Molecular Evolution of Drosophila Metallothionein Genes

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ABSTRACT

The metallothionein genes of *Drosophila melanogaster*, *Mtn* and *Mto*, may play an important role in heavy metal detoxification. Several different tandem duplications of *Mtn* have been shown to increase cadmium and copper tolerance, as well as *Mtn* expression. In order to investigate the possibility of increased selection for duplications of these genes in natural populations exposed to high levels of heavy metals, we compared the frequencies of such duplications among flies collected from metal-contaminated and non-contaminated orchards in Pennsylvania, Tennessee and Georgia. Restriction enzyme analysis was used to screen 1666 wild third chromosomes for *Mtn* duplications and a subset (327) of these lines for *Mto* duplications. The frequency of pooled *Mtn* duplications found ranged from 0% to 20%, and was not significantly higher at the contaminated sites. No *Mto* duplications were identified. Estimates of sequence diversity at the *Mtn* locus among a subsample (92) of the duplication survey were obtained using four-cutter analysis. This analysis revealed a low level of polymorphism, consistent with both selection at the *Mtn* locus, and a fairly recent origin for the duplications. To further examine this hypothesis, we sequenced an *Mtn* allele of *Drosophila simulans* and measured the amount of nucleotide sequence divergence between *D. simulans* and its sibling species *D. melanogaster*. The levels of silent nucleotide polymorphism and divergence in the *Mtn* region were compared with those in the *Adh* region, using the neutrality test of R. R. Hudson, M. Kreitman and M. Aguadé.

THE metallothioneins (MTs) and metallothionein-like proteins are small, cysteine-rich, metal-binding proteins, examples of which have been studied in vertebrates, invertebrates, plants, fungi and prokaryotes. The function of MTs is not entirely clear: however, the fact that they bind to and are induced by heavy metals such as cadmium, copper and zinc suggests that MTs may serve to reduce the toxic effects of such metals on living cells. While zinc and copper are essential trace metals which are toxic only in high concentrations, cadmium has no known biological function, and is toxic in very small amounts. Several experiments describing MT gene amplification in metal-resistant strains of mice, yeast and cultured mammalian cells also support the hypothesis that MTs serve to protect organisms from deleterious effects of metal contamination in their environments (BEACH and PALMITER 1981; FOGEL and WELCH 1982; WELCH *et al.* 1983; KARIN *et al.* 1984; KOROPATNICK *et al.* 1985).

Two different MT genes, designated *Mtn* (LASTOWSKI-PERRY, OTTO and MARONI 1985; MARONI and WATSON 1985; MARONI, OTTO and LASTOWSKI-PERRY 1986) and *Mto* (DEBEC, MOKDAD and WEGNEZ 1985; MOKDAD, DEBEC and WEGNEZ 1987), are known to exist in *Drosophila melanogaster*. Both genes are located on the right arm of the third chromosome. The *Mtn* locus is in the region 85E10-15 (MARONI, OTTO and LASTOWSKI-PERRY 1986), while the *Mto* locus is in the region 92E (M. WEGNEZ, personal communication). For both *D. melanogaster* MT genes, there is evidence to suggest that duplication of the gene may give a selective advantage in metal-contaminated environments. In the case of *Mto*, a presumed duplication was found in a cadmium-resistant *D. melanogaster* cell line producing elevated levels of metallothionein (MOKDAD, DEBEC and WEGNEZ 1987). In the case of *Mtn*, four different tandem duplications found in natural populations have been associated with increased metal tolerance in laboratory experiments (MARONI *et al.* 1987). The fact that these different duplications exist in appreciable frequencies in natural populations suggests that they may provide flies with some selective advantage.

If such selection were the result of industrialization, and had therefore been acting only during the last few centuries, then the presence in a population of several different duplications in appreciable frequencies would indicate that selection must be quite strong. An alternative explanation–that of high *Mtn* duplication rates–requires that these rates be much higher

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than at other loci, since the observation of functional gene duplications segregating in natural populations of Drosophila is such a rare occurrence. While TAKANO *et al.* (1989) have reported a polymorphism of an apparently nonfunctional duplication of *Gpdh* and LYCKEGAARD and CLARK (1989) have demonstrated variation in copy number of the *Stellate* multigene family on the *Y* chromosome, the segregation of four duplications of *Mtn* found by MARONI *et al.* (1987) remains the only case of polymorphism for functional euchromatic duplications segregating in natural populations of *D. melanogaster* (LANGLEY 1990).

Motivated by these findings, we looked for evidence of selection for these MT gene duplications in natural populations of *D. melanogaster* exposed to heavy metal pollution by comparing their frequencies in populations from metal-contaminated and from noncontaminated sites. Six-cutter restriction enzyme analysis (MANIATIS, FRITSCH and SAMBROOK 1982) was used to identify duplications. If duplications were also found for *Mto* this would be further evidence of selection associated with heavy metal contamination. If significant correlations were found between metal contamination levels and duplication frequencies at *Mtn* (and *Mto* if such were found), then we would have evidence of selection for duplications–in fact, selection strong enough to overcome the homogenizing effects of migration on gene frequencies over a distance of only about 25 miles. If no significant correlations were found, then we would conclude that migration is more important than any selective differences in determining the distribution of *Mtn* gene duplications in these local subpopulations. However, average selection pressures might still be quite substantial and have an important effect on *Mtn* gene duplication frequencies. To be able to investigate this latter possibility, we collected data on nucleotide variation at *Mtn* using four-cutter analysis (KREITMAN and AGUADÉ 1986a). Furthermore, we sequenced the *Mtn* region of *Drosophila simulans* and measured the amount of nucleotide divergence between *D. melanogaster* and *D. simulans*. This allowed us to apply the test of HUDSON, KREITMAN and AGUADÉ (1987) to identify possible departures from selective neutrality.

MATERIALS AND METHODS

Collection: From September 5 to 12, 1986, *Drosophila melanogaster* plus soil, leaf and fruit samples were collected from nine different sites (orchards or gardens), in the vicinities of three point sources of heavy metal pollution. There were six metal-contaminated sites (within 5 miles of the source) and three control sites (about 25 miles upwind from the source). Two of the heavy metal pollution sources were the two zinc smelters which were known to have caused significant cadmium pollution in the surrounding areas. One, located in Palmerton, (eastern) Pennsylvania, has been active since 1848 (but with decreased and cleaner production since 1980). The other, in Beaver, (western) Pennsylvania, has been active since 1931. Both smelters have caused heavy local accumulations of cadmium in the soil–up to approximately 35 ppm in Palmerton (WASHINGTON 1985) and up to approximately 25 ppm in Beaver (Beaver County Cooperative Extension 1988). The third contaminated area was Copperhill, Tennessee, a region where copper has been mined and processed since 1850.

Soil, leaf, fruit and fly samples from each site were analyzed for heavy metal content using atomic absorption spectrophotometry by Radian, Inc., in Morrisville, North Carolina. The relative contamination of sites close to the PA zinc smelters was confirmed, but no evidence of metal pollution was found for either of the Copperhill, Tennessee, collection sites. A second collection was made September 7–13, 1988 at the Pennsylvania sites.

Duplication survey: The duplication survey was conducted over three separate six-cutter experiments. In experiment I, third chromosomes were genetically extracted from the iso-female lines set up at the time of collection in 1986. The laboratory stock used in these crosses was *Df(3R) by^{10}, red e/In(3LR)TM3, p^{p} Sb Ser e^{s}* (KEMPHUES, RAFF and KAUFMAN 1983). Approximately one third of the chromosomes were homozygous lethal or sterile, and were maintained as *Mtn* deficiency (*Df(3R)by^{10}*) heterozygotes. One homozygous or hemizygous line was made from each of the 327 iso-female lines: over 50 "control" and 50 "contaminated" lines for each of the three point sources of metal pollution.

