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Recent evolutionary history of the metallothionein gene *Mtn* in *Drosophila*

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Summary

A new allele of one of the metallothionein genes of *D. melanogaster*, $Mtn^{\cdot 3}$, sheds light on the recent evolution of this gene. In comparison to the previously studied Mtn^{1} allele found in Canton S, this new allele, $Mtn^{\cdot 3}$, produces a transcript that is 49 bases longer and 65–70% less abundant. We detected $Mtn^{\cdot 3}$ in several laboratory strains as well as in isofemale lines derived from natural populations. Sequence comparison showed that $Mtn^{\cdot 3}$ differs from Mtn^{1} in that it has: (*a*) base-pair substitution and an extra 49 bp-segment in the 3′ untranslated region, (*b*) a substitution in the coding region that replaces the terminal Glu40 in Mtn^{1} with Lys40, and (*c*) two base-pair substitutions in the promoter region. The $Mtn^{\cdot 3}$-type was detected in six species of the *melanogaster* group by restriction analysis, and this result was confirmed by sequencing the *D. simulans Mtn* gene. Thus $Mtn^{\cdot 3}$, which produces a less abundant transcript, appears to be the oldest of the two alleles. We also found that the duplications previously isolated from natural populations all derived from Mtn^{1}, the more recent allele. Thus, two evolutionary steps: $Mtn^{\cdot 3}$ to Mtn^{1} and Mtn^{1} to $Dp(Mtn^{1})$, are accompanied by an overall 5- to 6-fold increase of RNA accumulation. The two changes seem to have occurred in non-African populations since $Mtn^{\cdot 3}$ but not Mtn^{1} was detected in our sample from tropical Africa, while Mtn^{1} and *Dp* (Mtn^{1}) are prevalent in European and North American samples.

1. Introduction

Metallothioneins (MT) are metal-binding, cysteine-rich, low-molecular-weight proteins present in most eukaryotes (Maroni, 1989; Hamer, 1986). There are two metallothionein genes in *Drosophila melanogaster*, *Mtn* (Lastowski-Perry, Otto & Maroni, 1985), and *Mto* (Mokdad, Debec & Wegnez, 1987). The *Mto* and *Mtn* proteins are quite different at the level of their primary structure (only 25% amino-acid identity), in contrast to MT gene families in other species. In addition, the basal transcript level is higher for *Mtn* than for *Mto*, and the two genes have different expression patterns during development (Silar *et al.* 1990). Despite these differences, similarity in the gene structure suggests that the two MT genes of *Drosophila* are derived from a common ancestor by a gene duplication event (Maroni, Otto & Lastowski-Perry, 1986*b*; Erraiss *et al.* 1989).

MT genes have served as a model for studies of basal and induced gene transcription, but little is known about their physiological function(s). The two roles most commonly suggested for this protein are metal homeostasis and metal detoxification (Karin, 1985).

MTs have the capacity to transfer zinc to metalloenzymes *in vitro* (Seagrave *et al.* 1986, Churchich *et al.* 1989). Both zinc and copper are trace metals essential for the activity of polymerases, transcription factors and metalloenzymes (Hanas *et al.* 1983; Evans & Hollenberg, 1988). Thus, through control of zinc and copper intracellular homeostasis, MTs may play key roles in cell proliferation and differentiation processes. In fact, MTs are temporally and tissue-specifically regulated during development in mammals, sea urchin, and *Drosophila* (Andrews *et al.* 1987; Wilkinson & Nemer, 1987; Nishimura, Nishimura & Toyama, 1989; Silar *et al.* 1990).

Also, the metal-binding capacity and inducibility of MTs suggest that they may be involved in protection against metal toxicity. In *Drosophila*, copper and cadmium exert a pleiotropic toxic effect that reduces

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growth rates and viability (Jacobson *et al.* 1981; Maroni & Watson, 1985; Lauvergeat, Ballan-Dufrançais & Wegnez, 1989). Among the possible genetic determinants of metal resistance, the *Mtn* gene is a good candidate. This is suggested by the observation that four duplications of the *Mtn* gene isolated from natural populations confer increased tolerance to copper and cadmium when compared to strains containing a single copy of the *Mtn* gene (Otto, Young & Maroni, 1986; Maroni *et al.* 1987). While *Mtn* duplications occur frequently in natural populations, no *Mto* duplications have been found (Lange, Langley & Stephan, 1990, and this report). The geographic distribution of *Mtn* duplications is intriguing. They occur at high frequency in European and North American samples, but they are absent from African samples (Maroni *et al.* 1987).

In this report we characterize the molecular organization and expression of a new allele of *Mtn* and its frequency in populations of *D. melanogaster* from different geographic regions. Our study included restriction analysis of sibling species of *D. melanogaster* and sequencing of the *Mtn* gene of one of these species (*D. simulans*) to determine the evolutionary relationship between this new allele and the previously identified *Mtn* alleles.

2. Materials and methods

(i) Drosophila *strains*

All the genetic markers and balancers mentioned are described in Lindsley & Zimm (1985, 1990). *Drosophila* lines from natural populations used in this study were derived from single, fertilized females ('isofemale' lines) captured in the wild.

