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Expression of metallothionein genes during the post-embryonic development of *Drosophila melanogaster*

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Expression of the two *Drosophila melanogaster* metallothionein genes, *Mtn* and *Mto*, has been analyzed by *in situ* hybridization during post-embryonic development. *Mtn* and *Mto* transcripts were detected exclusively in the digestive tract of larvae, pupae and adults reared on standard medium. *Mtn* and *Mto* expression domains overlap, but each gene is also expressed at unique sites. *Mtn* mRNA levels are approximately 10 and 20 times higher than those of *Mto* in larvae and adults, respectively. Copper and cadmium ions strongly induce *Mtn* and *Mto* mRNA accumulation in the midgut. Zinc is a weaker inducer, acting only at high concentrations. *Mtn* gene expression is induced by these three metals in Malpighian tubules, while *Mto* gene expression in this organ is induced only by zinc. Iron is a poor inducer of metallothionein mRNA accumulation. Functions of MTN and MTO proteins in metal homeostasis and detoxification are considered.

Keywords: metallothionein, development, metal induction, *Drosophila*

Introduction

Metallothioneins are small, cysteine-rich proteins that bind metal ions with high affinity. Amplifications (Beach & Palmiter 1981) and mutations (Michalska & Choo 1993, Masters *et al.* 1994) of mouse metallothionein genes clearly demonstrated their implication in metal detoxification. The function of metallothioneins may also be related to other aspects of metal metabolism, such as absorption, storage, transport and exchange with metalloproteins (Karin 1985). Furthermore, many data show that metallothioneins can play a role in protection against free radicals (Sato & Bremner 1993, Tamai *et al.* 1993). The vertebrate metallothionein isoform MT III, surprisingly, was first characterized as a growth inhibitory factor (GIF). It is repressed in the brain of patients suffering from Alzheimer's disease and inhibits the neurite outgrowth of human neurons in culture (Uchida *et al.* 1991, Tsuji *et al.* 1992). Finally, metallothioneins may function in developmental processes since their genes are expressed during early development of *Drosophila* (Silar *et al.* 1990), sea urchins (Nemar *et al.* 1991) and vertebrates (Andrews *et al.* 1991).

The two metallothionein proteins of *D. melanogaster*, MTN (40 amino acids) and MTO (43 amino acids), differ in so many features that we previously hypothesized that they would play different functions. Metallothionein purification from metal-intoxicated flies was successful only for the MTO protein, suggesting that the MTN protein is sequestered in an insoluble and probably polymerized form (Silar *et al.* 1990). Such polymerized metallothioneins have been found in the lysosomes of *Blatella germanica* ileum (Bouquegneau *et al.* 1985). In *Drosophila*, involvement of metallothioneins in detoxification processes is better demonstrated by the characterization of *Mtn* gene duplications in natural and laboratory strains. These strains produce more *Mtn* mRNA than those carrying one single *Mtn* gene, and tolerate increased cadmium and copper concentrations (Otto *et al.* 1986, Maroni *et al.* 1987, Lange *et al.* 1990). Duplications of the *Mto* gene have never been observed (Lange *et al.* 1990, Silar *et al.* 1990).

Expression of both *Mtn* and *Mto* genes can be increased by adding various metals to the culture medium (Debec *et al.* 1985, Maroni & Watson 1985, Erraïss *et al.* 1989). Copper and cadmium are strong inducers of metallothionein expression while zinc would be significantly less efficient

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(Silar *et al.* 1990). This situation contrasts with that of the mammalian metallothionein system where zinc is a strong inducer (Hamer 1986). Iron effects on *Drosophila* metallothionein regulation have never been investigated until now.

The digestive tract is the major site of metallothionein gene expression in *Drosophila* embryos (Bonneton & Wegnez, 1995). It is thus interesting to look for correlations between sites of *Mtn* and *Mto* gene expression and sites of metal accumulation in the gut. The localization of heavy metals in insects, both in normal conditions and after intoxication, has been the subject of numerous studies (Maroni 1989). Copper is distributed throughout the midgut and in the Malpighian tubules of larvae (Poulson & Bowen 1952, Lauverjat *et al.* 1989) and adults (Marchal-Ségault *et al.* 1990). In the middle midgut of *Drosophila* larvae, copper is localized in the lysosomes of the 'cuprophilic cells', which also contain high levels of sulfur (Poulson *et al.* 1952, Filshie *et al.* 1971; Tapp & Hockaday 1977, Lauverjat *et al.* 1989). Similar results were obtained with *Musca domestica* (Sohal *et al.* 1976, Sohal & Lamb 1979). The main site of zinc accumulation seems to be the Malpighian tubules, both in *Drosophila hydei* (Zierold & Wessing 1990) and in *M. domestica* (Sohal *et al.* 1976, Sohal & Lamb 1979). Iron was detected in the 'iron cells' of the middle midgut and, after induction, throughout the midgut of *D. melanogaster* larvae (Poulson & Bowen 1952). Finally, most of the ingested cadmium is localized in the midgut of *D. melanogaster* (Maroni & Watson 1985, Lauverjat *et al.* 1989).

In insects, the different functions related to nutrition are carried out by specialized structures. Secretion, for example, is assigned to cells of the anterior and middle midgut, while absorption is performed by posterior midgut cells (Dimitriadis & Kastritsis 1984, Terra *et al.* 1988). Detoxification mainly occurs in the Malpighian tubules which are considered to be excretory and osmoregulatory organs analogous to the vertebrate kidney. They filter the hemolymph and produce urine that is driven into the lumen of the hindgut (Wessing & Eichelberg 1978). Ions are transferred through the epithelium of the tubules and form cytoplasmic concretions that dissolve into the lumen. In *M. domestica*, these concretions contain significant amounts of sulfur, calcium and zinc (Sohal & Lamb 1979).