Unless otherwise indicated, the molecular techniques used in these experiments were from MANIATIS, FRITSCH and SAMBROOK (1982), with some modifications. DNA was prepared from the 327 homozygous and hemizygous lines. Each DNA sample was then subjected to two single digests: one with *Bam*HI and one with *Hin*dIII. These were also the enzymes used in the survey of MARONI *et al.* (1987) to assess the frequencies and sizes of the various *Mtn* duplications. Using this method, duplications are detected by the appearance on an autoradiograph of an extra band(s).

The six-cutter filters were probed with a pUC19 plasmid containing a 5-kb genomic *Mtn* fragment, Dm131. Dm131 had been kindly provided by G. MARONI and recloned into pUC19. The filters were then stripped and reprobed with an *Mto*-containing phage clone, IC3 recovered from a genomic library (made from one of the non-duplication isochromosomal lines–"BL21") using a cDNA kindly provided by M. WEGNEZ. The library was made according to SHRIMPTON, MONTGOMERY and LANGLEY (1986). Restriction maps of the *Mto* region and the clone, IC3, are shown in Figure 1.

In experiment II, rather than using extracted homozygous or hemizygous lines as was done in experiment I, 187 single flies, one from each of the Palmerton and Beaver isofemale lines not yet screened, were used. (These iso-female lines had been maintained in the laboratory for over 20 months.) Additional Copperhill lines were not screened, since there had been no clear evidence from the metals analysis of heavy metal pollution in Copperhill. DNA from these single flies was prepared according to STELLER (1986) and digested with *Sal*I. It had been discovered that *Sal*I cuts outside the breakpoints of all duplications encountered thus far (except in the cases in which an insertion contains a *Sal*I site, such as the CA35 and CA6 duplications which will be described below), so that probing with the *Mtn* clone shows one band for a homozygote, and the size of this band increases with the size of the duplicated region (see Figure 2). Heterozygotes are unambiguously detected (with the possible exception of CA6 duplication heterozygotes), because the two alleles give bands of different sizes, making it possible to use single flies which may be heterozygous.

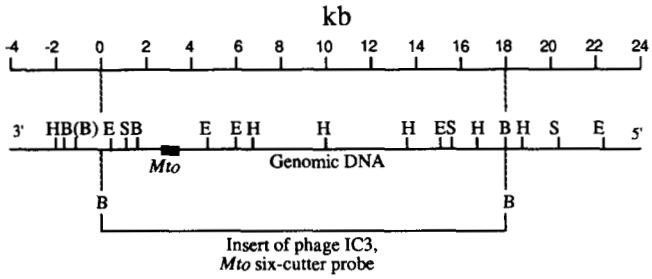

FIGURE 1.—Restriction map of the *Mto* region and the phage clone, IC3, used as a probe in the six-cutter duplication survey. The abbreviations are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. This clone was recovered from a library made from one of the isochromosomal lines (BL 21), using a cDNA provided by M. WEGNEZ. The *Bam*HI site in parentheses was polymorphic in the sample.

Apparent duplications were confirmed by digesting DNA from 20 flies from the corresponding iso-female line with *Bam*HI and *Hin*dIII.

For the six-cutter experiment III, as in experiment II, single flies were screened for duplications using *Sal*I. In this experiment, one F_1 offspring from each of the 576 wild females collected in 1988 were assayed.

Four-cutter analysis: Of the 327 wild third chromosomes originally surveyed by six-cutter analysis (experiment I), 92 were included in a study of four-cutter restriction fragment length polymorphism (KREITMAN and AGUADÉ 1986a) in a 1430-bp region including the *Mtn* locus. 43 chromosomes with unusual *Mtn* six-cutter restriction patterns were analyzed (including 26 of the 27 duplication lines, plus 18 others–all with likely insertions outside the four-cutter probe region). In addition, 48 other chromosomes, all with the "wild-type" six-cutter restriction pattern (non-duplicated and without insertions) were chosen at random (16 from each geographic region–eight from contaminated orchards, and eight from control orchards) and analyzed. The probe used was a 1430-bp *Eco*RI-*Acc*I fragment (in pUC19), which was the largest portion of the 1543 bp region sequenced by MARONI, OTTO and LASTOWSKI-PERRY (1986). The nine four-cutter enzymes used in this experiment were: *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I and *Taq*I. These are essentially all of the available four-cutters which cut within the probed region more than once (see the restriction map in Figure 2). Together, they cut at a total of 41 sites in the fragment, with some overlapping of sites and enable one to detect approximately 18.5% of all base pair substitutions and virtually all insertion/deletion variation within the 1430-bp region probed (KREITMAN and AGUADÉ 1986a).

Cloning and sequencing of the *Mtn* region of *D. simulans*: A lambda-phage library was constructed in lambda-ZAP (Stratagene) from a complete *Sac*I-restriction digest of genomic DNA from a RAs5 stock kindly provided by G. SIMMONS. This library was screened with the 1430-bp *Eco*RI-*Acc*I fragment containing the *Mtn* gene of *D. melanogaster* (see above). The sequence was determined by the dideoxy method.

RESULTS

No *Mto* duplications: Figure 1 shows the restriction map of the *Mto* region and the genomic clone used to probe the Southern blots. Duplications of the gene should be readily detected with this probe and the restriction enzymes used. The *Mto* region was found to contain a polymorphic *Bam*HI site and substantial insertion/deletion variation, but no duplications of the *Mto* gene were found.

***Mtn* duplication frequencies:** Selection in favor of *Mtn* duplications in areas of heavy metal contamination might lead to geographic differentiation in the frequencies of duplications that correlated with heavy metal contamination. The relative contamination of the collecting sites near the two smelters in Pennsylvania was confirmed. Indeed the contamination of fallen fruit and *D. melanogaster* themselves was documented (LANGE 1989). The Copperhill, Tennessee, sites did not differ in observed levels of cadmium contamination. Thus the Copperhill populations were not surveyed beyond the initial experiment I.

The *Mtn* duplication survey results from experiment I are shown in Table 1. Of the 327 chromosomes screened, 27 carried *Mtn* duplications. Four of the six duplication types found had the same six-cutter restriction pattern as four found by MARONI *et al.* (1987): their 2.2-, 3.5-, 4.6- and 6.0-kb duplications (Figure 2). Their "i35" and "i60" types, which include inserts into the 3.5- and 6.0-kb types, respectively, were not seen in this study. However, the two new duplication types discovered–CA35 and CA6, both from the Apple Valley Orchard near Copperhill, Tennessee–bear some resemblance to i35 and i60. Like i35, CA35 is a 3.5-kb duplication with an insertion. The CA35 insertion appears to be a *roo* transposable element inserted 3′ of the 3′ copy of the gene, based on restriction mapping and comparison with the *roo* map in FINNEGAN and FAWCETT (1986). CA6 appears to be an i6.0 chromosome with an additional approximately 8-kb insert within the original i60 insert. The restriction map of this second insert (no *Bam*HI sites, at least one *Bgl*II site, and at least one *Sal*I site) is consistent with the restriction map of the transposable element *springer* (FINNEGAN and FAWCETT 1986).