Drosophila melanogaster. Thirty lines from a fruit market in Loua (Congo) and 40 lines from vineyards in Grande Ferrade, near Bordeaux (France) were provided by Dr Jean David. *Drosophila simulans*. Twenty-six lines from Australia, South Africa, the Congo, Tunisia, France and Japan (4–6 lines from each location) were provided by Dr Cathy Laurie and six lines from Grande Ferrade were provided by Dr David. Other species of the melanogaster group: *D. mauritiana*, *D. sechellia*, *D. tessieri*, *D. yakuba*, *D. orena*, *D. erecta* samples were provided by Dr Laurie. The balancer *TM3* and the multiply marked chromosome *rucuca* were used to extract the third chromosome from a *red* strain and to prepare stocks homozygous for third chromosomes that had undergone recombination between the markers *st* (3–44) and *cu* (3–50·0).

(ii) *Bacterial strains and plasmids*

The *DH5-alpha E. coli* strain was used for propagation and cloning of all plasmids. Competent cells were obtained from BRL (Gaithersburg, MD, USA) and bacteria were grown following the manufacturer's instructions. Plasmid DNA was prepared as described previously; the plasmid cDm51 contains Mtn^1 cDNA (Lastowski-Perry *et al.* 1985).

(iii) *Polymerase chain reaction (PCR) and DNA analysis*

Fly DNA was extracted as previously described (Maroni *et al.* 1987). Two 30-mer oligonucleotides were designed for the amplification of a 1·2 kb *Mtn* genomic fragment. Primer B44 matches the Mtn^1 sequence from coordinate 1–30 and primer B123 matches the Mtn^1 sequence from 1155 to 1125 (Fig. 2). B123 also introduced an *Xho* I site at 1145 to allow easier subcloning when required. The reaction mixture contained 10 ng/μl template DNA, 8 ng/μl of each primer, 0·025 unit/μl *Taq* polymerase (AmpliTaq, Perkin-Elmer Cetus, Norwalk, CT, USA) in Tris-Cl pH 8·3 (9·2 mM), KCl (46 mM), $MgCl_2$ (1·75 mM), gelatin (0·01 %). Seakem agarose 0·5 %, Nusieve 2·5 % (FMC, Rockland, ME, USA) in Tris-acetic acid (40 mM), EDTA (1·0 mM) pH 7·8 (TAE buffer) were used for gel electrophoresis of the PCR products. Southern blot analyses were performed as previously described (Maroni *et al.* 1987).

(iv) *DNA sequencing*

To avoid sequence errors inherent to *Taq* polymerase mis-incorporation during the amplification process, genomic DNA of the *red* strain was subjected to 20 cycles of amplification in three independent experiments. The amplified genomic fragments were pooled and extracted with 1 vol. of phenol, fractionated on a 0·7 % agarose gel in TAE buffer, purified with GeneClean (Bio 101, La Jolla, CA, USA), and digested with a combination of *Pst* I, *Bgl* II, *Hin*d III and *Xho* I to generate 200–350 bp fragments suitable for cloning in the pBS/SK – vector (Stratagene, La Jolla, CA, USA). For each fragment, 4–8 clones were isolated, mixed and both strands sequenced. The same protocol was used for the *D. simulans Mtn* gene except that subclones were generated using the enzymes *Pst* I, *Hin*d III, *Stu* I, *Bgl* II, *Bam*H I and *Xho* I. The method of Sanger (1977) was used to sequence the double-stranded templates using the Sequenase Version 2 kit (USB, Cleveland, USA). Substitution of dITP (USB) for dGTP helped resolve the compression occurring at positions 871–876 (Fig. 3) in both species.

(v) *RNA analyses*

RNA assays were carried out as previously described (Otto *et al.* 1986; Maroni *et al.* 1987).

3. Results

(i) *A new pattern of* Mtn *expression was found in a laboratory strain*

Northern blot analysis of a *D. melanogaster* laboratory stock carrying the marker *red* showed differences in both the mobility and the abundance of *Mtn* mRNA (Fig. 1). Compared with *Mtn* mRNA from other previously characterized strains, e.g. Canton S, *Mtn* mRNA in *red* was larger by 50–100 nucleotides and its abundance was reduced by 65–70% (Table 1). In order to determined the linkage between the new phenotype and the *Mtn* locus, heterozygotes were constructed by crossing flies from the *red* stock with the multiply marked third chromosome *rucuca* stock (homozygote *rucuca* flies have the same *Mtn* expression pattern as Canton S). Transcript analysis of eight lines derived from gametes in which there had been a recombination event between *st* (3–44·0) and *cu* (3–50·0), is consistent with the existence of linkage between the genetic determinants for both the size and abundance of the *Mtn* transcript and the structural gene *Mtn* (3–49·0) (data not shown).

