformant stocks (T-1s, T-2s), a double-insert transformant stock (DBL), Wa-s, Wa-f, or the *copia* variant line RI42 (COP). Lane +, RNA from flies homozygous for a wild-type allele; lane fn23, RNA from flies homozygous for *fn23*. (*Inset*) Linear regression of number of cpm in the wild-type (263-base) band on cpm in the *fn23* (225-base) band for three of the dilution series: T-1s (triangles), T-2s (crosses), and DBL (squares).

two time blocks. Heterozygotes were produced by crossing either Adh^{fn23} *pr cn* (RNase protection assay) or *b* Adh^{nLA248} *cn bw* (RNA gel blot) females to males from various different homozygous Adh^S or Adh^F lines. In two experiments, mock heterozygotes were produced by mixing equal numbers of flies of mutant and wild-type homozygotes. Male progeny were aged 7–8 days posteclosion before homogenization for either RNA or protein assays.

RESULTS

Experiment 1: RNA Gel Blot Analysis. In this experiment ADH activity, protein (CRM), and RNA levels were estimated for six pairs of F and S alleles derived from different geographic locations. The results were essentially the same for each allele pair (Table 1). Although the F member of each pair had much higher ADH activity and considerably more ADH CRM, the difference in RNA levels went consistently in the opposite direction. ANOVA of the RNA estimates showed that this allozyme difference is significant ($P = 0.004$).

ANOVA of the RNA slopes from this experiment showed a significant gel effect ($P < 0.05$), which makes comparison of samples run in different gels difficult. Gel-to-gel differences can be as high as 2-fold, even though the relative ranking of samples within a gel remains essentially the same

Table 1. F/S ratios of ADH enzyme activity, CRM, and RNA for alleles paired by geographic origin

	Exp. 1 (RNA gel blot)			Exp. 3 (RNA gel blot)			Exp. 3 (RNase protection)		
Pair	Activity	CRM	RNA	Activity	CRM	RNA	Activity	CRM	RNA
Ja	2.34	1.38	0.87	2.34	1.20	0.97	2.24	1.32	1.12
Wa	2.48	1.53	0.96	2.24	1.13	0.93	2.31	1.25	0.98
Fl	2.37	1.19	0.90	1.93	1.20	1.02	2.30	1.46	1.05
Fr	1.92	1.08	0.64	2.65	1.35	0.89	2.41	1.43	0.87
KA	2.97	1.92	0.95	—	—	—	—	—	—
WI	2.62	1.61	0.75	—	—	—	—	—	—
CA	—	—	—	3.02	1.64	0.86	3.14	1.97	1.03
Average	2.45	1.45	0.85	2.44	1.30	0.93	2.48	1.49	1.01
n*	8	8	2	4	4	4†	4	4	4
P‡	<0.0001	<0.0001	0.004	<0.0001	0.09	0.08	<0.0001	<0.0001	0.48
d.f.§	1/25	1/25	1/8	1/11	1/1¶	2.45/5.0‖	1/11	1/11	1/9

*Number of observations averaged for each line within each pair. Each observation derives from a different set of flies.
†Except for CA lines, for which $n = 3$.
‡Probability level of F-test for allozymes from analysis of variance (ANOVA).
§Degrees of freedom for F-test (numerator/denominator).
¶There is only 1 d.f. for the denominator here because of a significant block–allozyme interaction. This causes a great reduction in the power of the test.
‖Satterwaite's approximate F-test.

and the coefficient of variation calculated from the residual variance is relatively low (9.1%). This gel effect is the reason why Table 1 summarizes the results in terms of F/S ratios for pairs of alleles that were always run together in the same gel, rather than in terms of absolute values. Because of this undesirable gel effect, because the RNA gel blot method is very time-consuming, and because we felt that the unexpected result of experiment 1 required confirmation, the RNase protection method was developed.

Experiment 2: RNase Protection Assay. A variety of genotypes were analyzed to explore the precision of the RNase protection assay. They were constructed by crossing Adh^{fn23} *pr cn* homozygotes to the following homozygous stocks: Wa-s, Wa-f, two single-insert transformant stocks of each of two types (containing fragments from the Wa-s and Wa-f clones), a double-insert transformant stock constructed by combining each of the two Wa-s-type single inserts through traditional genetic methods, and the isogenic stock RI42, which contains a *copia* element in the 5′ flanking region of *Adh* (16).