In this paper, we asked whether *Mtn* and *Mto* genes are implicated in the *D. melanogaster* metal metabolism. *In situ* hybridization was used to localize metallothionein transcripts throughout post-embryonic development. Analyses were done using flies reared on standard and metal-contaminated culture media.

Materials and methods

Drosophila *strains and metal treatment*

The *D. melanogaster* wild-type Oregon R strain was used in all experiments. This strain carries the Mtn^1 allele (Théodore *et al.* 1991). Flies were reared at 25°C on *Instant Drosophila Medium* (Carolina Biological Supply Company, 1 g/4 ml of deionized water). Induction of metallothionein synthesis by metals was tested by substituting deionized water with 0.1 mM $CdCl_2$, 0.5 mM $CuSO_4$, 0.5 mM $FeSO_4$, 2.5 mM $ZnSO_4$, 5 mM $ZnSO_4$, 2.5 mM $ZnCl_2$ or 5 mM $ZnCl_2$. Third instar larvae were grown on this medium for larval and pupal stage analyses. Wandering third instar larvae were collected after a 16 h treatment and pupae 3 days after pupation. Five day old adults were transferred on metal-contaminated medium and collected after 3 days.

Histology

Third instar larvae were washed in Ringer solution and fixed for 2 h at 4 C with a 6:3:1 ethanol:chloroform:acetic acid mixture containing 4% glucose. It was necessary to inject this mixture into the abdomen with a microfine syringe in order to improve the fixation of internal structures. After dehydration in an ascending ethanol series, then in butanol, larvae were embedded in Paraplast and sectioned (7 μm slices). An identical procedure was used with pupae and adults, but the fixation time was increased to 18 h.

Synthesis of single-stranded RNA probes

Mtn and *Mto* cDNAs inserted into Bluescript vectors (Stratagene) (Silar *et al.* 1990) were used to synthesize antisense and sense radioactive RNA probes by incorporation of 5′ [α-^{35}S]UTP (Amersham). Specific activity: *Mto* = $1–2 \times 10^8$ d.p.m. μg^{-1}, *Mtn* = $4–5 \times 10^7$ d.p.m. μg^{-1}.

In situ *hybridization*

Slides coated with a solution containing 1% gelatin and 0.5% chrome alum were kept overnight at 80°C before use. After deparaffinization and hydration, samples were post-fixed for 20 min with 4% paraformaldehyde in PBS, pH 7.4, washed twice with PBS for 30 min and then treated for 15 min at 37°C with proteinase K (1 $\mu g\ ml^{-1}$). The probes were diluted in the hybridization buffer (50% deionized formamide, 0.3 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 × Denhardt's solution, 8% dextran sulfate, 10 mM DTT, 100 $\mu g\ ml^{-1}$ yeast tRNA) and heated at 80°C for 2 min. Hybridization was performed for 4 h at 52°C with a probe concentration of 2 ng μl^{-1}. Subsequently, slides were washed twice in 2 × SSPE (20 × SSPE is 3 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 10 mM DTT) at room temperature, overnight in the same conditions, then in 2 × SSPE, 50% formamide, 10 mM DTT at 60°C for 1 h. After a treatment with 40 $\mu g\ ml^{-1}$ RNAse A and 2 IU ml^{-1} RNAse T1 in 4 × SSPE at 37°C for 30 min, slides were washed in 4 × SSPE at 37°C for 30 min, in 2 × SSPE at room temperature for 1 h, then in 1 × SSPE, 0.5 × SSPE and 0.1 × SSPE, each at room temperature for 1 h.

After dehydration, slides were coated with Amersham LM1 emulsion (1:1 dilution in distilled water) and stored in the dark at 4°C. Slides were developed (4 min, 18°C) with Kodak D 19 after a 7 day exposure, rinsed in water and then fixed with Ilford Hypam (1:4 dilution, 5 min, 18°C). Preparations were stained with 0.2% toluidine blue for 10 s,

differentiated, dehydrated through an ascending ethanol series and mounted in eukitt (Prolabo).

RNA analyses

Total RNA of control and intoxicated larvae and adults was purified using the RNA Quick TM II kit (Bioprobe Systems). RNA samples (20 μg lane^{-1}) were fractionated on 1.3% agarose/formaldehyde gels and blotted onto nitrocellulose Hybond N membranes (Amersham). *Mto* and *Mtn* cDNAs were purified by gel electrophoresis and labeled by random priming with [^{32}P]ATP. The cDNA of *D. melanogaster* ribosomal protein *RP49* was also labeled by random priming and used as an internal standard to control RNA concentrations. Specific activity of probes: *Mtn* and *Mto* = 1.5×10^8 d.p.m. μg^{-1}, *RP49* = 10^8 d.p.m. μg^{-1}. Prehybridizations were carried out for 2 h at 42 C in a solution containing 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution, 100 μg ml^{-1} yeast tRNA and 0.5% SDS. Hybridizations were performed overnight at 42 C in the same solution. Membranes were washed three times for 60 min at 42 C in 2 × SSC, 0.5% SSD, then three times for 60 min at 42 C in 0.2 × SSC, 0.5% SDS.

Results and discussion

Expression of metallothionein genes in larvae, pupae and adults was analyzed by *in situ* hybridization using ^{35}S-labeled RNA probes. There was no difficulty in discriminating between *Mtn* and *Mto* transcripts since the sequences are only 50% similar (Mokdad *et al.* 1987). Serial sections from the three post-embryonic developmental stages were used to look for *Mtn* and *Mto* mRNAs in all morphological structures. Hybridizations were carried out with both antisense and sense RNA probes. Background hybridization signals were only observed in the cuticle and in the alimentary bolus. Reproducibility of the results was checked by performing three independent series of experiments. Identical results were obtained with male and female flies.