Pooling the results for the contaminated sites within each of the three geographic regions, the contaminated *vs.* control site *Mtn* duplication frequencies for Palmerton, Beaver and Copperhill were 3.5% *vs.* 0.0%, 13.6% *vs.* 7.4%, and 10.9% *vs.* 13.7%, respectively (Figure 3). There was a slight, but not statistically significant, increase in the frequency of duplications from the Palmerton and Beaver cadmium-contaminated orchards, as compared to their corresponding control orchards ($P = 0.22$). The statistic used was the Mantel-Haenszel statistic (FLEISS 1981).

The results of experiment II are shown in Table 2. Note that each chromosome was weighted by one half. In other words, each fly was scored as if it were a haploid. This is because the iso-female lines were highly inbred after having been maintained in the laboratory for over 20 months, and random drift may

TABLE 1

1986 *Mtn* duplication frequencies: experiment I

Orchard	*n*	No. of duplications of each size (kb)					Frequency (%) of duplications		
		2.2	3.5	4.6	6.0	Total	In each orchard	Control *vs.* pooled contam	In each region
Palmerton, Pennsylvania									
Christy (control	51					0	0.0	0.0	
									1.9
Daniels	50		2			2	4.0	3.5	
Zinc City	7					0	0.0		
Beaver, Pennsylvania									
Dawson (control)	54		2		2	4	7.4	7.4	
Curran	25		1	1	3	5	20.0		10.6
Lohry	29	1	1		1	3	10.3	13.6	
Van Buren	5					0	0.0		
Copperhill, Tennessee									
A. Valley (control)	51	1		1	3	7[a]	13.7	13.7	
									12.3
Mountain View	55	2	2		2	6	10.9	10.9	
Total	327	4	8	2	11	27			8.3

[a] Two novel duplication types were found in the Apple Valley population (CA 6 and CA 35). See text for an explanation.

have changed the original frequencies within each line (perhaps in a biased way with respect to *Mtn*, due to relaxation of selection pressure for heavy metal tolerance, but presumably in an unbiased way with respect to collection site). In the extreme case, each line would have become homozygous for one of the original chromosomes. Therefore, it was more conservative to sample, in essence, "one" chromosome from each iso-female line.

Thus, 12 "chromosomes" carried a *Mtn* duplication, out of 187 screened. These duplications were all of the four basic types (2.2, 3.5, 4.6 and 6.0 kb without inserts) seen previously by MARONI *et al.* (1987) and in experiment I. The pooled contaminated *vs.* control site *Mtn* duplication frequencies for Palmerton and Beaver were 4.2% *vs.* 4.2% and 10.9% *vs.* 5.5%, respectively. The data from experiment II, and from both experiments taken together, were again suggestive but inconclusive. The Mantel-Haenszel *P* for experiment II alone was 0.67, and for both I and II together, it was 0.16.

The results of experiment III are shown in Table 3. Of the 1152 chromosomes screened, seven could not be scored, as explained below. A total of 117 *Mtn* duplications were found, including examples of the 2.2-, 3.5-, 4.6- and 6.0-kb and CA35 types, plus one new type. This new type appears to have an insert between the two gene copies, just 3′ of the 5′ coding region.

Eight flies, which were heterozygous for an *Mtn* insertion or duplication initially could not be scored as either. The corresponding iso-female line showed no evidence of any rearrangements when checked with *Bam*HI and *Hin*dIII which would explain the *Sal*I restriction pattern. This could mean that either (1) the increase in the size of the *Sal*I fragment was due to an insertion 5′ of the probed *Bam*HI and *Hin*dIII fragments, or (2) the chromosome carrying the rearrangement had been lost from the iso-female line in the 6 weeks since the F_1 flies were frozen for screening, or at any rate, was not carried by any of the 20 flies sampled. It was possible to distinguish between these two possibilities by restricting with *Sal*I some of the same DNA sample which had been digested with *Bam*HI and *Hin*dIII. This revealed that one line had an insert, while the other seven had lost the rearrangement chromosome, and could not be scored.

Of 125 iso-female lines checked with *Bam*HI and *Hin*dIII, 7 had lost the chromosome of interest. This suggests that although the potential for loss of duplications in culture did not affect the data analysis for this largest experiment, it may have been important in experiments I and II, where iso-female lines were maintained for 4 and 20 months, respectively, before being screened for duplications. Note, however, that a comparison of Tables 1, 2 and 3 does not support any substantial and consistent loss of duplications in the laboratory.

The pooled contaminated sites *vs.* control site duplication frequencies for Palmerton and Beaver were 5.4% *vs.* 7.8%, and 12.3% *vs.* 8.4%, respectively. The Mantel-Haenszel *P* for this experiment alone was $\leq$ 0.71 or 0.78 (counting the seven unscorable chromosomes as single-copy in the first case and as duplication chromosomes in the second case); for all three experiments together *P* was $\leq$ 0.31 (0.35, second case). Looking at Beaver alone, including the results of all three experiments, the *P* value was $\leq$ 0.051 (0.069, second case). For Palmerton alone, the value was $\leq$