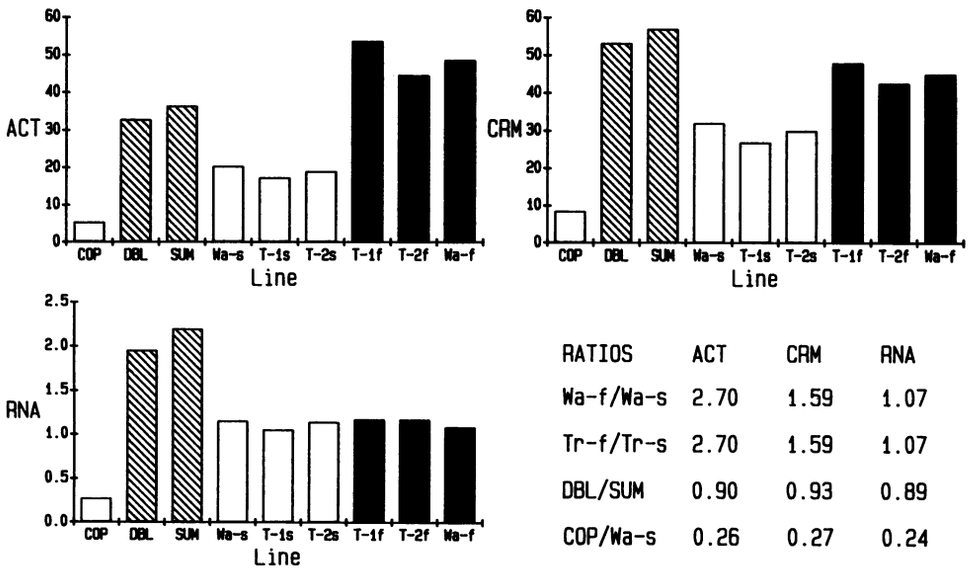
Fig. 3 summarizes the results in terms of genotypic means and ratios of those means for certain pairwise comparisons (see also Fig. 2). As expected in the absence of dosage compensation, the double insert was nearly equal to the sum of the two single inserts for RNA, CRM, and enzyme activity. The three ratios were also very similar for the RI42 *copia*/Wa-s comparison (about 0.25 in each case), indicating that the low activity of RI42 (relative to the typical S line Wa-s) is accounted for by a low steady-state CRM level, which in turn is accounted for by a low steady-state RNA level. In contrast, the three ratios are quite different for the F/S comparisons. For both the Wa and transformant comparisons, F has about 2.5-fold higher activity than S and about 1.5-fold higher CRM, and the RNA levels are essentially the same. ANOVA of the Wa and single-insert transformant data showed no significant difference in RNA level between allozymes, whereas the difference was highly significant for enzyme activity ($P < 0.001$) and CRM ($P < 0.005$). It is notable that the comparison between transformant lines, in which an allele of each type was inserted into a common genetic background, gave results very similar to those of comparisons between wild-type S and F lines, which may differ in genetic background.

In experiment 2 the four independent RNA preparations from each of the four transformant stocks were each run in two different gels. ANOVA of these 32 observations showed no significant gel effect and a coefficient of variation of 5.5% (calculated from the residual variance). A small pilot experiment gave similar results: for a single RNA sample run in three dilution series in each of two gels, there was no significant gel effect and the coefficient of variation was 3.2%. Thus, the RNase protection assay is a very precise and convenient method for comparing RNA levels for *Adh* alleles.

Experiment 3: Survey of F/S Lines by Both Methods. Because experiment 1 gave the unexpected result that F alleles appear to produce a slightly lower RNA level than S alleles, both RNA-assay methods were used in experiment 3 to determine whether that result could be method-specific. Four of the same pairs of alleles included in experiment 1 were analyzed again. Experiment 3 also included a pair of alleles (CA) that were analyzed by Anderson and McDonald (15) to determine whether any difference between their results and ours could be attributed to the particular alleles studied.

Table 1 shows that the results of the two methods were essentially the same. For all five pairs of alleles, F had a much higher enzyme activity and a substantially higher CRM, but the differences in RNA levels were small and not consistent in direction. In ANOVAs of the RNA estimates, the allozyme effect was not significant for either method. The average F/S ratio was essentially the same and very close to unity for both methods: 0.93 for RNA gel blots and 1.01 for the RNase protection assay.