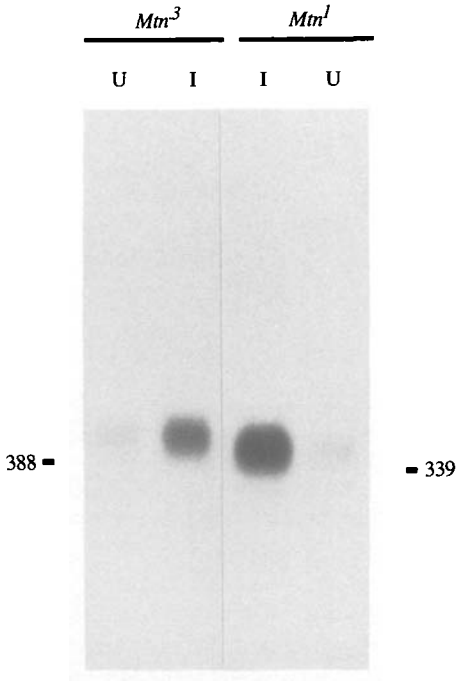

Fig. 1. Autoradiograph of a Northern blot of RNA from *Mtn*$^{\cdot 3}$ and *Mtn*^{1} larvae hybridized to a *Mtn*^{1} cDNA probe. The larvae were fed for 24 h on 0·16 mM $CdCl_2$-supplemented food to induce MT biosynthesis (I, induced), or on normal food (U, uninduced). Equal amounts of total RNA (6 μg) were loaded in each lane. To obtain the data for Table 1, portions of the membranes containing the signal were cut out, and radioactivity was measured by liquid scintillation counting. Similar results were obtained for three other *Mtn*$^{\cdot 3}$ strains and several *Mtn*^{1} strains.

Table 1. Mtn *RNA levels in* D. melanogaster *larvae homozygous for three different alleles*

	MTN$^{\cdot 3}$	*Mtn*^{1}	*Dp*(*Mtn*^{1})
Uninduced	26·1 ± 5	39·9 ± 1	51·6 ± 19
Induced	161·8 ± 8	442·1 ± 58	695·8 ± 39

The values (^{32}P cpm) are averages of data from three independent extractions of larval RNA (see Fig. 1 legend). Similar results were obtained in other experiments using RNA from adults or where induction was carried out with copper instead of cadmium salts.

(ii) *The molecular characteristics of the* Mtn *locus in* red *and Canton S are different*

Comparison of the restriction patterns of *Mtn* in *red* and Canton S by Southern analysis showed a difference in a *Bgl* II–*Ssp* I fragment (Fig. 2) that includes the 3′ transcribed but untranslated region of *Mtn*: in *red*, this segment was slightly longer than in Canton S (data not shown). We designate the *Mtn* allele found in *red* *Mtn*$^{\cdot 3}$ to reflect its lower abundance. The previously described allele (Maroni *et al.* 1986*b*) will now be designated as *Mtn*^{1}.

We used PCR to amplify the *Mtn* region; the amplified segment extended from position 1, 373 bp upstream of the transcription start site, to position 1155, 303 bp downstream of the polyadenylation site (Fig. 2). The amplification product from *red* genomic DNA was cloned and sequenced (Fig. 3). *Mtn*^{1} and *Mtn*$^{\cdot 3}$ sequences are distinguished by four single base-pair substitutions: two in the promoter region, one in the 3′ untranslated region, and one that leads to the substitution of the terminal Glu40 of *Mtn*^{1} with Lys40 in *Mtn*$^{\cdot 3}$. Further, the 3′ untranslated region of *Mtn*$^{\cdot 3}$ is 49 bp longer than in *Mtn*^{1}. The latter would account for the size difference between mRNAs.

(iii) *The* Mtn$^{\cdot 3}$*-type restriction pattern is found in sibling species of* Drosophila melanogaster

In order to determine which of the two *Mtn* alleles may be more similar to the ancestral allele, we examined seven other species in the melanogaster group using a PCR-based method with *D. melanogaster* primers. To distinguish between the *Mtn*^{1} and the *Mtn*$^{\cdot 3}$ alleles, the amplification products were digested with *Bam*H I; this enzyme cuts in the second exon (see Fig. 2) and gives rise to two bands, one corresponding to an 814-bp 5′ fragment and the second to either a 284-bp (*Mtn*^{1}) or a 333-bp (*Mtn*$^{\cdot 3}$) fragment. Thus, clear allelic typing of the *Mtn* locus is possible since the 49-bp difference in the 3′ fragment of *Mtn*^{1} and *Mtn*$^{\cdot 3}$ can be resolved by agarose gel electrophoresis. Figure 4 presents the results of this analysis. For all eight species, using *D. melanogaster* primers, there is enough sequence conservation in the flanking region of the *Mtn* gene to generate signals that are clearly distinguishable. Four species have patterns very similar to *D. melanogaster*: these are