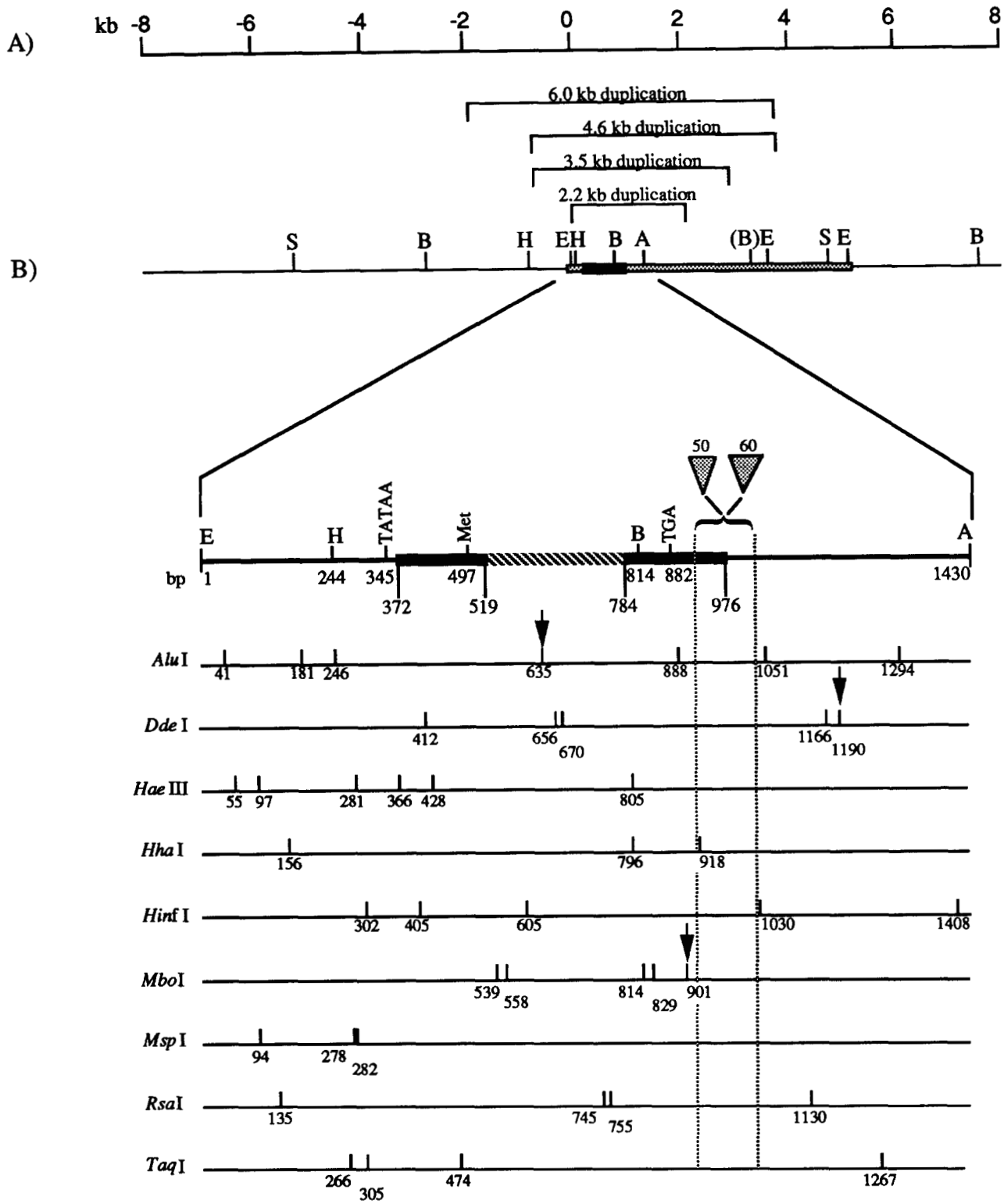

FIGURE 2.—Six-cutter (A) and four-cutter (B) restriction maps of *Mtn*. The abbreviations used for the six-cutters are: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*II; S, *Sal*I. A, six-cutter restriction map of the *Mtn* region. The transcribed portion of *Mtn* is designated by a black box, and the 5-kb *Eco*RI-*Eco*RI fragment homologous to the probe (Dm131 in pUC19) is shaded. The approximate breakpoints of the four known *Mtn* duplications are indicated above the map. B, four-cutter restriction maps of the 1430- bp *Mtn* fragment used as a probe (in pUC19) in the four-cutter analysis. This probe was subcloned from the Dm131-containing pUC19 plasmid used as a probe in the duplication survey. The transcribed portion of *Mtn* is boxed, with the intron hatched. Arrows point to the polymorphic restriction sites. The *Alu*I results suggest that all chromosomes differ from the published sequence at at least two cut sites. The most likely consensus map and polymorphic site location are shown here, including new *Alu*I sites at positions 181 and 635 (polymorphic) and loss of a site at position 1058. The polymorphic *Mbo*I site is either at 901, as shown, or at 1509. Some chromosomes have a 50-bp or a 60-bp insertion between sites 918 and 1030, as indicated.

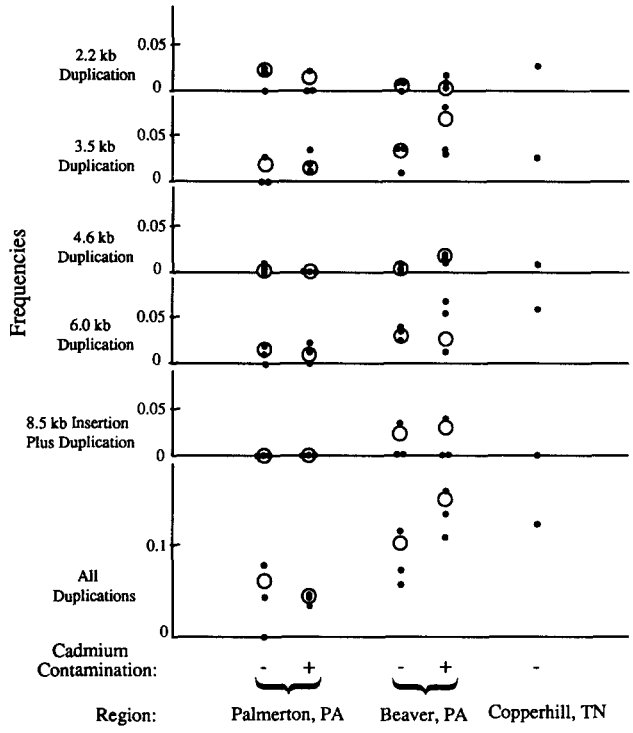

FIGURE 3.—Frequencies of the *Mtn* duplications. The frequencies of each duplication and "all duplications" are plotted for control (−) and contaminated (+) populations in Palmerton and Beaver. Contaminated and control populations were pooled for the one experiment from Copperhill (see text). The small filled dots represent the frequencies in individual experiments (see Tables 1–3). The open circles represent the weighted averages for these experiments.

0.21 (0.24, second case; with the frequency higher at the control site).

Four-cutter restriction map variation: The results of the four-cutter analysis are shown in Table 4. Six different haplotypes were found, including the one predicted by the published sequence. Only one of the *Mtn* duplication chromosomes (a 4.6-kb duplication from the Apple Valley Orchard near Copperhill) had an unusual four-cutter restriction pattern–loss of an *Mbo*I site in one of the two copies of the gene. This was a unique change, not seen in any other line, including the other 4.6-kb duplication chromosome scored in experiment I (Table 1). Two other polymorphic sites were found: a *Dde*I site was lost in two lines from Beaver; and an *Alu*I site was lost in three lines, two from Beaver and one from Copperhill. In addition to these three polymorphic sites, two insertions were found. Like the polymorphic sites, the insertions fall outside of the coding region. They are both in the 114-bp noncoding fragment between positions 918 and 1030. One is a unique, approximately 60-bp insertion, seen in one Zinc City, Pennsylvania, line. The other is an approximately 50-bp insertion found in twelve lines, including some lines from each of the three geographic regions. A summary of the complete data on both four-cutter and six-cutter (experiment I) restriction variation is presented in Table 5.

The 92 chromosomes examined in the four-cutter study do not constitute a random sample. In order to be able to compare the estimates of heterozygosity obtained here with those obtained for other loci, the sample was reduced to a representative sample. Included were the 48 chromosomes chosen at random, the 18 chromosomes chosen on the basis of large insertions found in the six-cutter survey, and 6 of the 26 duplication chromosomes (so that the frequency of duplications in the sample would reflect that found in six-cutter experiment I), for a total sample size (n) of 71 chromosomes. One of the 26 duplications had lost an *Mbo*I site, a unique change. This duplication would be among the six chosen about 23.1% of the time. In other words, there is a 23.1% chance that the random sample would have three segregating sites, and a 76.9% chance that the random sample would have two segregating sites.

TABLE 2

1986 *Mtn* duplication frequencies: experiment II

Orchard	n	No. of duplications of each size (kb)[a]					Frequency (% of duplications		
		2.2	3.5	4.6	6.0	Total	In each orchard	Control *vs.* pooled contam	In each region
Palmerton, Pennsylvania									
Christy (control)	48	1.0		0.5	0.5	2.0	4.2	4.2	
									4.2
Daniels	48		1.0		1.0	2.0	4.2	4.2	
Beaver, Pennsylvania									
Dawson (control)	45	0.5	0.5	0.5	1.0	2.5	5.6	5.6	
									8.2
Curran	25		1.5		1.0	2.5	10.0	10.9	
Lohry	21	0.5		0.5	1.5	2.5	11.9		
Total	187	2.0	3.0	1.5	5.0	12.0			6.4

[a] A duplication seen in a heterozygote (as all but one were) was scored as 0.5, as explained in the text.

