Metallothionein genes in *Drosophila melanogaster* constitute a dual system

(cDNA sequence/differential regulation)

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ABSTRACT We have selected a metallothionein (MT) cDNA clone from a cadmium-resistant *Drosophila melanogaster* cell line. This clone includes an open reading frame coding for a 43-amino acid protein whose characteristics are a high cysteine content (12 cysteines, 28% of all residues) and a lack of aromatic amino acids. This protein differs markedly from the *Drosophila* MT (Mtn gene) previously reported [Lastowski-Perry, D., Otto, E. & Maroni, G. (1985) J. Biol. Chem. 260, 1527-1530]. The MT system of *Drosophila* thus consists of at least two distantly related genes, in sharp contrast with vertebrate MT systems, in which the different members of MT gene families display high similarity. The gene corresponding to our MT cDNA (M10) is inducible in *Drosophila* cell lines and in both larval and adult flies.

Metallothioneins (MTs) are small metal-binding proteins found in eukaryotic cells and probably involved in zinc ion homeostasis. These proteins are mainly described as copper MTs in lower eukaryotes (*Neospora, Saccharomyces*) and as zinc or cadmium MTs in higher eukaryotes (vertebrates), in reference to the predominant type of metallic ions that are bound to these molecules *in vivo* (1). The significance of this difference is not yet clear, since the exact role of MT is not known (2). Most of our knowledge about MT comes from work done on vertebrates. A better insight into the function of these ubiquitous proteins could be achieved by studying species such as *Drosophila melanogaster* that offer an easy generational approach.

We have previously established a stable cadmium-resistant *Drosophila* cell line whose cells produce high levels of MT (3). Starting with mRNA from these cells, a cDNA clone has been obtained and sequenced; it displays the main characteristics of MT cDNA but differs markedly from another *Drosophila* MT cDNA (4). The two MT cDNAs code for proteins of different sizes (40 and 43 amino acids, respectively) that are very different in sequence. We compare in this paper the MT system of *Drosophila* with the systems of other eukaryotes.

The terminology used in sequence comparisons is very often ambiguous. In this paper, we use the term homology (homologous) to indicate that different sequences have evolved from a common ancestral sequence. When comparing and quantifying the degree of similarity between DNA or protein sequences, we use the term isology (isologous), which lacks implications about evolutionary relationships. This term was initially used for the comparison of chemical structures (5).

MATERIALS AND METHODS

*D. melanogaster* Cell Lines. The two cell lines used in this study, the D line (cadmium sensitive) and the Cd R200 line (cadmium resistant), are characterized in the preceding paper (3).

Nucleic Acid Isolation. Total cellular RNA was prepared according to Auffray and Rougeon (6) by lysis of cells in 8 M urea/4 M LiCl/10% NaDodSO4/10 mM sodium acetate, pH 5.0. RNA was precipitated from this solution at 4°C for 48 hr. RNA from larvae and adults was extracted from batches of 50 individuals disrupted at 0°C in 1 ml of 100 mM NaCl/10 mM EDTA/0.5% NaDodSO4/10 mM Tris HCl, pH 7.4, and then extracted with phenol/chloroform (7). RNA was precipitated at 4°C by addition of LiCl to 4 M. The method of Aviv and Leder (8) was used to purify poly(A) RNA. Genomic DNA was prepared according to the method of Junakovic (9).

In Vitro Translation. Both total cellular RNA and poly(A) RNA were translated in a wheat germ cell-free system as described by the supplier (Amersham). [35S]Cysteine (1000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) was used as the radioactively labeled amino acid. After translation, the temperature was raised to 65°C for 10 min. Thermostable proteins were then carboxymethylated and electrophoresed on 8 M urea/0.1% NaDodSO4/20% polyacrylamide gels (10).

Construction and Screening of a Cd R200 cDNA Library. A cDNA library was constructed starting from 5 μg of poly(A) RNA purified from Cd R200 cells (11). Insertion of the cDNA at the EcoRI site of vector plasmid pHC8 was performed after addition of EcoRI linkers (12). The protocol of Mandel and Higa (13) was used to transform *Escherichia coli* strain JA21 (14). The colony hybridization method of Grunstein and Hogness (15) was employed to identify cDNA plasmids exhibiting hybridization with a Cd R200[32P]cDNA probe but not with a D[32P]cDNA probe.