Differential expression of metallothionein genes during post-embryonic development of D. melanogaster

Expression of *Mtn* and *Mto* genes in *Drosophila* larvae was observed in the middle and posterior midgut (Figures 1 and 2). The 'cuprophilic' and the 'iron' cells belong to the middle midgut (Poulson & Bowen 1952). However, specific sites of expression were also detected in other structures. *Mtn* is expressed in the proventriculus (Figure 1) and in the Malpighian tubules (not shown), while *Mto* is expressed in the salivary glands (Figure 2).

Analysis of *Mtn* and *Mto* gene expression in pupae was more difficult because the digestive tract is totally restructured during metamorphosis. Organs undergoing histolysis are progressively replaced by proliferating cells of the salivary glands, foregut and hindgut imaginal rings, and of midgut imaginal islands (Bryant & Levinson 1985). Expression of *Mtn* and *Mto* was observed in the imaginal midgut as soon as this structure differentiates (Figure 3). Poulson & Bowen (1952) suggested that the accumulation of metals in the larval midgut is a form of excretion since histolysis of this organ forms the yellow body which is eliminated in the meconium after emergence of the adult. We did not detect any metallothionein transcript in the cells forming the yellow body. However, metallothionein proteins may be present in that structure.

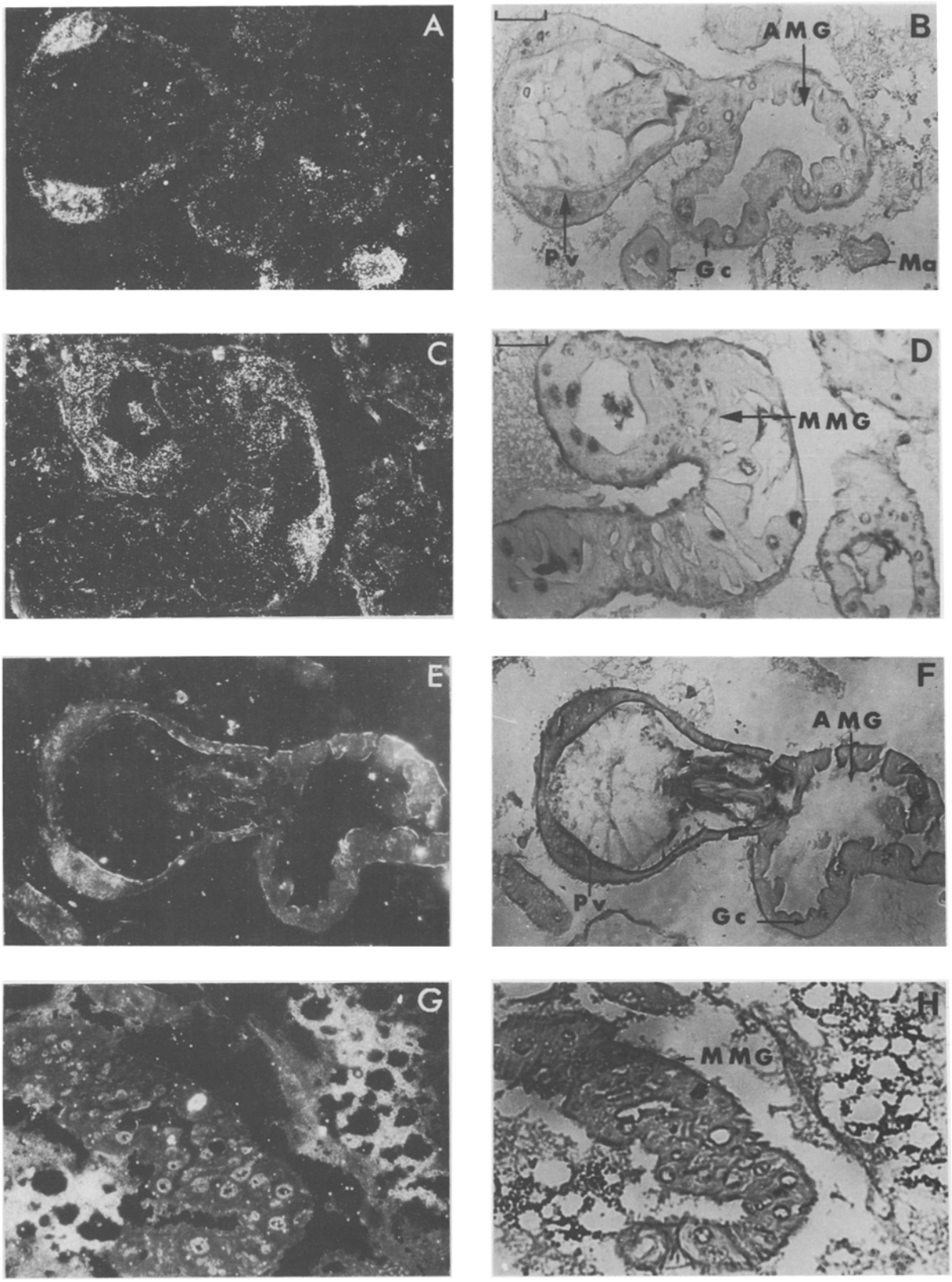
In adults, *Mtn* is expressed in cardia, in Malpighian tubules (Figure 4) and in the midgut (not shown). Expression of *Mto* is restricted to a part of ventriculus (Figure 2), the adult equivalent of the larval midgut (Miller 1965). All *in situ* hybridizations performed with larvae, pupae and adults thus show that *Mtn* and *Mto* transcripts accumulate preferentially in the digestive tract. We did not detect any expression of the two metallothionein genes in other tissues. All *in situ* hybridization results are summarized in Figure 5.