TABLE 3

1988 *Mtn* duplication frequencies: experiment III

Orchard	n[a]	No. of duplications of each type: 2.2 kb	3.5 kb	4.6 bk	6.0 kb	CA35	New[b]	Total	Frequency (%) of duplications: In each orchard	Control *vs.* pooled contam	In each region
Palmerton, Pennsylvania											
Christy (control)	296	8 (9)[c]	8	1 (2)	6			23	7.8	7.8	
											6.3
Daniels	138	6	1		2			9	6.5	4.6	
Zinc City	122		2 (3)		1 (2)			3	2.5		
Beaver, Pennsylvania											
Dawson (control)	296	3	11	1	10		9 (11)	34	11.5	11.5	
											13.8
Curran	120	0 (1)	10	2	1	2	8	23	19.3		
Lohry	120	1	9	1	1	1	3	16	13.3	16.0	
Van Buren	60		3	3	2		1	9	15.0		
Total	1152	18	44	8	23	3	21	117			10.2

[a] Here, n is the number of chromosomes screened.
[b] This new duplication type is described in the text.
[c] The numbers in parentheses refer to the counts if the seven nonscorable chromosomes are considered to be duplications. They looked to have possible duplications of the sizes indicated, but this could not be confirmed (see text).

TABLE 4

Four-cutter analysis results

Orchard	n	Insertion between sites 918 and 1030: 60 bp	50 bp	No. of lines exhibiting the following changes: Loss of *Alu*I site 635	Loss of *Dde*I site 1190	Loss of *Mbo*I site 901 or 1509[a]
Palmerton, Pennsylvania						
Christy (control)	11		2			
Daniels	9					
Zinc City	1	1				
Beaver, Pennsylvania						
Dawson (control)	16		2	1	2	
Curran	8		1			
Lohry	10		2	1		
Van Buren	3					
Copperhill, Tennessee						
A. Valley (control)	17		2	1		1
Mountain View	17		3			
Total	92	1	12	3	2	1

[a] It could not be determined which of these two *Mbo*I sites was lost in this chromosome (actually in one tandem repeat of a 4.6-kb duplication chromosome).

Three different estimates of sequence heterozygosity are shown in Table 6, and compared to the corresponding estimates obtained for other loci of *D. melanogaster* from four-cutter data. The estimated heterozygosity per nucleotide site H (KREITMAN and AGUADÉ 1986a) was in this case approximately 0.0007 or 0.0006 for the cases of 3 and 2 segregating sites (S), respectively. This value is below the range found by these authors for the *Adh* coding region and flanking sequences (0.002 − 0.006). The estimate is not changed much ($H = 0.00046$ or 0.0005) if the sample size is taken to be 77, which is the number of copies of the *Mtn* gene surveyed (including *two* from each of the six duplication chromosomes), as opposed to 71, the number of *chromosomes* surveyed. The estimates of θ (HUDSON 1982) for the *Mtn* locus are $\hat{\theta} = 0.002 \pm 0.001$ or $\hat{\theta} = 0.001 \pm 0.0009$ ($S = 3$ or $S = 2$), and are the same whether $n = 71$ or $n = 77$. The index of nucleotide diversity, or π (NEI and LI 1979; NEI and TAJIMA 1981) was calculated to be 0.0005 ± 0.0008 or 0.0004 ± 0.0007 ($n = 71$, $S = 3$ or $S = 2$) for *Mtn*. For the case of $n = 77$, the estimates are 0.0005 ± 0.0007 or 0.0004 ± 0.0007 ($S = 3$ or $S = 2$).

At face value, the estimates obtained here for these measures of sequence diversity are lower than those obtained for other loci. The index of nucleotide diversity and H, in particular, are much lower for *Mtn*, reflecting the fact that the three *Mtn* restriction site

TABLE 5

The *Mtn* haplotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Haplotypes																								
Loci																								
Ins(a)	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
*Alu*I	ns	ns	+	+	+	+	+	ns	+	+	+	+	−	+	+	+	ns	+	+	+	+	−	+	+
*Mbo*I	ns	ns	+	+	+	+	+	ns	+	+	+	+	+	+	+	−	ns	+	+	+	+	+	+	+
Ins(b)	ns	ns	−	−	−	+	−	ns	−	−	−	−	−	−	−	−	ns	−	−	−	−	+	+	+
Ins((c)	ns	ns	−	−	−	−	−	ns	−	−	−	−	−	−	−	−	ns	−	−	+	−	−	−	−
*Dde*I	ns	ns	+	+	+	+	+	ns	+	+	+	+	+	−	+	+	ns	+	+	+	+	+	+	+
Ins(d)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
*Bam*HI	+	−	−	+	−	+	−	ns	+	−	−	ns	+	−	−	−	ns	−	−	+	−	+	−	ns
Ins(e)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ins(f)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
2.2-kb duplication	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3.5-kb duplication	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
Ins(3.5 kb duplication)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
4.6-kb duplication	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
6.0-kb duplication	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Ins(6.0-kb duplication)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Numbers																								
Collection sites																								
PC (51)	27	13	6	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
PD (50)	19	18	4	3	0	0	1	3	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
PZ (7)	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
BD (54)	18	19	2	1	2	1	2	1	3	0	2	0	0	2	0	0	0	0	0	0	0	1	0	0
BC (25)	5	12	0	1	3	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
BL (29)	7	12	3	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0
BV (5)	0	2	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CA (51)	21	12	5	2	3	2	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0
CM (55)	19	16	6	0	2	2	2	3	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	1
Total (327)	121	105	26	12	11	9	7	8	5	4	3	2	2	2	1	1	1	1	1	1	1	1	1	1

Key: PC, Palmerton-Christy; PD, Palmerton-Daniels (cadmium-contaminated); PZ, Palmerton-Zinc City (cadmium-contaminated); BD, Beaver-Dawson; BC, Beaver-Curran (cadmium-contaminated): BL, Beaver-Lohry (cadmium-contaminated) BV, Beaver-Van Buren (cadmium-contaminated); CA, Copperhill-Apple Valley; CM, Copperhill-Mountain View. +, present; −, absent; ns, not scored.

TABLE 6

Estimates of heterozygosity per nucleotide from four-cutter data for four loci of *D. melanogaster*

Locus	H^a (×10³)	$\hat{\theta}^b$ (×10³)	π^c (×10³)
Adh[d]	2 to 6		
white[e]		4 ± 2	4 ± 2
Zw[f]		2.89	3.41
Mtn			
$n = 71, S = 3$	0.7	2 ± 1	0.5 ± 0.8
$n = 71, S = 2$	0.6	1 ± 0.9	0.4 ± 0.7
$n = 77, S = 3$	0.6	2 ± 1	0.5 ± 0.7
$n = 77, S = 2$	0.5	1 ± 0.9	0.4 ± 0.7

[a] KREITMAN and AGUADÉ (1986a).
[b] HUDSON (1982).
[c] NEI and LI (1979); NEI and TAJIMA (1981).
[d] KREITMAN and AGUADÉ (1986b).
[e] MIYASHITA and LANGLEY (1988).
[f] MIYASHITA (1990)
Key: n, sample size; S, number of segregating sites.

variants are all in low frequency in the sample. The test of HUDSON, KREITMAN and AGUADÉ (1987) was used to compare the $\hat{\theta}$ value of *Mtn* with that of the *Adh* region.

DNA sequence of the *Mtn* region of *D. simulans* and application of the HUDSON-KREITMAN-AGUADÉ (HKA) test: Figure 4 shows the alignment of the *D. simulans* sequence with that of *D. melanogaster* (MARONI, OTTO and LASTOWSKI-PERRY 1986) in the region probed in the four-cutter survey. The total sequence obtained for the *Mtn* region of *D. simulans* (1684 nucleotides) will be available in GenBank (accession number: M55407). While the intron and 3′ regions are highly diverged the coding and 5′ regions are very conserved. Only one nucleotide difference was observed in the coding region. Surprisingly this is a nonsynonomous difference changing a lysine to a glutamine. Note also the conservation of the sequences 5′ of the TATA box that are thought to be important in the heavy-metal-induced transcription of eukaryotic metallothioneins (MARONI, OTTO and LASTOWSKI-PERRY 1986).

