Recent evolutionary history of the metallothionein gene \textit{Mtn} in \textit{Drosophila}

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(Received 5 March 1991)

Summary
A new allele of one of the metallothionein genes of \textit{D. melanogaster}, \textit{Mtn}′, sheds light on the recent evolution of this gene. In comparison to the previously studied \textit{Mtn}′ allele found in Canton S, this new allele, \textit{Mtn}′3, produces a transcript that is 49 bases longer and 65–70% less abundant. We detected \textit{Mtn}′3 in several laboratory strains as well as in isofemale lines derived from natural populations. Sequence comparison showed that \textit{Mtn}′3 differs from \textit{Mtn}′ 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 \textit{Mtn}′ with Lys40, and (c) two base-pair substitutions in the promoter region. The \textit{Mtn}′3-type was detected in six species of the \textit{melanogaster} group by restriction analysis, and this result was confirmed by sequencing the \textit{D. simulans} \textit{Mtn} gene. Thus \textit{Mtn}′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 \textit{Mtn}′, the more recent allele. Thus, two evolutionary steps: \textit{Mtn}′3 to \textit{Mtn}′ and \textit{Mtn}′ to \textit{Dp(Mtn)′}, 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 \textit{Mtn}′3 but not \textit{Mtn}′ was detected in our sample from tropical Africa, while \textit{Mtn}′ and \textit{Dp(Mtn)′} 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 \textit{Drosophila melanogaster}, \textit{Mtn} (Lastowski-Perry, Otto & Maroni, 1985), and \textit{Mto} (Mokdad, Debec & Wegnez, 1987). The \textit{Mto} and \textit{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 \textit{Mtn} than for \textit{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 \textit{Drosophila} are derived from a common ancestor by a gene duplication event (Maroni, Otto & Lastowski-Perry, 1986b; 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 \textit{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 \textit{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 \textit{Drosophila}, copper and cadmium exert a pleiotropic toxic effect that reduces...
growth rates and viability (Jacobson et al. 1981; Maroni & Watson, 1985; Lauvergeat, Balland-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. tesserii, D. yakuba, D. orena, D. erecta were designed for the amplification of a 1.2 kb Mtn genomic fragment. Primer B44 matches the Mtn' sequence from coordinate 1–30 and primer B123 matches the Mtn' 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 (92 mM), KCl (46 mM), MgCl₂ (1-75 mM), gelatin (0-01 %), Seakem agarose 0-5 %, Nusieve 2-5 % (FMC, Rockland, ME, USA) in Tris-acetic acid (40 mM), EDTA (10 μM) 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).

(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' 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' sequence from coordinate 1–30 and primer B123 matches the Mtn' 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 (92 mM), KCl (46 mM), MgCl₂ (1-75 mM), gelatin (0-01 %), Seakem agarose 0-5 %, Nusieve 2-5 % (FMC, Rockland, ME, USA) in Tris-acetic acid (40 mM), EDTA (10 μM) 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, Hind 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, Hind III, Sst I, Bgl II, BamH 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 (homoygote 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).

(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'1 to reflect its lower abundance. The previously described allele (Maroni et al. 1986b) 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'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’3. Further, the 3’ untranslated region of Mtn’3 is 49 bp longer than in Mtn’. The latter would account for the size difference between mRNAs.

(iii) The Mtn'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’3 alleles, the amplification products were digested with BamH 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’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’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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Table 1. Mtn RNA levels in D. melanogaster larvae homozygous for three different alleles

|       | $M^N$ | $Mn'$ | $Dp(Mn')$ |
|-------|-------|-------|-----------|
| 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.

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Fig. 1. Autoradiograph of a Northern blot of RNA from Mtn'1 and Mtn' 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'2 strains and several Mtn'1 strains.

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Fig. 1. Autoradiograph of a Northern blot of RNA from Mtn'2 and Mtn' 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'2 strains and several Mtn'1 strains.

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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 BamHI 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"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"3

The allelic typing of 32 isofemale lines of D. simulans from various geographic origins showed that all of them had the Mtn"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"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"3. A comparison of the three sequences is shown in Fig. 3.

(v) The frequencies of the Mtn"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"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"3 allele (data not shown). We found that only four out of 40 isofemale lines collected in France were heterozygous for Mtn"3 while the remainder carried Mtn 1 or its duplications. In contrast, all of 30 lines from the Congo were homozygous for Mtn"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.
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Fig. 3. Comparison of Mtn', Mtn'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'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). The four single-base substitutions and the 49-bp deletion in Mm' are indicated above the Mtn'3 sequence. The TATA box is underlined and the putative capping and polyadenylation sites deduced from sequence similarity with Mtn' are indicated as in Fig. 2. The 3'-end sequence (downstream of coordinate 823) of Mtn alleles was determined in two Mtn'3 strains and one Mtn' strain (the latter agreed with the published Mtn' 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'3, M69015; 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'. In D. melanogaster strains carrying Mtn', the Mtn transcript is larger and less abundant than in strains carrying the previously described allele Mtn (Lastowski-Perry et al. 1985). The molecular basis for increased accumulation of Mtn mRNA in Mtn' flies has not been determined. The stability of the Mtn' RNA might be increased by the changes in the transcribed region, or the transcription rate of Mtn' might be modified by the base substitutions in the promoter or by changes in the RNA transcript.
outside of the sequenced segment. The size difference between \( Mtn'3 \) and \( Mtn' \) 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 \( Mtn'3 \) is probably the more primitive allele. (i) Five out of the seven sibling species of \( D. \) melanogaster contain a restriction pattern similar to \( Mtn'3 \) (the other two species were uninformative). (ii) All 32 lines of \( D. \) simulans from various geographic areas contain the same \( Mtn'3 \)-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 \( Mtn'3 \). Thus, it appears reasonable to conclude that \( Mtn'3 \) is closer to the ancestral allele than \( Mtn' \) and that \( Mtn' \) 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 \( Mtn'3 \) (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 \( Mtn' \) 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 \( Mtn'3 \). This suggests that \( Mtn'3 \) is also present at a low frequency in America.

The very high frequency of \( Mtn' \) 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 |
|--------------------------------------------------|--------|--------|--------|
| Grande Ferrade (France) | \( Dp(\text{Mtn})^{b} \) | \( \text{Mtn} \) | \( \text{\text{Mtn}'} \) | Total |
| 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 \( Dp(\text{Mtn})H35 \) and \( Dp(\text{Mtn})H46 \), 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.
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 \( Mtn^3 \)) in non-African populations is strengthened.

(iii) \textit{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 \textit{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 \( Mtn^2 \) 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 \( Mtn^3 \).

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 \( Mtn^3 \) 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 \textit{D. melanogaster}, (i) there were no duplications of \( Mio \) (Lange, et al. 1990; and this work), and (ii) in \textit{D. simulans}, where \( Mtn \) is of the monomorphic \( Mtn^3 \) 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 \( Mio \) have diverged and specialized in function, \( Mio \) functioning in metal homeostasis and \( Mtn \) being involved in metal tolerance. One might speculate that the ancestral \( Mtn \) gene, probably of the \( Mtn^4 \) 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 \textit{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.

We thank A. M. Pret, D. Maroni, S. N. Peterson and R. H. Schiestl for critical reading of the manuscript. We are also grateful to Y. F. Yang for her technical assistance. Supported by Public Health Service grant ES02654 from the National Institutes of Health.

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