We observed, in accordance with previous results (Silar *et al.* 1990), that *Mtn* mRNA levels are always higher than those of *Mto*. A quantitative analysis reveals that there are approximately 10 and 20 times more *Mtn* than *Mto* mRNA molecules in larvae and adults, respectively (Figure 6, compare *Mtn* and *Mto* control values in 1, 2 and 3). These results confirm that the *Mto* gene is expressed at a relatively low rate, from the embryo to the larval and adult stages (Silar *et al.* 1990).

In summary, *Mtn* and *Mto* genes are differentially expressed during post-embryonic development, both at the spatial (Figure 5) and at the quantitative level (Figure 6). It is difficult to assign specific roles for the two *Drosophila* metallothioneins from these data. The presence of the two types of transcripts in the midgut is compatible with the hypothesis of a similar role in that structure. High *Mtn* mRNA levels in the Malpighian tubules are possibly related to the uniform distribution of copper observed in this organ by Poulson & Bowen (1952). Zinc is also known to accumulate in vacuoles of *Drosophila hydei* Malpighian tubules (Zierold & Wessing 1990). The abundance of *Mtn* transcripts in Malpighian tubules thus suggests that the MTN protein may play a role in the accumulation of zinc and copper in these structures. In order to study further the involvement of *Mtn* and *Mto* genes in the regulation of metal exchanges, we tested by *in situ* hybridization the effect of various metal treatments on the spatial distribution of both gene transcripts during post-embryonic *Drosophila* development.

Metal-induced expression of metallothionein genes in post-embryonic stages of D. melanogaster

We tested whether copper, cadmium, zinc and iron affect the same regions of the *Drosophila* gut by examining *Mtn* and *Mto* transcript distribution in metal-intoxicated larvae, pupae and adults. Copper, zinc and cadmium have a dramatic effect on *Mtn* and *Mto* mRNA accumulation in the digestive tract, while iron has a weaker effect. The four metals tested affect metallothionein expression



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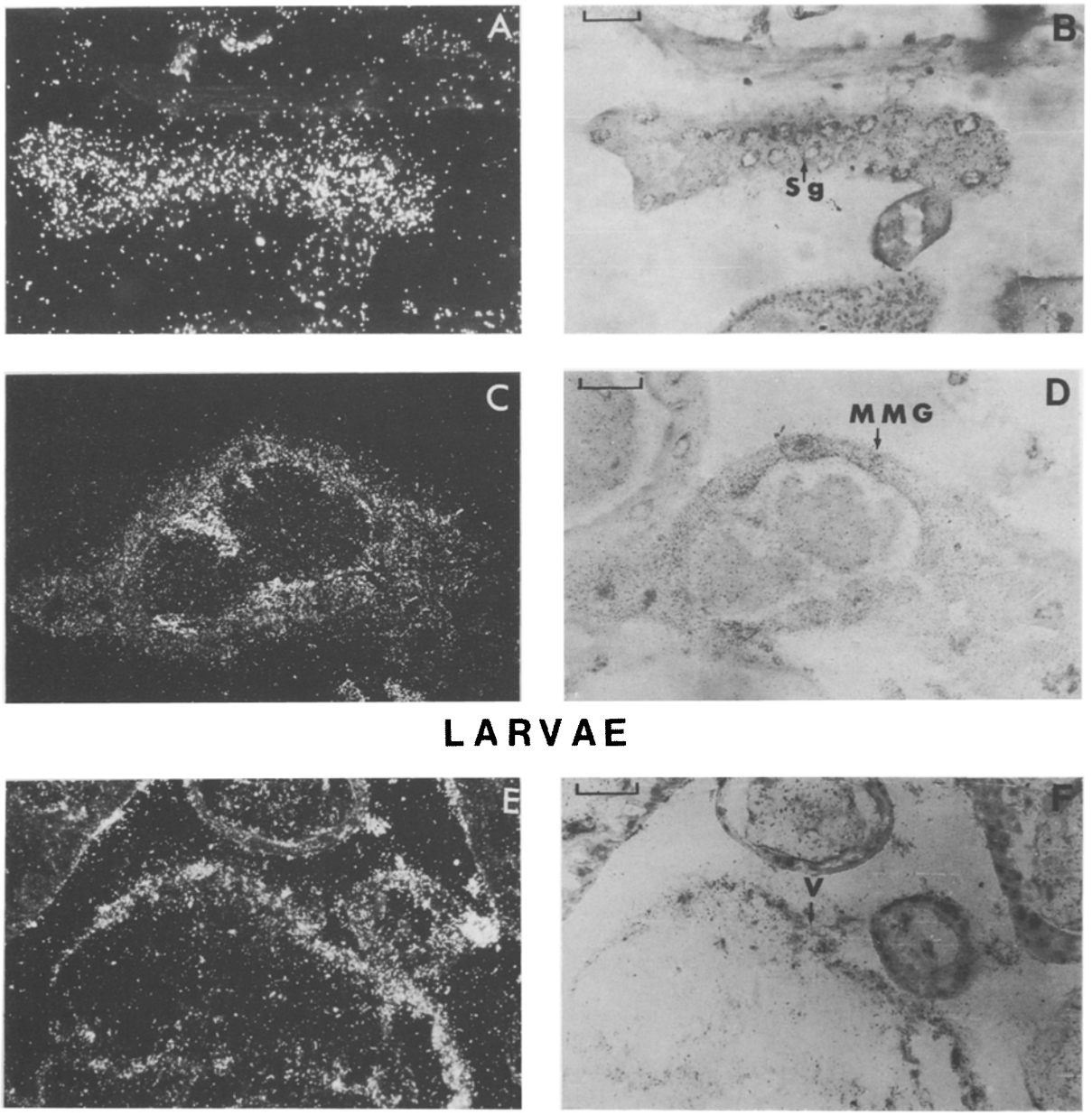