To apply the HKA test of selective neutrality to data on silent variation in both the *Mtn* and *Adh* regions, we used the intraspecific data obtained from our four-cutter analysis of the *Mtn* region and KREIT-

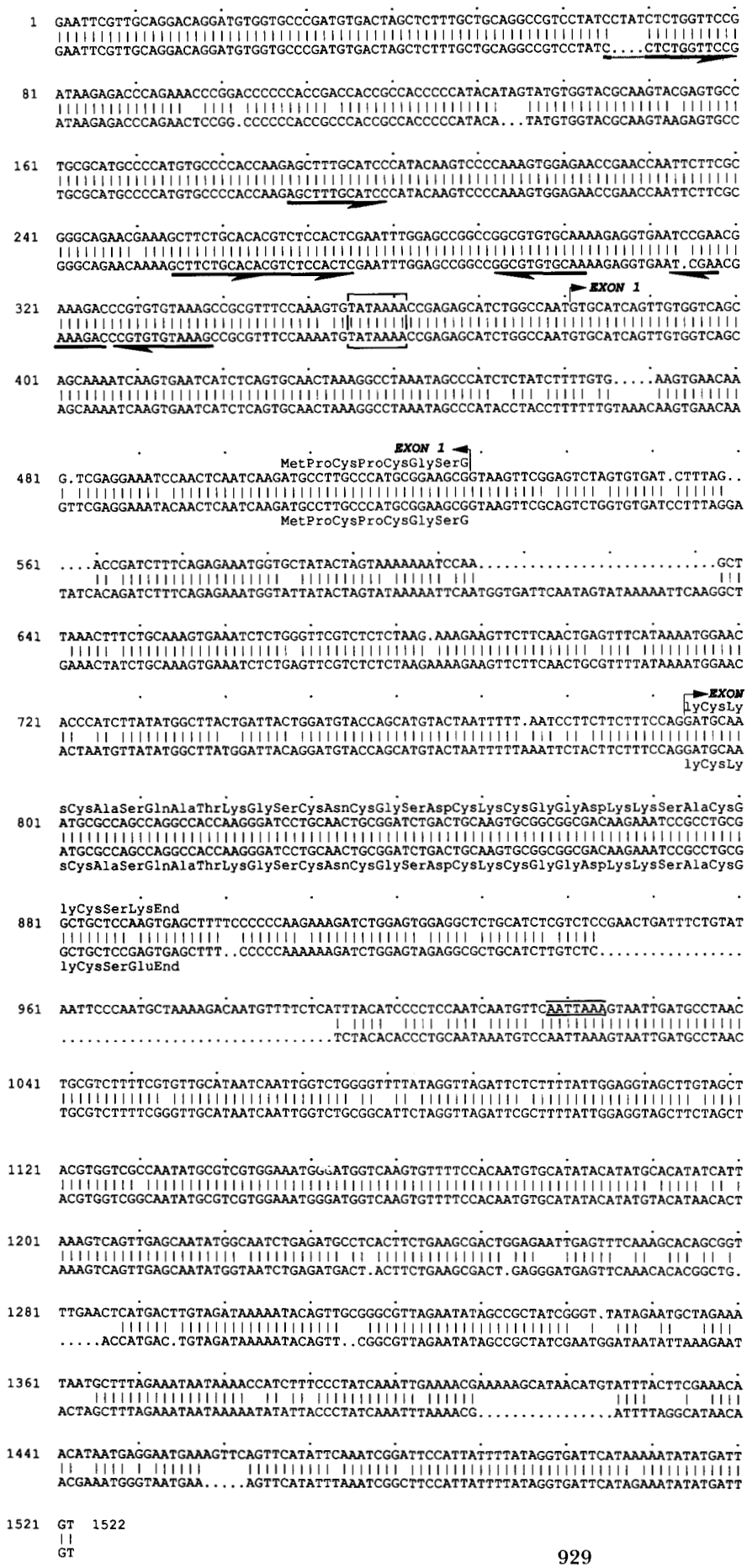

FIGURE 4.—The aligned DNA sequence of the *Mtn* region of *D. simulans* (top) and *D. melanogaster* (bottom). The first nucleotide position corresponds to position 1 in the *Mtn* sequence of *D. melanogaster* in GenBank, accession number M12964 (position 190 corrected to C). The sequence sequences were aligned using the *Align* algorithm (DEVEREUX, MAEDERLI and SMITHIES 1984) in the Sequence Analysis Software, Version 6.0 from the Genetics Computer Group, University of Wisconsin, Madison. Sequences in the 5′ region that are underlined by arrows are typical of conserved sequences found in the 5′ regions of metallothionein genes in most eukaryotes (MARONI, OTTO and LASTOWSKY-PERRY 1986). The presumptive TATA box and polyadenylation site are enclosed in boxes. The boundaries of the two exons are indicated. The amino acid difference (Lys to Glu) is attributable to the only nucleotide difference in the coding region (A to G) at position 890.

TABLE 7

DNA polymorphism at *Mtn* and *Adh* in *D. melanogaster* and divergence between *D. melanogaster* and *D. simulans* at *Mtn* and between *D. melanogaster* and *D. sechellia* at *Adh*

		Adh regions		
	Mtn region	5′-Flanking	*Adh* locus	3′-Flanking
Within species:				
Size	1430	4335	1858	801
Silent site equivalents	233	425	250	141
Polymorphic sites (obs.)	2	10	14	2
Sample size	71	81	81	81
Between species:				
Silent sites aligned	1298	4202	1316	784
Silent site differences (obs.)	93	285	58	32
HUDSON-KREITMAN-AGUADÉ tests:				
θ per region [*Mtn*]		1.56 [0.88]	1.45 [1.82]	0.22 [0.60]
Polymorphic sites (exp.) [*Mtn*]		7.76 [4.24]	7.20 [8.80]	1.08 [2.92]
Silent site differences (exp.) [*Mtn*]		287.22 [90.78]	64.80 [86.20]	32.92 [92.08]
X^2 [d.f. = 1]		1.39	8.23	1.02

The data on the *Adh* locus and its 3′-flanking region in *D. melanogaster* were obtained from the consensus sequence in KREITMAN (1983). The *Adh* locus spans from coordinate 1 to 1858 of KREITMAN's (1983) sequence. Included into the *Adh* 5′-flanking region in *D. melanogaster* (KREITMAN and AGUADÉ 1986b) were 63 bp of the 5′-flanking region in KREITMAN (1983). The information on the *Adh* region in *D. sechellia* is based on sequence data of COYNE and KREITMAN (1986) and M. AGUADÉ and M. KREITMAN (unpublished results; see HUDSON, KREITMAN and AGUADÉ 1987). The intraspecific data on silent variation in the *Adh* regions are based on the four-cutter analysis of KREITMAN and AGUADÉ (1986b). The number of silent site differences in the 1430-bp *Eco*RI-*Acc*I fragment of *Mtn* (coordinates 1 to 1430 in Figure 2) are obtained from a sequence comparison of one *D. simulans* allele (Figure 4) with one allele from *D. melanogaster* (MARONI, OTTO and LASTOWSKI-PERRY 1986). The lower part of the table shows the results of the HUDSON-KREITMAN-AGUADÉ test, as applied to *Mtn* and each of the regions of *Adh*. The numbers in parentheses denote the corresponding values for *Mtn*.