Mock Heterozygotes. The results reported here stand in contrast to those of Anderson and McDonald (15), who used a dot blot hybridization assay without an internal control to analyze homozygous F and S lines. Since both the gel blot assay and the protection assay that we used involved heterozygous flies with an internal mutant control, it is possible that some trans-acting effects or interallelic interactions account for the discrepancy with the results of Anderson and McDonald. This possibility was tested by generating mock heterozygotes by mixing equal numbers of flies of each of the two homozygous types (the *Adh* control mutant, *fn23* or *nLA248*, and a wild-type S or F *Adh* allele) before homogenization and extraction of RNA. Mock heterozygotes were compared with true heterozygotes in two experiments. Ex-



RATIOS	ACT	CRM	RNA
Wa-f/Wa-s	2.70	1.59	1.07
Tr-f/Tr-s	2.70	1.59	1.07
DBL/SUM	0.90	0.93	0.89
COP/Wa-s	0.26	0.27	0.24

FIG. 3. Experiment 2: genotypic means of ADH enzyme activity (ACT), CRM, and RNA levels for flies produced by crossing *fn23* homozygotes to lines homozygous for various wild-type alleles (or *P*-element inserts; see text). Each mean is based on four observations. RNA levels were estimated by RNase protection assay. Solid bars, ADH-F; open bars, ADH-S; hatched bars, values for the double-insert transformant (DBL) and for the sum of the means of the two constituent single-insert lines (SUM). COP, *copia* variant line RI42.

Table 2. F/S ratios of line means of ADH enzyme activity, CRM, and RNA for true heterozygotes (Het) and mock heterozygotes (Mix)

Pair	Type	F/S ratio		
		Activity	CRM	RNA
Exp. 4 (RNA gel blot)				
Wa	Het	2.19	1.44	1.09
Wa	Mix	2.67	1.68	1.02
CA	Het	2.79	1.44	1.19
CA	Mix	2.76	1.66	1.08
Exp. 3 (RNase protection)				
Wa	Het	2.31	1.25	0.98
Wa	Mix	2.10	1.32	0.91

Each line mean is based on four observations derived from different sets of flies.

periment 4 analyzed two allele pairs (Wa and CA) by the gel blot method, and experiment 3 analyzed the Wa allele pair by the RNase protection method.

Table 2 shows that, for each allele pair and for both methods, the mock heterozygotes had essentially the same RNA levels as the true heterozygotes. ANOVAs showed no significant effect of allozyme or of the interaction between allozyme and type (mock vs. true heterozygote) for RNA level. Thus, there is no evidence that the degree of difference between allozymes depends on whether the wild-type alleles are homozygous or heterozygous with the *Adh* control mutants.

DISCUSSION

Seven pairs of S and F lines derived from different geographic locations were analyzed in this study, and each gave essentially the same result. F lines have, on the average, about 2.5-fold higher levels of enzyme activity and about 1.5-fold higher levels of CRM than S lines. Thus, the difference in the amount of ADH protein (CRM) accounts for a substantial fraction, but clearly not all, of the activity difference between allozymes. As mentioned above, ADH-F probably has a higher catalytic efficiency than ADH-S, as well as being more concentrated in the fly (14). However, both RNA gel blots and RNase protection assays show that F lines do not have higher ADH mRNA levels than S lines.

Our RNA results stand in contrast to those of Anderson and McDonald (15), who reported that their F1 strain has a >2-fold higher ADH RNA level than the S1 strain, which parallels the differences in ADH activity and CRM. We analyzed the same two strains (the CA pair provided by McDonald) and found no difference in RNA level even though the differences in enzyme activity and CRM were large. The reasons for this discrepancy are not clear, since there are many differences in the methods used to measure RNA. However, one methodological difference can be ruled out: the use of heterozygotes with control *Adh* mutants in our study compared with the use of homozygotes by Anderson and McDonald. Our mock-heterozygote experiments showed that the lack of difference between allozymes is not affected by whether the *Adh* mutant control occurs within the same fly as the wild-type allele (true heterozygotes) or whether it is added by mixing mutant and wild-type homozygous flies prior to RNA extraction.

We conclude that the difference in concentration of ADH protein between allozymes is not due to a difference in RNA template concentration, which leaves two possibilities. Either there is a difference in the translation rates of the two RNAs or there is a difference in the rates of degradation of the two proteins *in vivo*.

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