Figure 2. *In situ* localization of *Mto* mRNA transcripts in *D. melanogaster* larvae and adults. An antisense *Mto* [^{35}S]RNA probe was hybridized to RNA on sagittal sections of *D. melanogaster* larvae and adults. Panels (**A**) and (**C**): dark field pictures showing hybridization signals in the salivary glands and in a restricted part of the middle midgut. No signals are visible in the loop of the middle midgut on the left of (**C**). Panels (**B**) and (**D**): bright field pictures of the same sections. Panels (**E**) and (**F**): similar experiment performed with an adult section. Strong hybridization signal is displayed in the area of the ventriculus shown in this section. Symbols: MMG = middle midgut; Sg = salivary glands; V = ventriculus. Autoradiographic exposure: 7 days. Scale bar = 50 μm.

Figure 1. *In situ* localization of *Mtn* mRNA transcripts in *D. melanogaster* larvae. Antisense and sense *Mtn* [^{35}S]RNA probes were hybridized to RNA on sagittal sections of third instar larvae. Panels (**A**) and (**C**): dark field pictures showing hybridization signals of the antisense probe in proventriculus and Malpighian tubules (**A**) and in a portion of the middle midgut (**C**). Panels (**B**) and (**D**): bright field pictures of the same sections. Panels (**E**) and (**G**): dark field pictures showing the background signals obtained with the sense probe. Panels (**F**) and (**H**): bright field pictures of the same sections. Symbols: AMG = anterior midgut; Gc = gastric caeca; Ma = Malpighian tubules; MMG = middle midgut; Pv = proventriculus. Autoradiographic exposure: 7 days. Scale bar = 50 μm.