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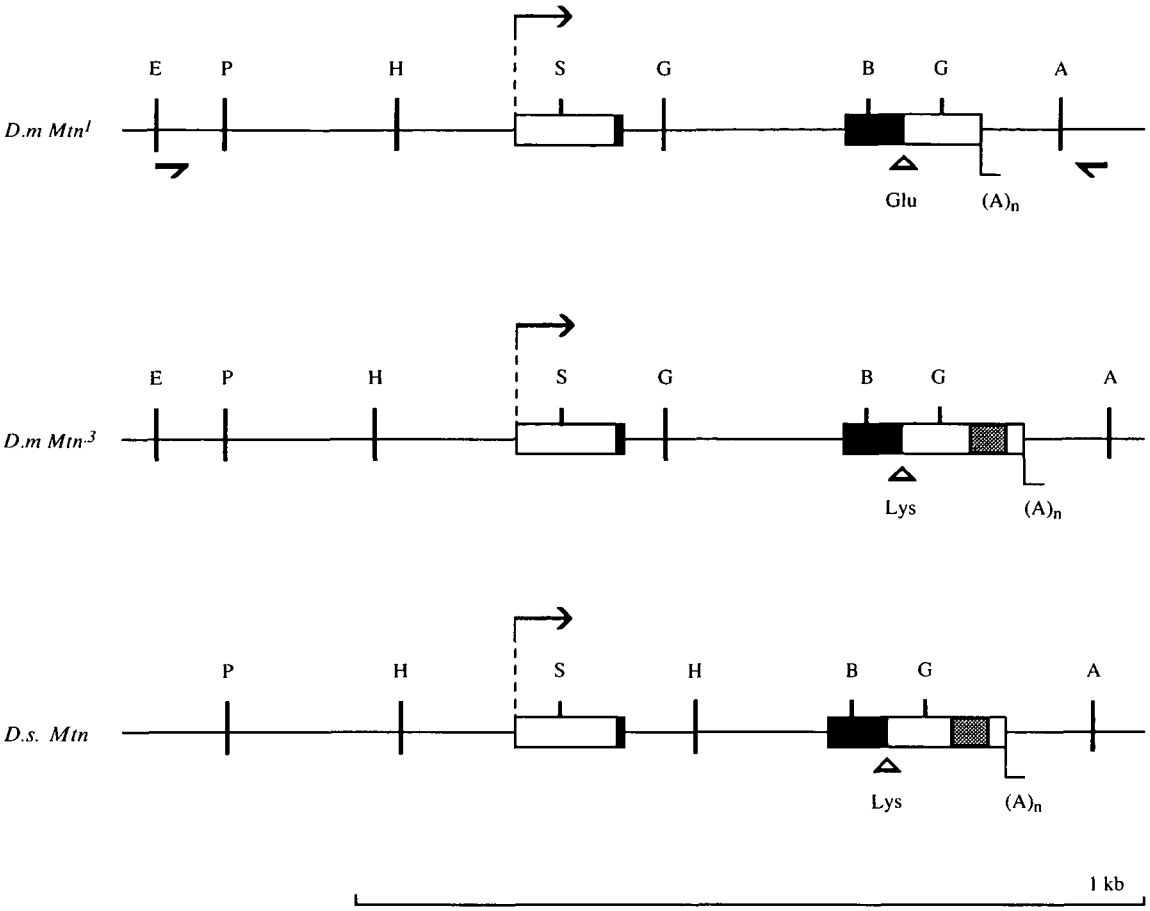

Fig. 2. Structure of the *Mtn* gene. Restriction sites: A, *Alu* I; B, *Bam*H I; G, *Bgl* II; H, *Hin*d III; S, *Stu* I. The position of capping and polyadenylation sites as deduced from sequence analysis are indicated by a bent arrow and $(A)_n$ respectively. Transcribed but untranslated sequences (open boxes) the coding sequence (filled boxes) and the segment not present in *Mtn*^{1} (stippled boxes) are shown. The position of the oligonucleotides used in the PCR analysis are indicated by arrows below the line.

D. simulans, *D. yakuba*, *D. tessieri*, and *D. sechellia*. Undigested PCR products from *D. erecta* and *D. orena* contained two bands, but only one hybridized with an *Mtn* probe (data not shown). An extra *Bam*H I site in the 5′ fragments of *D. mauritiana*, *D. erecta* and *D. orena* gives rise to a third band in each of these three species. The identity of each band marked in Fig. 4 was confirmed by Southern analysis. It appears from these results that in at least 5 out of the 7 sibling species tested, the *Mtn* gene resembles *Mtn*$^{\cdot 3}$ rather than *Mtn*^{1}. The 3′ fragments of *D. erecta* and *D. orena* are intermediate in size between the *D. melanogaster* alleles.

The cladogram in Fig. 4 is based on *Adh* sequences and polytene chromosome banding patterns (Bodmer & Ashburner, 1984) and our results are in general agreement with it.

(iv) *The sequencing of* Mtn *in* D. simulans *confirms its similarity with* Mtn$^{\cdot 3}$

The allelic typing of 32 isofemale lines of *D. simulans* from various geographic origins showed that all of them had the *Mtn*$^{\cdot 3}$-like 3′ fragment (data not shown). To confirm this typing at the nucleotide level, PCR products of *D. simulans* DNA were cloned and sequenced. The 49−bp fragment of *Mtn*$^{\cdot 3}$ was found to be conserved in *D. simulans*. Also, the last codon in *D. simulans* (Lys40) was identical to the one found in *Mtn*$^{\cdot 3}$. A comparison of the three sequences is shown in Fig. 3.