MAN and AGUADÉ's (1986b) data on the *Adh* regions. A summary of these data is shown in Table 7. The interspecific data on the *Mtn* region are based on a sequence comparison of one *D. melanogaster* allele (MARONI, OTTO and LASTOWSKI-PERRY 1986) and one *D. simulans* allele (Figure 4). Sequence comparisons at *Adh* were done between consensus sequences of *D. melanogaster* and *D. sechellia* (see Table 7). *D. sechellia* is more closely related to *D. simulans* than to *D. melanogaster* (COYNE and KREITMAN 1986). The application of the HKA test in this case assumes a more recent common ancestor for *D. simulans* and *sechellia* and that the neutral mutation rates have been the same in both lines of descent.

In the original application of the HKA test the various regions of the *Adh* region were shown to be inconsistent with the simple assumptions of the tests (HUDSON, KREITMAN and AGUADÉ 1987). For this reason we compared by the HKA test the polymorphism and divergence at *Mtn* with those at each region of *Adh* (5′-flanking, coding and 3′-flanking). Table 7 shows the observations and results for each of these tests. The X^2 for each test is approximately distributed as a χ^2 with one degree of freedom (HUDSON, KREITMAN and AGUADÉ 1987). By this criterion only the comparison between *Mtn* and the coding region of *Adh* is statistically significant. Since the coding region of *Adh* is thought to demonstrate more silent site polymorphism than predicted under neutral mutation and genetic drift (HUDSON and KAPLAN 1988), the large X^2 of the comparison of *Mtn* with the coding region at *Adh* can be primarily attributed to *Adh*.

It should be noted that the HKA test has little statistical power to detect a reduction in the polymorphism at *Mtn* in the comparison with the 5′ flanking region of *Adh*. If only one segregating site had been observed at *Mtn* this would also not have been statistically significant. If zero segregating sites had been observed this would have only been marginally significant ($X^2 = 4.22$; $P < 0.05$).

DISCUSSION

The observation of cosmopolitan gene duplications of *Mtn* that confer resistance to heavy metal toxicity suggests that the other Drosophila metallothionein gene, *Mto*, may also be duplicated in natural populations. Indeed in MOKDAD, DEBEC and WEGNEZ (1987) increased copy numbers of *Mto* were associated with heavy metal resistance in Drosophila tissue culture cells. However, the survey by Southern blot analysis of 327 chromosomes from several natural populations (contaminated and control) found no duplicated *Mto* genes. The contribution of *Mto* to heavy metal tolerance may be specific to cell culture. Resistance in whole animals may depend largely on *Mtn*.

The central goal of the research reported here was to attempt to associate the frequencies of *Mtn* duplications with environmental variation in heavy metal

contamination. The thesis underlying this project was that strong selection in favor of these duplications may have generated increased frequencies in local populations exposed to these toxic elements. Three sequential surveys were carried out on the two Pennsylvania populations where heavy metal contamination of the flies and their environment was actually demonstrated. Despite some heterogeneity among populations we failed to detect any consistent association of the frequencies of the *Mtn* duplications with heavy metal exposure.

Two obvious explanations for this lack of correlation of *Mtn* duplication frequencies with heavy metal contamination are (1) that the rates of migration from the many less contaminated regions into the heavily polluted study sites are so large that any differentiation in duplication frequencies caused by selection is swamped out or (2) that genetic variation at many other loci confer heavy metal tolerance, *i.e.*, the contribution of *Mtn* duplications in natural populations to heavy metal tolerance is minimal. However, cadmium tolerance tests performed on the F_2 descendants of females collected in 1988 failed to detect differences between control and contaminated sites (LANGE 1989). These results support the idea that migration rates are high, which is in agreement with the results of four-cutter surveys of the *Adh* locus, suggesting that *D. melanogaster* populations separated by very large distances can be genetically homogeneous (KREITMAN and AGUADÉ 1986a; SIMMONS *et al.* 1989). HANSON (1966) and SLATKIN (1973) have investigated the ability of a local "pocket" of selection in favor of an alternative allele to cause a stable differentiation in gene frequency. The quantitative application of their theory to the frequencies of the *Mtn* duplications in the relatively highly polluted areas downwind of the two Pennsylvania smelters in this study would require knowledge of the *difference* in selection in the polluted and unpolluted regions (s), knowledge of the standard deviation of the distance migrated (l) and the size of the polluted region. In the absence of any actual measurements of these quantities we note that the ability of such local selection to create any differentiation in gene frequency requires that the size of the region of local selection be quite large ($l/\sqrt{s}$).

While our results indicate that *differences* in the selection on *Mtn* duplications in local populations may be obscured by migration they do not rule out significant selection in the *total* population. Heavy metal pollution is largely associated with industrial development. An attractive hypothesis about the evolution of the *Mtn* locus is that industrialization has led to strong selection for increased expression of the locus and that certain alleles (haplotypes) have recently risen to high frequencies (MARONI *et al.* 1987). One scenario is that increased levels of heavy metals (perhaps during the last 200 years) lead to selection favoring a particular allele with the highest activity. The selective advantage of this allele would have to be greater than 7×10^{-3} if its initial frequency were 10^{-3}, assuming that there are 10 generations per year and that it has reached a frequency greater than 0.999 (KAPLAN, HUDSON and LANGLEY 1989). The continued selection for increased expression of *Mtn* may have drawn rare duplications into intermediate frequencies and allowed them to spread throughout the species distribution (MARONI *et al.* 1987). Polymorphism for this type of duplication is unknown in Drosophila. Such duplications can be expected to suffer a net loss each generation due to intrachromosomal recombination. Thus it is reasonable to assume that selection for increased *Mtn* function continues. If this scenario is correct the recent selective events have drastically changed the distribution of molecular polymorphism in the *Mtn* region. Two expected consequences of recent strong selection at such a locus are linkage disequilibrium and reduced polymorphism (MAYNARD SMITH and HAIGH 1974; KAPLAN, HUDSON and LANGLEY 1989). In our data there are three obvious cases of linkage disequilibrium the polymorphic *Alu*I site with the 50-bp insertion ($P < 0.005$); [the polymorphic *Bam*HI site with the 6.0-kb duplication (Beaver and Copperhill pooled, $P < 0.005$; Palmerton and Copperhill pooled, $P < 0.05$), and the polymorphic *Bam*HI site with the 50-bp insertion (Palmerton and Copperhill pooled, $P < 0.005$)]. However all polymorphisms were relatively rare (except that at *Bam*H I) affording very little power to detect any other linkage disequilibrium.

To judge the significance of the apparently low level of molecular polymorphism at the *Mtn* locus (see Table 6) we cloned and sequenced the *Mtn* gene from *D. simulans* to allow the application of the HKA test, which compares the polymorphism and divergence at two loci under the assumption of selective neutrality and genetic drift (HUDSON, KREITMAN and AGUADÉ 1987). We compared *Mtn* with each of the three regions of the *Adh* locus studied by KREITMAN and AGUADÉ (1986b). The only comparison that proved statistically significant was that between *Mtn* and the coding region of the *Adh* locus (see Table 7). Since the excess polymorphism in the coding region of *Adh* is suspected to be due to a balanced polymorphism, this result can not be interpreted as demonstrating reduced polymorphism at *Mtn* (HUDSON and KAPLAN 1988). The comparison of *Mtn* with the 5′ flanking region of *Adh* was not statistically significant (see Table 7), but it should be noted that there was little power to detect the expected reduction. A much larger experiment based on DNA sequencing of alleles from natural populations will be needed to confidently test this prediction of the proposed scenario.