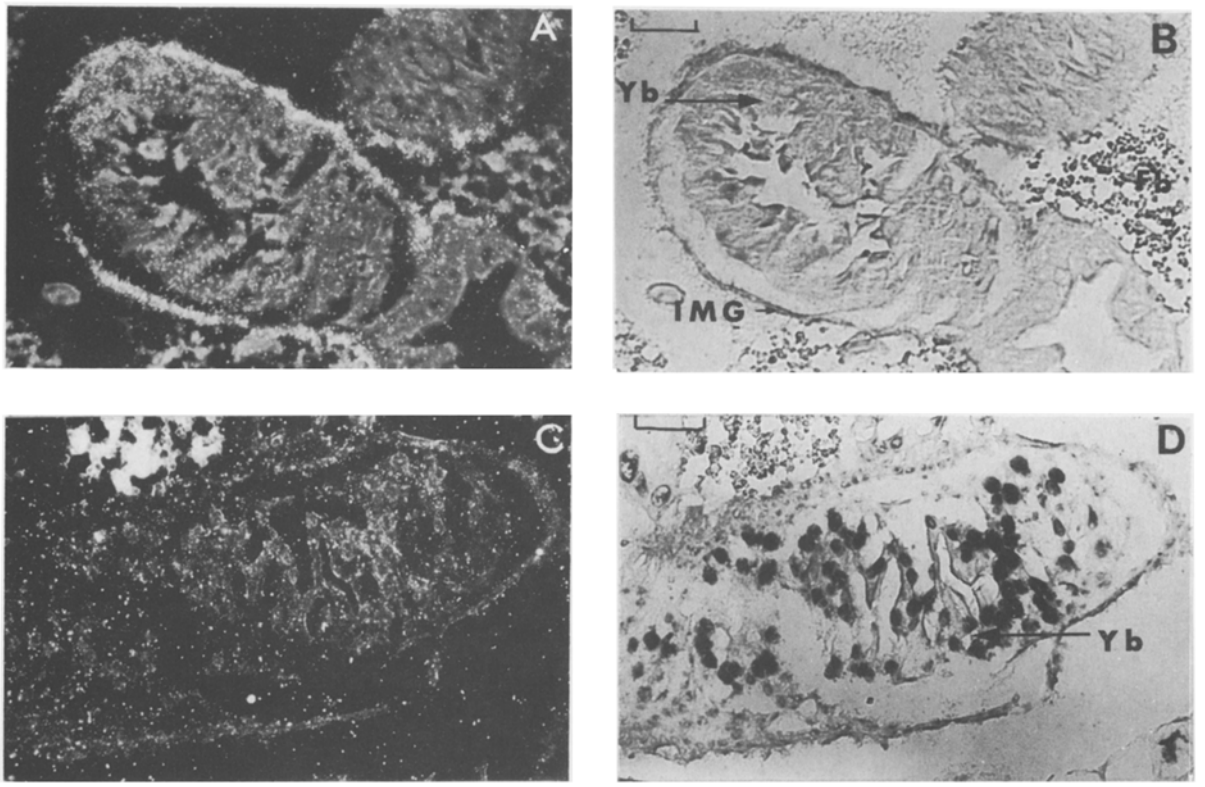


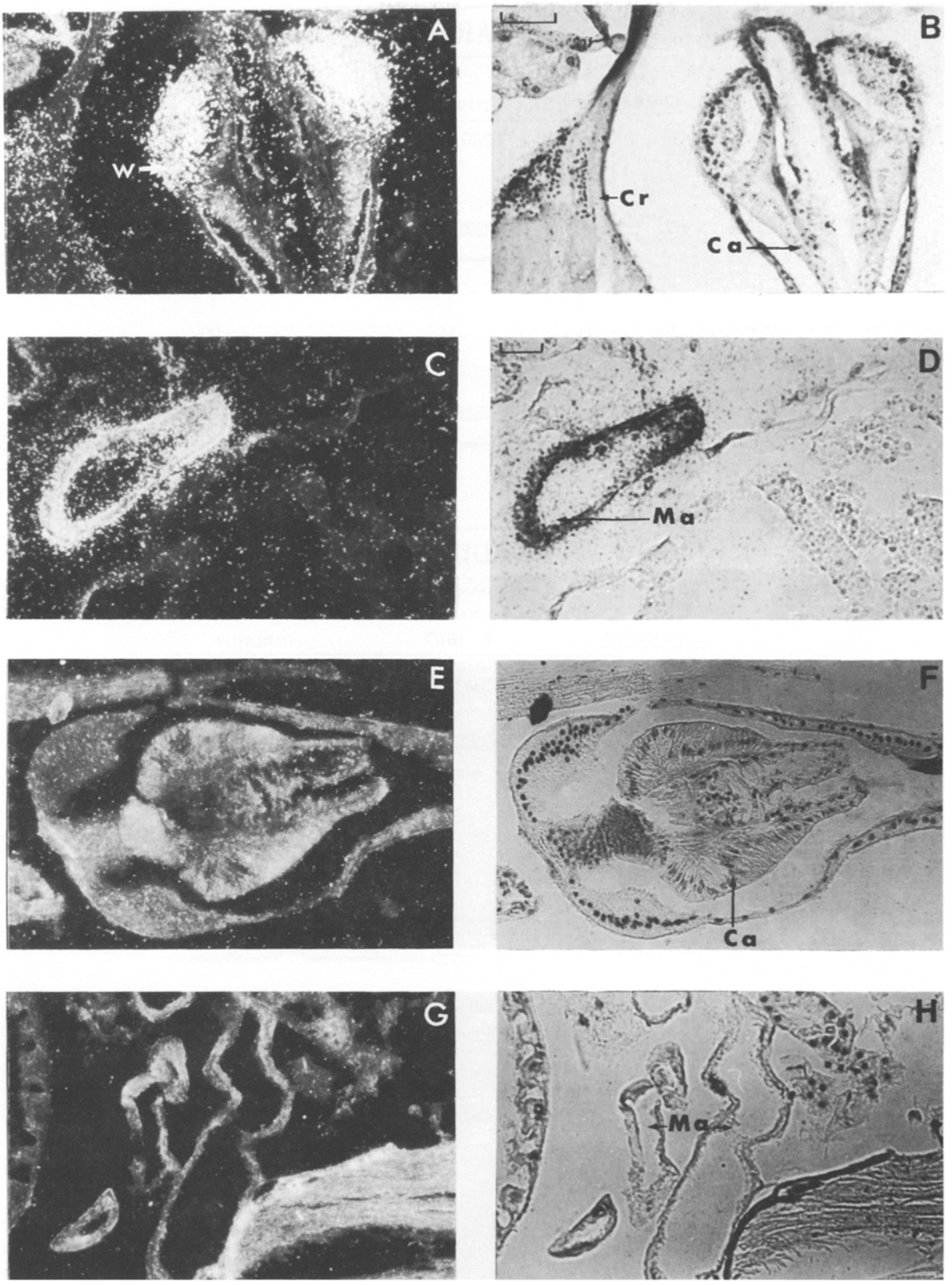
Figure 3. *In situ* localization of *Mto* mRNA transcripts in *D. melanogaster* pupae. Antisense and sense *Mto* [^{35}S]RNA probes were hybridized to RNA on sagittal sections of *D. melanogaster* 3 day old pupae. Panel (**A**): dark field picture showing hybridization signals of the antisense probe in the imaginal midgut. Panel (**B**): bright field picture of the same section. Panel (**C**): dark field picture showing the background signals obtained with the sense probe in the imaginal midgut. Panel (**D**): bright field picture of the same section. Similar results were obtained with a *Mtn* probe. Symbols: IMG = imaginal midgut; Yb = yellow body. Autoradiographic exposure: 7 days. Scale bar = 50 μm.

in two respects. First, they induce an accumulation of metallothionein mRNA in the structures where metallothionein genes are expressed in standard culture conditions. Second, metals induce the expression of metallothionein genes in regions displaying no signals under standard culture conditions. These changes in metallothionein expression patterns differ depending on the metal tested. Although *Mtn* and *Mto* expression patterns following metal treatment are similar, some specific changes are observed in certain regions of the midgut. Zinc, cadmium and copper, for example, have a significant effect on *Mto* mRNA accumulation in larval salivary glands, while they have no effect on *Mtn* expression in the same structures. All *in situ* hybridization data are integrated in the Figure 7.

Copper and cadmium effects. Copper and cadmium both extend the *Mtn* expression domain to the whole midgut of larvae, pupae and adults. In the case of *Mto*, copper induces a wider extension of expression than cadmium. In larvae, this extension reaches the anterior midgut while in adults *Mto* transcripts are found throughout the ventriculus. Extension of *Mto* expression domain by cadmium is restricted to the middle and posterior midgut (Figure 7).