(v) *The frequencies of the* Mtn$^{\cdot 3}$, Mtn^1 *and* Dp(Mtn) *alleles in* D. melanogaster *samples from two geographic areas are very different*

We used the PCR-based method to distinguish between *Mtn*$^{\cdot 3}$ and *Mtn*^{1}, and Southern analysis to detect duplications. These methods demonstrated that all previously described *Mtn* duplications (Maroni *et al.* 1987; Lange *et al.* 1990) carry the *Mtn*^{1} allele (data not shown). We found that only four out of 40 isofemale lines collected in France were heterozygous for *Mtn*$^{\cdot 3}$ while the remainder carried *Mtn*^{1} or its duplications. In contrast, all of 30 lines from the Congo were homozygous for *Mtn*$^{\cdot 3}$ (Table 2).

We also tested for the occurrence of *Mtn* duplications in *D. simulans* but found none in 32 lines, including 6 lines from the same French vineyard where the *Mtn* duplications were so numerous (Table

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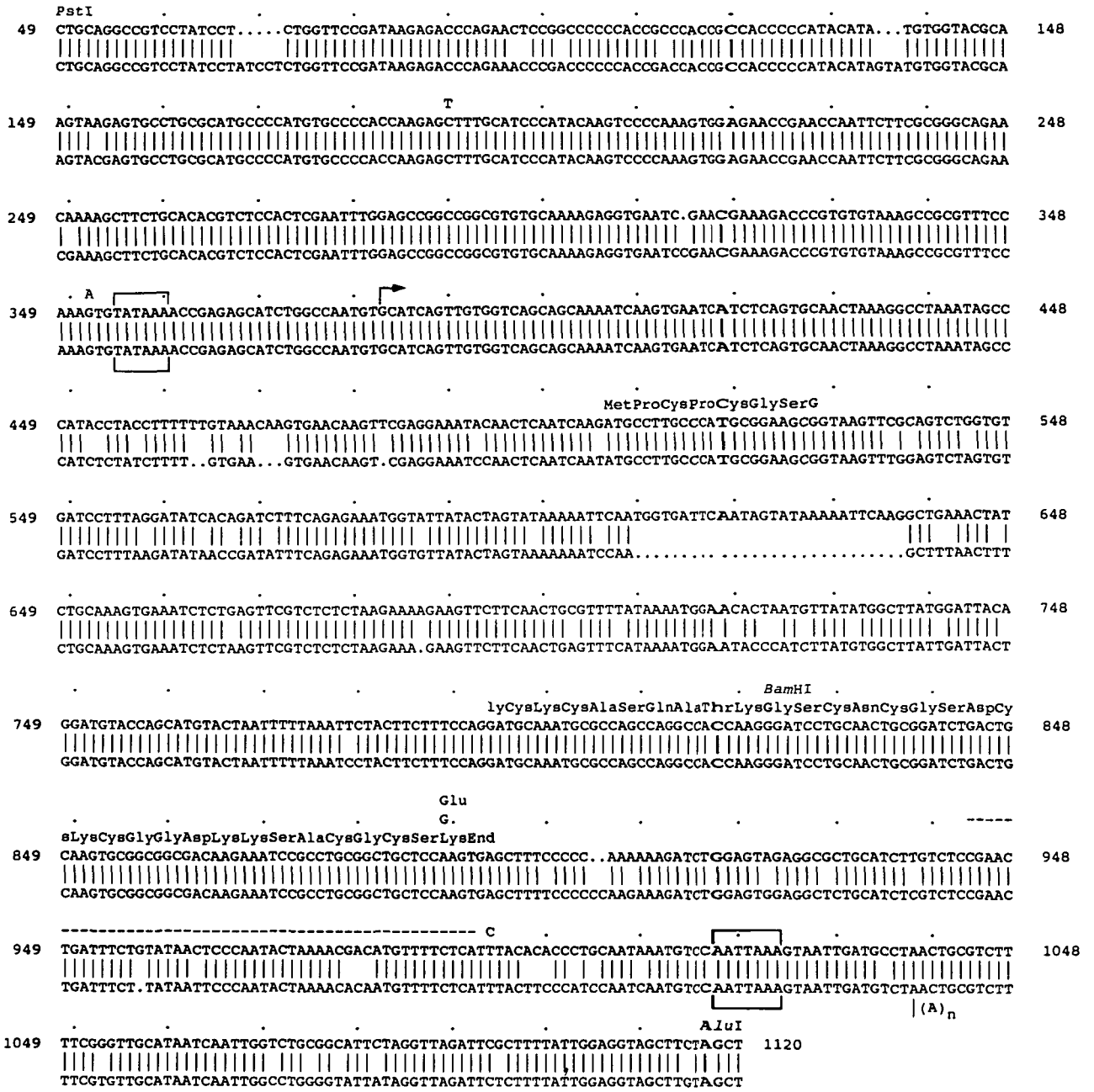