LITERATURE CITED

BEACH, L. R., and R. D. PALMITER, 1981 Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. Proc. Natl. Acad. Sci. USA **78:** 2110–2114.

Beaver County Cooperative Extension, 1988 College of Agriculture, U.S. Department of Agriculture and Pennsylvania Counties Cooperating, 1988 Heavy Metals in Beaver County Soils.

COYNE, J. A., and M. KREITMAN, 1986 Evolutionary genetics of two sibling species, *Drosophila simulans* and *D. sechellia*. Evolution **40:** 673–691.

DEBEC, A., R. MOKDAD and M. WEGNEZ, 1985 Metallothioneins and resistance to cadmium poisoning in Drosophila cells. Biochem. Biophys. Res. Commun. **127:** 143–152.

DEVEREUX, J., P. MAEDERLI and O. SMITHIES, 1984 A comprehensive set of sequence analysis programs for the Vax. Nucleic Acid Res. **12:** 384–395.

FINNEGAN, D. J., and D. H. FAWCETT, 1986 Transposable elements in *Drosophila melanogaster*, pp. 1–62 in *Oxford Surveys on Eucaryotic Genetics*, Vol. 3, edited by N. MACLEAN. Oxford University Press, New York.

FLEISS, J. L., 1981 *Statistical Methods for Rates and Proportions*. John Wiley & Sons, New York.

FOGEL, S., and J. W. WELCH, 1982 Tandem gene amplification mediates copper resistance in yeast. Proc. Natl. Acad. Sci. USA **79:** 5342–5346.

HANSON, W. D., 1966 Effects of partial isolation (distance), migration, and different fitness requirements among environmental pockets upon steady state gene frequencies. Biometrics **22:** 453–468.

HUDSON, R. R., 1982 Estimating genetic variability with restriction endonucleases. Genetics **100:** 711–719.

HUDSON, R. R., and N. L. KAPLAN, 1988 The coalescent process in models with selection and recombination. Genetics **120:** 831–840.

HUDSON, R. R., M. KREITMAN and M. AGUADÉ, 1987 A test of neutral molecular evolution based on nucleotide data. Genetics **116:** 153–159.

KAPLAN, N. L., R. R. HUDSON and C. H. LANGLEY, 1989 The "hitchhiking effect" revisited. Genetics **123:**887–899.

KARIN, M., R. NAJARIAN, A. HASLINGER, P. VALENZUELA, J. WELCH and S. FOGEL, 1984 Primary structure and transcription of an amplified genetic locus: the *CUP1* locus of yeast. Proc. Natl. Acad. Sci. USA **81:** 337–341.

KEMPHUES, K. J., E. C. RAFF and T. C. KAUFMAN, 1983 Genetic analysis of *B2t*, the structural gene for a testis-specific β-tubulin subunit in *Drosophila melanogaster*. Genetics **105:** 345–356.

KOROPATNICK, J., R. WINNING, E. WIESE, M. HESCHL, L. GEDAMU and J. DUERKSEN, 1985 Acute treatment of mice with cadmium salts results in amplification of the metallothionein-1 gene in liver. Nucleic Acids Res. **13:** 5423–5439.

KREITMAN, M., 1983 Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature **304:** 412–417.

KREITMAN, M., and M. AGUADÉ, 1986a Genetic uniformity in two populations of *Drosophila melanogaster* as revealed by filter hybridization of four-nucleotide-recognizing restriction enzyme digests. Proc. Natl. Acad. Sci. USA **83:** 3562–3566.

KREITMAN, M., and M. AGUADÉ, 1986b Excess polymorphism at the *Adh* locus in *Drosophila melanogaster*. Genetics **114:** 93–110.

LANGE, B. W., 1989 *Drosophila melanogaster* metallothionein genes: selection for duplications? Ph.D. thesis, Duke University, Durham, North Carolina.

LANGLEY, C. H., 1990 The molecular population genetics of Drosophila, pp. 75–91 in *Population Biology of Genes and Molecules*, edited by N. TAKAHATA and J. F. CROW. Biafukan, Tokyo.

LASTOWSKI-PERRY, D., E. OTTO and G. MARONI, 1985 Nucleotide sequence and expression of a Drosophila metallothionein. J. Biol. Chem. **260:** 1527–1530.

LYCKEGAARD, E. M. S., and A. G. CLARK, 1989 Ribosomal DNA and *stellate* gene copy number variation on the *Y* chromosome of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **86:** 1944–1984.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MARONI, G., E. OTTO and D. LASTOWSKI-PERRY, 1986 Molecular and cytogenetic characterization of a metallothionein gene of Drosophila. Genetics **112:** 493–504.

MARONI, G., and D. WATSON, 1985 Uptake and binding of cadmium, copper and zinc by *Drosophila melanogaster* larvae. Insect Biochem. **15:** 55–63.

MARONI, G., J. WISE, J. E. YOUNG and E. OTTO, 1987 Metallothionein gene duplications and metal tolerance in natural populations of *Drosophila melanogaster*. Genetics **117:** 739–744.

MAYNARD SMITH, J., and J. HAIGH, 1974 The hitchhiking effect of a favorable gene. Genet. Res. **23:** 23–35.

MIYASHITA, N., 1990 Molecular and phenotypic variation of the *Zw* locus region in *Drosophila melanogaster*. Genetics **125:** 407–419.

MIYASHITA, N., and C. H. LANGLEY, 1988 Molecular and phenotypic variation of the *white* locus region in *Drosophila melanogaster*. Genetics **120:** 199–212.

MOKDAD, R., A. DEBEC and M. WEGNEZ, 1987 Metallothionein genes in *Drosophila melanogaster* constitute a dual system. Proc. Natl. Acad. Sci. USA **84:** 2658–2662.

NEI, M., and W.-H. LI, 1979 Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA. **76:** 5269–5273.

NEI, M., and F. TAJIMA, 1981 DNA polymorphism detectable by restriction endonucleases. Genetics **97:** 145–163.

SHRIMPTON, A. E., E. A. MONTGOMERY and C. H. LANGLEY, 1986 *OM* mutations in *Drosophila ananassae* are linked to insertions of a transposable element. Genetics **114:** 125–135.

SIMMONS, G. M., M. E. KREITMAN, W. F. QUATTLEBAUM and N. MIYASHITA, 1989 Molecular analysis of the alleles of alcohol dehydrogenase along a cline in *Drosophila melanogaster*. I. Maine, North Carolina, and Florida. Evolution **43:** 393–409.

SLATKIN, M., 1973 Gene flow and selection in a cline. Genetics **75:** 733–756.

STELLER, H., 1986 Rapid small scale isolation of *Drosphila* DNA and RNA, p. 10.1 in *Rubin Lab Methods Book*.

TAKANO, T., S. KUSAKABE, A. KOGA and T. MUKAI 1989 Polymorphism for the number of tamdemly multiplicated glycerol-3-phosphate dehydrogenase genes in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **86:** 5000–5004.

WASHINGTON, J. W., 1985 Metallic contamination surrounding a zinc smelter. M.S. thesis, Pennsylvania State University.

WELCH, J. W., S. FOGEL, G. CATHALA and M. KARIN, 1983 Industrial yeasts display tandem gene iteration at the *CUP1* region. Mol. Cell. Biol. **3:** 1353–1361.

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