The expression of metallothionein genes in the larval middle midgut includes the region containing the 'cuprophilic cells', also called 'cup-shaped' cells. It is known that these cells accumulate metal ions following an intoxication with copper or cadmium salts (Poulson & Bowen 1952, Filshie *et al.* 1971). Maroni & Watson (1985) tested the absorption of ^{109}Cd by second instar larvae and found that the whole midgut was involved in the process, the cuprophilic cells exhibiting the highest absorption capacity. Lauverjat *et al.* (1989) also demonstrated that accumulation of metals is not

Figure 4. *In situ* localization of *Mtn* mRNA transcripts in *D. melanogaster* adults. Antisense and sense *Mtn* [^{35}S]RNA probes were hybridized to RNA on sagittal sections of *D. melanogaster* flies. Panels (**A**) and (**C**): dark field pictures showing hybridization signals of the antisense probe in the wall of cardia (**A**) and in the Malpighian tubules (**C**). Panels (**B**) and (**D**): bright field pictures of the same sections. Panels (**E**) and (**G**): dark field pictures showing the background signals obtained with the sense probe. Panels (**F**) and (**H**): bright field pictures of the same sections. Symbols: Ca = cardia; Cr = crop; Ma = Malpighian tubules; w = wall of cardia. Autoradiographic exposure: 7 days. Scale bar = 50 μm.



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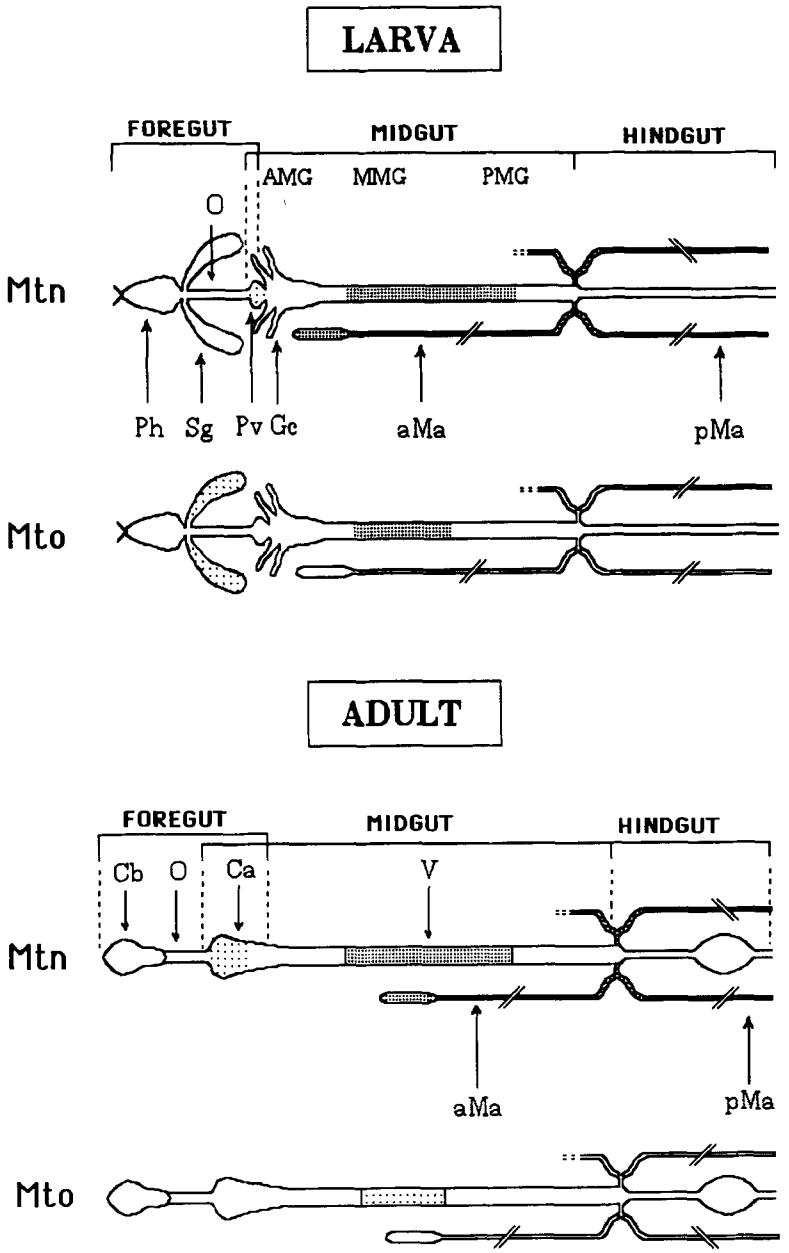

Figure 5. Distribution of *Mtn* and *Mto* transcripts in the digestive tract of *D. melanogaster*. Schematic drawing of the digestive tract of third instar larvae and adults. The length of each region is represented to scale. The crop and the salivary glands of adults, that never display hybridization signals, were omitted. Intensity of hybridization signals, that was estimated visually, is symbolized by three grades: white for the absence of signal, lightly or darkly stippled for light and strong signals, respectively. Abbreviations: AMG = anterior midgut, MMG = middle midgut, PMG = posterior midgut, aMa = anterior Malpighian tubules, pMa = posterior Malpighian tubules, Ca = cardia, Cb = cibarium, Gc = gastric caeca, O = oesophagus, Ph = pharynx, Pv = proventriculus, Sg = salivary glands, V = ventriculus.

restricted to cuprophilic cells but involves the entire midgut. Our *in situ* hybridization data confirm that the whole midgut responds to copper and cadmium intoxication and that metallothioneins may play a role in this process.