Fig. 3. Comparison of *Mtn*^{1}, *Mtn*$^{\cdot 3}$, and *D. simulans Mtn* sequences. Sequences were aligned using the Sequence Analysis Software Package from the Genetics Computer Group, University of Wisconsin, Madison (Devreux, Haeberli & Smithies, 1984). Numbered strand: *D. melanogaster Mtn*$^{\cdot 3}$; *bottom strand*: *D. simulans Mtn*. The numbering system is that used for the *Mtn* sequence of *D. melanogaster* in GenBank (Drometg, accession number: M12964; Maroni, *et al.* 1986*b*). The four single-base substitutions and the 49-bp deletion in *Mtn*^{1} are indicated above the *Mtn*$^{\cdot 3}$ sequence. The TATA box is underlined and the putative capping and polyadenylation sites deduced from sequence similarity with *Mtn*^{1} are indicated as in Fig. 2. The 3′-end sequence (downstream of coordinate 823) of *Mtn* alleles was determined in two *Mtn*$^{\cdot 3}$ strains and one *Mtn*^{1} strain (the latter agreed with the published *Mtn*^{1} sequence). Differences with the *D. simulans Mtn* sequence published in Lange *et al.* (1990) could be due to polymorphisms. The sequences can be found under the following GenBank/EMBL Accession numbers: *D. melanogaster Mtn*$^{\cdot 3}$, M6901 5; *D. simulans Mtn*, M69016.

2). The 40 French lines yielded no duplications for the other metallothionein gene, *Mto*.

4. Discussion

(i) *Identification of the ancestral* Mtn *allele in* Drosophila

A new pattern of *Mtn* expression was found to be associated with a new allele, *Mtn*$^{\cdot 3}$. In *D. melanogaster* strains carrying *Mtn*$^{\cdot 3}$, the *Mtn* transcript is larger and less abundant than in strains carrying the previously described allele *Mtn*^{1} (Lastowski-Perry *et al.* 1985). The molecular basis for increased accumulation of *Mtn* mRNA in *Mtn*^{1} flies has not been determined. The stability of the *Mtn*^{1} RNA might be increased by the changes in the transcribed region, or the transcription rate of *Mtn*^{1} might be modified by the base substitutions in the promoter or by changes

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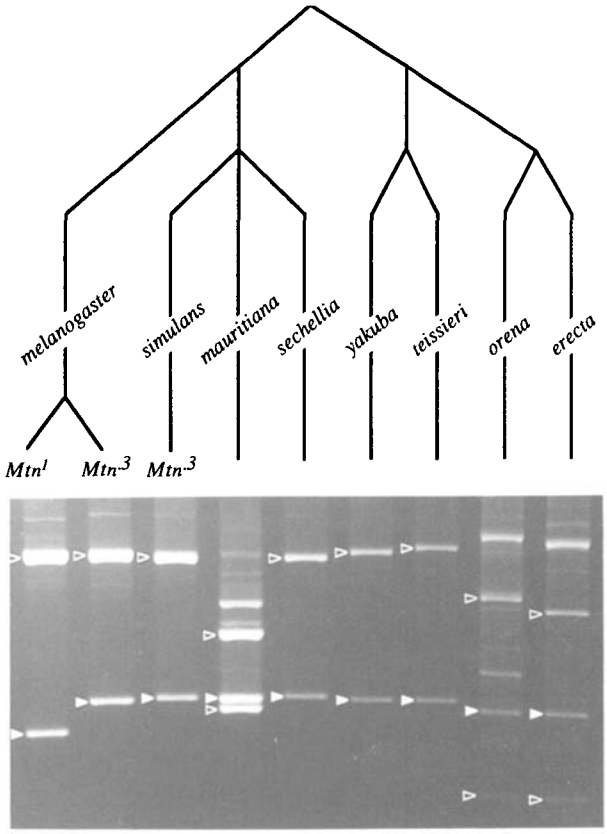

Fig. 4 Analysis of the *Mtn* locus in sibling species of *D. melanogaster*. Agarose gel electrophoresis of PCR amplification products after digestion with *Bam*H I. For each species, the *Bam*H I fragment corresponding to the 3′-end (filled arrow head) and the 5′-end fragment(s) of the gene (open arrow head) were determined by Southern blot hybridization (data not shown). In the top part of the figure, a partial phylogenetic tree of the *melanogaster* group is presented.

outside of the sequenced segment. The size difference between $Mtn^{\cdot 3}$ and Mtn^{1} could be the result of either an insertion or a deletion. In an attempt to establish which was the case we investigated closely related species and our results suggest that $Mtn^{\cdot 3}$ is probably the more primitive allele. (i) Five out of the seven sibling species of *D. melanogaster* contain a restriction pattern similar to $Mtn^{\cdot 3}$ (the other two species were uninformative). (ii) All 32 lines of *D. simulans* from various geographic areas contain the same $Mtn^{\cdot 3}$-type restriction pattern. (iii) The *Mtn* locus of *D. simulans* contains the 49-bp segment in its 3′ region and the same terminal codon as found in $Mtn^{\cdot 3}$. Thus, it appears reasonable to conclude that $Mtn^{\cdot 3}$ is closer to the ancestral allele than Mtn^{1} and that Mtn^{1} was derived from the ancestral *Mtn* gene by a deletion event in *D. melanogaster*; even though the data do not completely refute alternative explanations.

(ii) *Evolutionary history of the* Mtn *locus in* D. melanogaster

The studies reported here confirm earlier observations on the distribution of *Mtn* duplications: they are absent from African samples (verified in three independent samples from Botswana, Zambia [(Maroni *et al.* 1987), and Congo (this report)], and they appear at high frequency in European samples. The estimated 40-60% allelic frequency of duplications in the sample from Grande Ferrade (France) is more than twice as high as those observed earlier (Maroni *et al.* 1987; Lange *et al.* 1990).