RNA and DNA Gel Blotting and Hybridization. RNA was electrophoresed on 1.2% agarose/formaldehyde gels and blotted as described by Thomas (16) onto Schleicher & Schuell BA 85 nitrocellulose membranes. DNA was electrophoresed on 0.7% agarose gels and blotted as described by Smith and Summers (17). Hybridization conditions were those of Wahl et al. (18). nick-translated probes (108 cpm/μg) were used at 30 ng/ml.

DNA Sequencing. Plasmid DNA was purified by the method of Birnboim and Doly (19). Restriction fragments to be sequenced were dephosphorylated, fractionated on 5% polyacrylamide gels, and labeled at their 5' ends by using T4 polynucleotide kinase (20). Labeling at the 3' end was done with the Klenow fragment of DNA polymerase (11). Fragments with a unique 32P-labeled end were obtained by either

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strand separation or restriction digestion. Sequencing reactions were performed according to the Maxam and Gilbert procedure (20).

RESULTS

Cloning and Sequencing of a Drosophila MT. The cadmium-resistant Cd R200 cell line derives from the cadmium-sensitive D line (3). Poly(A)+ RNA purified from both cell lines was assayed in a wheat germ translation system for the presence of MT mRNA. We used [35S]cysteine, the predominant amino acid component of MT, as a radioactive label. A low molecular weight protein, approximately 5000, was shown to be the major translational product of the Cd R200 mRNA (Fig. 1, lane 2). This protein was not detected in the control experiment with mRNA from D cells (Fig. 1, lane 4). Addition of CdCl2 (30 μM) to the incubation mixture increased the amount of [35S]cysteine incorporated into MT by approximately 3-fold (Fig. 1, lane 3). An “induction” at the translational level has already been observed in the case of the rat MT mRNA (21). This may conceivably result from an increase in the rate of release of MT from ribosomes upon binding of cadmium ions to nascent polypeptide chains. Cadmium could also stabilize MT against oxidation and degradation.

Putative MT clones were isolated by differential screening of a Cd R200 cDNA library constructed in the pUC8 plasmid. Five out of 1000 clones gave a very intense signal when probed with Cd R200 [32P]cDNA and no signal when probed with D [32P]cDNA (data not shown). Two of the five clones, all of which cross-hybridized, were selected for sequencing. The strategy of sequencing and the derived sequence are shown in Fig. 2. This 301-base-pair (bp) sequence is the combination of pCd2 and pCd14 sequences. Both clones share an identical segment of 191 bp, thus allowing the derivation of a single sequence that contains an open reading frame corresponding to a 43-amino acid protein (Fig. 2). This protein displays the main structural characteristics of MT—i.e., a high cysteine content (12 cysteines, 28% of all residues) and no leucine, isoleucine, histidine, or aromatic amino acids. This MT-encoding sequence will be referred to as Mto in the next part of the report.

Comparison of Mto with Other MT-Encoding Sequences.

We have used the program EPURE (22) to compare the amino acid sequence of Mto-encoded MT with the other reported MT sequences (23). From all binary comparisons, we found only two alignments of sequences that appear as significant. The first was found for a segment spanning 24

![Fig. 1. In vitro translation of poly(A)+ RNA from D and Cd R200 cells. The poly(A)+ RNA was translated, using [35S]cysteine, in a cell-free wheat germ system. The products were analyzed, after carboxymethylation, on a 20% polyacrylamide gel. Translation conditions: lane 1, control without added RNA; lane 2, 5 μg of Cd R200 poly(A)+ RNA; lane 3, 5 μg of Cd R200 poly(A)+ RNA + 30 μM CdCl2; lane 4, 5 μg of D poly(A)+ RNA. The arrow indicates the main translational product of Cd R200 poly(A)+ RNA.](image)

![Fig. 2. Primary structure of Mto cDNA. (A) Strategy used for determining Mto cDNA sequence. The upper line shows the combined restriction map of pCd2 (213 bp) and pCd14 (279 bp). The thicker line corresponds to the open reading frame. The arrows below both pCd14 and pCd2 indicate the direction and extent of sequenced fragments. (B) Combined nucleotide sequence (301 bp) of the antisense strand of pCd14 and pCd2. The deduced protein sequence is written below the open reading frame. The three restriction sites shown in A are underlined. The two AACAAAG (see the text) and the polyadenylation site are overlined.](image)
amino acids of the two Drosophila MTs (Cys\textsuperscript{6} to Cys\textsuperscript{28} in Mto product, Cys\textsuperscript{4} to Cys\textsuperscript{28} in Mtn product; Fig. 3A). The second alignment is between Drosophila and Scylla (crab) MTs (Fig. 3A).