Our results may give some hints as to the geographic context in which changes in the *Mtn* locus occurred. The simplest hypothesis to explain a monomorphic $Mtn^{\cdot 3}$ (the more primitive allele) in the Congolese sample [the site nearer to the original site of dispersal of species (David & Capi, 1988; Singh, 1989)] and the prevalence of Mtn^{1} in the French sample, is that the latter may have arisen outside of Africa and never managed to establish itself within tropical Africa. The '50-bp insertion' found at a frequency of 0·1 in American samples reported by Lange *et al.* (1990) almost certainly corresponds to $Mtn^{\cdot 3}$. This suggests that $Mtn^{\cdot 3}$ is also present at a low frequency in America.

The very high frequency of Mtn^{1} in Europe (and apparently in North America) can be explained either

Table 2. *Occurrence of the various* Mtn *alleles in samples from two geographic areas*

	$Dp(Mtn^{1})$[a]	Mtn^{1}	$Mtn^{\cdot 3}$	Total
Grande Ferrade (France)				
Number of lines[b]	36	40	4	40
Allelic frequency[c]	0·38/0·62	0·59/0·33	0·03/0·05	
Loua (Congo)				
Number of lines[b]	0	0	30	30
Allelic frequency[c]	0	0	1·00	

[a] Combines cases of *Dp(Mtn)H35* and *Dp(Mtn)H46*, 2 of 4 previously described duplications for the *Mtn* gene (Maroni *et al.* 1987) that were found in this sample.
[b] Number of lines in which the allele was detected.
[c] Allelic frequencies were estimated assuming that each isofemale line represents a sample of two (first number) or four (second number) chromosomes from the population.

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by natural selection for this allele or by a founder effect. Our results do not address this issue. However, if we consider the differential distribution of the *Mtn* alleles, together with the observation that four Mtn^1 duplications of independent origins exist, the hypothesis that natural selection has led to ever-increasing expression of *Mtn* (specifically Mtn^1) in non-African populations is strengthened.

(iii) *Metallothioneins and metal detoxification in* Drosophila

One source of selective pressure for Mtn^1 and its duplications might be environmental metals derived from industrial or agricultural pollution. For example, in the last hundred years treatment of fruit orchards with copper salts ('Bordeaux mix') has been a fairly widespread practice in Europe. In the U.S.A. there have been several reports of Cu-resistant strains of the plant pathogen *Pseudomonas syringae* isolated from fruit orchards (Anderson & Lindow, 1986). Likewise, *Mtn* duplications might have spread in the population because they confer increased resistance to metals; as was shown for four alleles of $Dp(Mtn^1)$ in laboratory tests (Maroni *et al.* 1987). A selective advantage of Mtn^1 over $Mtn^{\cdot 3}$ in flies reared in the presence of metal has not been demonstrated. However a tempting hypothesis is that Mtn^1 type flies have an increased resistance to metal toxicity, since they produce three times as much mRNA when compared to flies carrying the primitive allele $Mtn^{\cdot 3}$.

We should point out that transcript abundance is not the only relevant parameter to consider in comparing these two *Mtn* alleles. The proteins encoded by Mtn^1 and $Mtn^{\cdot 3}$ also differ at the terminal amino acid. The substitution of Lys40 for Glu40 introduces a charge difference in the neighborhood of the last Cys-X-Cys group of the molecule and this may have a significant effect on its properties. We would expect that it is a combination of the effectiveness of the protein and its amount that determines metal tolerance.

One possibility along those same lines is that the amino acid substitution might have led to the acquisition of a new function in metal detoxification due to a change in the affinity or the specificity of the *Mtn* protein. It is intriguing that in areas where Mtn^1 duplications are found at a high frequency in *D. melanogaster*, (i) there were no duplications of *Mto* (Lange, *et al.* 1990; and this work), and (ii) in *D. simulans*, where *Mtn* is of the monomorphic $Mtn^{\cdot 3}$ type, no duplications of this allele were found (this work). Silar *et al.* (1990) and Lange *et al.* (1990) have suggested that after the original duplication of their common ancestor occurred, *Mtn* and *Mto* have diverged and specialized in function, *Mto* functioning in metal homeostasis and *Mtn* being involved in metal tolerance. One might speculate that the ancestral *Mtn* gene, probably of the $Mtn^{\cdot 3}$ type, was not primarily involved in metal detoxification but in homeostasis and it is only the latest events in the history of the *Mtn* gene in *D. melanogaster*, the appearance of the recent Mtn^1 and $Dp(Mtn^1)$ alleles that have led to specialization of the *Mtn* gene in metal detoxification. This is a testable hypothesis.

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References

Andersen, G. L. & Lindow, S. e. (1986). Occurrence and control of copper tolerant strains of *Pseudomonas syringae* on almond and citrus in California. *Phytopathology* **76**, 1118.

Andrews, G. K., Huet, Y. M., Lehman, L. D. & Dey, S. K. (1987). Metallothionein gene regulation in the pre-implantation rabbit blastocyst. *Development* **100**, 463–469.

Beach, L. R. & Palmiter, R. D. (1981). Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proceedings of the National Academy of Sciences, USA* **78**, 2110–2114.