The program EPURE has also been used to compare the sequences of Mtn and Mto cDNAs at the nucleotide level (Fig. 3B). In this case, alignment was found to start at the codon corresponding to the first amino acid of the segment defined from protein comparisons and to fall one codon short of the TAA stop codon of the Mtn sequence and three codons short of the TAA stop codon of Mto (Fig. 3). No alignment of sequences can be proposed in the 5' and 3' noncoding regions. However, a tandemly duplicated 6-nucleotide box (AACAAG) does occur in the 5' noncoding region of both sequences. Respective positions, relative to ATG initiation codons, are −39 in Mtn (4) and −67 in Mto (Fig. 2).

Expression of Mtn. Inducibility of Mtn in cell lines, larvae, and adults. Expression of Mtn in Cd R200 cells was assayed by probing "Northern" blots (16, 18) with pCd14. An abundant RNA species, of approximately 400 nucleotides, was detected starting from either total or poly(A)\textsuperscript{+} cellular RNA (data not shown). This mRNA was not detectable in D cells grown under normal conditions but was clearly induced in these cells after addition of CdCl\textsubscript{2} to 10 \mu M in the culture medium (Fig. 4). The Mto mRNA concentration, low 4 hr after cadmium treatment, increased sharply between 4 and 8 hr and then rose slowly up to 48 hr. Mto mRNA levels in 10 \mu M CdCl\textsubscript{2}-induced D cells (48 hr) and in Cd R200 cells grown in the presence of 200 \mu M CdCl\textsubscript{2} were compared by dot-blot analysis (data not shown). In these conditions, Cd R200 cells were found to display 4 to 3 times more Mto mRNA than induced D cells. Wild-type Drosophila larvae and adults do not express Mto at a detectable level, but the gene was shown to be inducible in both stages after addition of cadmium to the culture medium (data not shown).

Differential response of Mto to cadmium and copper induction. The two distinct Drosophila MT cDNAs were cloned starting from very different systems, either copper-induced larvae (Mtn; ref. 4) or cadmium-resistant cells (Mto; this work). This suggested the possibility of specific induction for each of the two MT genes. To test this possibility directly, we looked for a differential induction of Mto in response to cadmium or copper ions. As shown in Fig. 5, Mto is poorly inducible by copper in D cells. The amount of Mto mRNA detectable in these cells grown in the presence of 1 mM CuSO\textsubscript{4} is negligible compared with the mRNA level in cells induced with 10 \mu M cadmium. There is also a significant, but less dramatic, difference in inducibility of Mto by cadmium or copper in adult Drosophila. In this case, similar levels of Mto mRNA could be detected when copper concentration was 5 times that of cadmium (data not shown).

DISCUSSION

We report in this paper the sequence of a Drosophila cDNA clone (referred to as Mto) that has an open reading frame coding for a protein possessing the main structural characteristics of MTs. The structure of this clone differs significantly from that reported by Lastowski-Perry et al. (4) for another Drosophila MT (referred to as Mtn). The genome of Drosophila thus contains at least two very different MT

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**Fig. 3.** Comparison of Mto and Mtn sequences and their deduced amino acid sequences. (A) Amino acid sequence comparison of Mto (this work), Mtn (4), and Scylla (24) MTs. Protein sequences are written with the single-letter code (25). The two upper arrows indicate the borders of the two Drosophila sequences, which were aligned according to EPURE (22). Asterisks between Mto and Mtn sequences mark shared residues. Triangles above Mto and Mtn indicate cysteine residues that are not shared by both sequences. Double asterisks between Scylla MT-I and Drosophila MT point to residues common to the three MTs. Single asterisks between Scylla and Drosophila MTs show the residues that are common to the crab and one of the two fly MTs. Triangles below the Scylla sequence indicate cysteine residues found only in that sequence. (B) cDNA sequence comparison of Mto and Mtn. Sequences are aligned according to the data yielded by EPURE. The two sequences correspond to the amino acid sequences underlined in A. Asterisks denote shared nucleotides.
genes. This result is in sharp contrast with the other MT systems studied so far, where MT gene families consist of members displaying high levels of isology.

Evolution of MT Genes in D. melanogaster. The two Drosophila MTs, Mtn and Mto, are remarkably different in sequence (Fig. 3). They differ in size (40 and 43 amino acids, respectively), number of cysteine residues (10 and 12, respectively), and amino acid composition (no valine residue in Mtn, two valine residues in Mto). Comparison of Mtn and Mto nevertheless allows us to align both sequences without the need of introducing gaps (Fig. 3). It can be calculated, from this alignment, that Mto and Mtn proteins are 26% isologous. This low level of isology explains why Maroni et al. (27) did not detect Mto by using Mtn as a probe and why we did not detect Mtn by using the Mto probe. It is, however, likely that the two genes are homologous. If so, then the duplication of the ancestral MT gene that led to Mtn and Mto was a very ancient evolutionary event. It is interesting to note that the Scylla MT-I (24) appears as intermediate between the two Drosophila MTs. As shown in Fig. 3, seven amino acids, all of which are cysteine residues, are common to the three sequences. Five additional amino acids are found at similar positions in Mtn and Scylla MT-I and four other in Mtn and Scylla MT-I (Fig. 3). A similar result is obtained when the Scylla MT-II (24) is compared with the two Drosophila sequences. This suggests that duplication of the ancestral Drosophila MT gene may have occurred after separation of the crustacean and insect evolutionary lines.

These results contrast with the data reported for vertebrates, in which the different MTs within any species are much alike, having the same number and the same positions of cysteine residues as well as exactly the same size (1, 23). Isology levels between the different MTs (61 amino acids) within any vertebrate species range from 77% to 92%. A similar situation is found for the two MT isoforms from the crustacean Scylla (24). In this case the two MTs slightly differ in size (57 and 58 amino acids, respectively) but are still 81% isologous.

Expression of Mto. We show in this paper that cadmium ions strongly induce Mto mRNA synthesis in the D cell line as well as in adults and larvae. As shown in Fig. 4, the time course of Mto mRNA accumulation in D cells, after cadmium induction, is similar to that observed in other MT systems (28). These results are in good agreement with the characterization in the same cells of a protein displaying all MT properties (3). The cadmium-resistant cell line (Cd R200) was shown to display MT levels 22 times higher than the cadmium-induced D line (3). Here we show that Mto mRNA levels are only 3 to 4 times higher in Cd R200 than in induced D cells. A duplication of Mto in Cd R200 cells (preliminary data) most probably accounts for the increased level of Mto mRNA in these cells. The much higher induction at the protein level could be due to some translational regulation or to a higher stability of MT in the presence of 200 μM CdCl2. Interpretation of the Cd R200 cadmium resistance phenotype will evidently also require the study of Mtn expression.

Copper ions, in contrast, are much less efficient Mto inducers than cadmium ions. This is particularly striking in the case of D cells, which respond very poorly to copper induction. These results are difficult to compare with those reported for Mtn, which is highly copper-inducible (4). Both genes have been studied in completely different conditions—i.e., mainly cell lines in the case of Mto and mainly larvae in the case of Mtn. At first sight, it could appear that Mto and Mtn are characterized by strongly different inducing properties. They have both been cloned by differential screening. Copper-induced larvae were the starting material used in the case of Mtn (4) and cadmium-induced cells in the case of Mto (this work). It was possible, in each case, to select only a single type of Drosophila MT. Lower eukaryotes, such as Neurospora and Saccharomyces, possess copper-inducible MT genes (29, 30). In contrast, higher eukaryotes such as crustaceans (24), sea urchins (23), and vertebrates (1) possess MT genes inducible mainly by cadmium or zinc. Drosophila, thus, could possibly possess both types of MT genes. The following data show that this view of the Drosophila MT system is perhaps too simple. As demonstrated by Lastowski-Perry et al. (4), cadmium ions are also able to induce Mtn in larvae. In the case of Mto, copper ions are very poor inducers in D cells but are more efficient in adults and larvae. The study of both Mtn and Mto expression in cell lines and in the different developmental stages is thus obviously required to reach a correct interpretation of the Drosophila MT system.

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