Churchich, J. E., Scholtz, G. & Kwork, K. (1989). Activation of pyridox kinase by metallothionein. *Biochemica et Biophysica Acta.* **996**, 181–186.

Crawford, B. D., Enger, M. D., Griffith, B. B., Griffith, J. K., Hanners J. L., Longmire, J. L., Munk, A. C., Stallings, R. L., Tesmer, J. G., Walters, R. A. & Hildebrand, C. E. (1985). Coordinate amplification of metallothionein I and II genes in cadmium-resistant Chinese hamster cells: implications for mechanisms regulating metallothionein gene expression. *Molecular and Cellular Biology* **5**, 320–329.

David, J. R. and Capy, P. (1988). Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genetics* 4: 106–111.

Devereux, J., Maederli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the Vax. *Nucleic Acids Research* **12**, 384–385.

Evans, R. M. & Hollenberg, S. M. (1988). Zinc finger: guilt by association. *Cell* **52**, 1–3.

Hamer, D. H. (1986). Metallothionein. *Annual review of Biochemistry* **55**, 913–951.

Hanas, J. S., Hazuda, D. J., Bogenhagen, D., Wu, F. Y. H. & Wu, C. W. (1983). *Xenopus* transcription factor A requires zinc for binding to 5S R gene. *Journal of Biological Chemistry* **258**, 14120.

Jacobson, K. B., Opresko, L., Owenby, R. K. & Christie, N. T. (1981). Effects of cadmium on *Drosphila*: toxicity, proteins, and transfer RN *Toxicology and Applied Pharmacology* **60**, 368–378.

Karin, M. (1985). Metallothionein: proteins in search of function. *Cell* **41**, 9–10.

Lange, B. W., Langley, C. H. & Stephan, W. (1990). Molecular evolution of Drosphila metallothionein genes. *Genetics* **126**, 921–932.

Lastowski-Perry, D., Otto, E. & Maroni, G. (1985). Nucleotide sequence and expression of a *Drosophila* metallothionein. *Journal of Biological Chemistry* **260**, 1527–1530.

Lauvergeat, S., Ballan-Dufrançais, C. & Wegnez, M. (1989). Detoxification of cadmium. Ultrastructural study and

electron-probe microanalysis of the midgut in a cadmium-resistant strain of *Drosophila melanogaster*. *Biology of Metals* **2**, 97–107.

Lindsley, D. L. & Zimm, G. (1985). The genome of *Drosophila melanogaster*. *Drosophila Information Service* **62** 1–227.

Lindsley, D. L. & Zimm, G. (1990). The genome of *Drosophila melanogaster*, *Drosophila Information Service* **68**, 1–382.

Maroni, G., & Watson, D. (1985). Uptake and binding of cadmium, copper and zinc by *Drosophila melanogaster* larvae. *Insect Biochemistry* **15**, 55–63.

Maroni, G., Otto, E. & Lastowski-Perry, D. (1986*b*). Molecular and cytogenetic characterization of a metallothionein gene of *Drosophila*. *Genetics* **112** 493–504.

Maroni, G., Wise, J., Young, J. E. & Otto, E. (1987). Metallothionein gene duplications and metal tolerance in natural populations of *Drosophila melanogaster*. *Genetics* **117**, 739–744.

Maroni, G. (1989). Animal metallothioneins, In *Heavy Metal Tolerance in Plants* (ed. by A. J. Shaw), pp. 215–232. Boca Raton: CRC Press.

Mokdad, R., Debec, A. & Wegnez, M. (1987). Metallothionein genes in *Drosophila melanogaster* constitute a dual system. *Proceedings of the National Academy of Sciences, USA* **84**, 2658–2662.

Nishimura, H., Nishimura, N. & Tohyama, C., (1990). Localization of metallothionein in the genital organs of the male rat. *Journal of Histochemistry and Cytochemistry* **38**, 927–933.

Otto, E., Young, J. E. & Maroni, G. (1986). Structure and expression of a tandem duplication of the *Drosophila* metallothionein gene. *Proceedings of the National Academy of Sciences, USA* **83**, 6025–6029.

Sanger, F., Nicklen, S. & Coulson, A. R. (1987). DNA sequencing with chain terminating inhibitors *Proceedings of the National Academy of Sciences* **74**, 54–63.

Seagrave, J. -C., Hanners, J. L., Taylor, W. & O'Brien, H. A. (1986). Transfer of copper from metallothionein to nonmetallothionein proteins in cultured cells. *Biological Trace Element Research* **10**, 163–173.

Silar, P., Theodore, L., Mokdad, R., Errais, N., Cadic, A. & Wegnez, M. (1990). Metallothionein *Mto* gene of *Drosophila melanogaster*: structure and regulation. *Journal of Molecular Biology* **215**, 217–224.

Singh, R. S. (1989). Population genetics and evolution of species related to *Drosophila melanogaster*. *Annual Review of Genetics* 23: 425–453.

Wilkinson, D. G. & Nemer, M. (1987). Metallothionein genes MTa and MTb expressed under distinct quantitative and tissue-specific regulation in sea urchin embryos. *Molecular and Cellular Biology* **7**, 48–58.