Zinc effects. Previous studies showed that zinc was not very efficient to induce metallothionein gene expression in *Drosophila*. Treatment of larvae during 48 h with 5 mM $ZnCl_2$ was shown to be inefficient to induce synthesis of a cadmium-binding protein possessing the properties of metallothioneins (Maroni & Watson 1985). At the transcriptional level, zinc was shown to be a poor inducer of *Mtn* in larvae, when compared with copper or cadmium (Lastowski-Perry *et al.* 1985, Maroni *et al.* 1986). Finally, no detectable increases of both *Mtn* and *Mto* transcripts were observed in adults treated for 1 day with concentrations

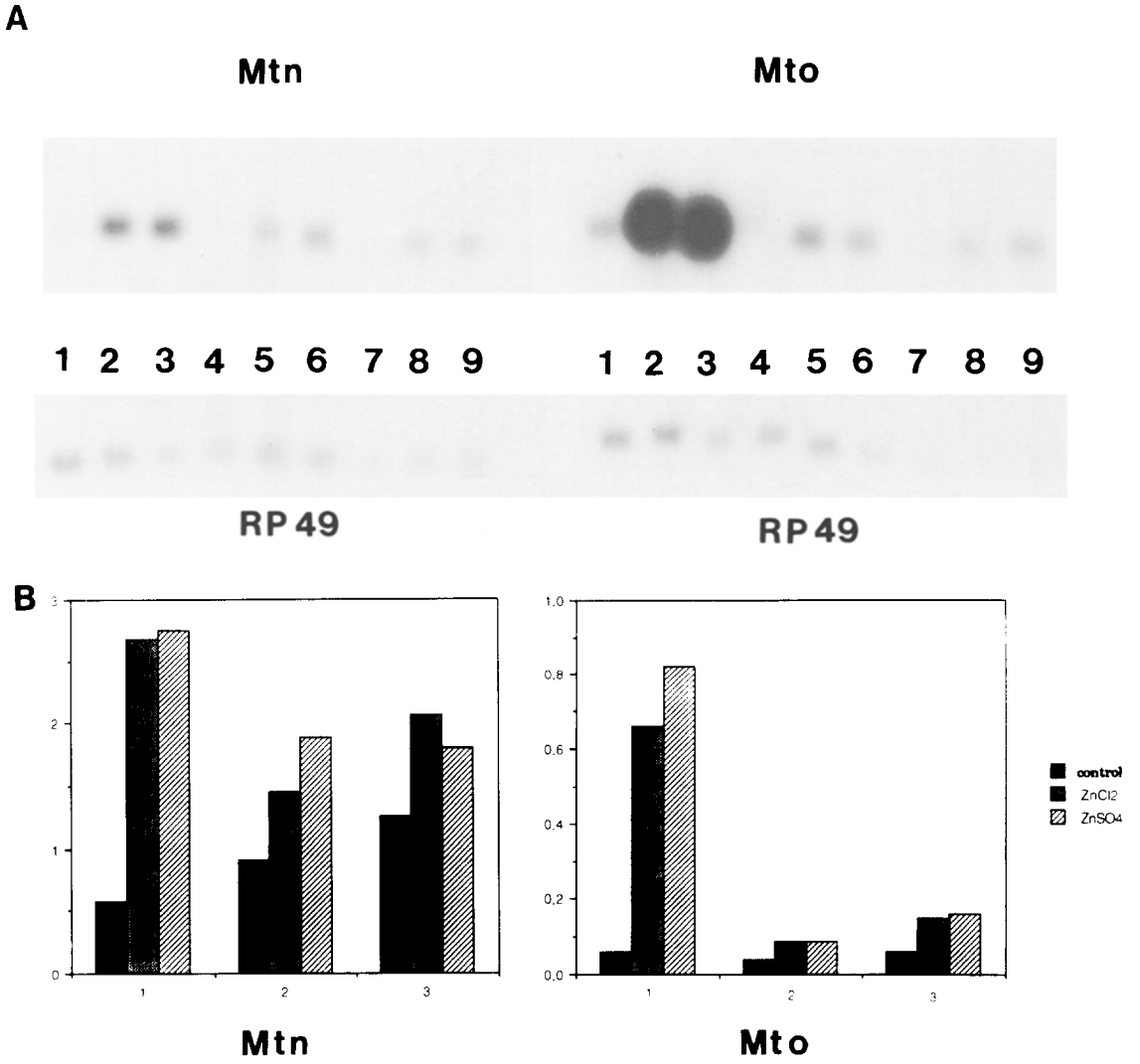

Figure 6. Induction of *Mtn* and *Mto* mRNA accumulation by zinc. Total RNA extracted from 10 animals (±20 μg) was fractionated on 1.3% agarose gels and probed with *Mtn*, *Mto* and *RP49* cDNAs. (**A**) Autoradiograms showing the effect of $ZnCl_2$ and $ZnSO_4$ on *Mtn* and *Mto* mRNA accumulation in third instar larvae and adults. Lanes 1–3 (third instar larvae): 1 = not treated control larvae, 2 = 5 mM $ZnCl_2$, 3 = 5 mM $ZnSO_4$; lanes 4–6 (1 day old adults): 4 = not treated control adults, 5 = 5 mM $ZnCl_2$, 6 = 5 mM $ZnSO_4$; lanes 7–9 (3 day old adults): 7 = not treated control adults, 8 = 5 mM $ZnCl_2$, 9 = 5 mM $ZnSO_4$. Specific activity of DNA probes: 10^8 d.p.m. μg^{-1}. Duration of exposure: *Mtn* = 3 h, *Mto* = 3 days, *RP49* = 18 h. (**B**) Quantification of *Mtn* and *Mto* mRNAs. We used the SOFI detector (Mastrippolito *et al.* 1991) to measure the hybridization signals shown in (**A**). 1 = third instar larvae, 2 = 1 day old adults, 3 = 3 day old adults. Black boxes: controls, spotted boxes: 5 mM $ZnCl_2$ treated animals, stippled boxes: 5 mM $ZnSO_4$ treated animals. *RP49* hybridization signals were used to adjust variations in RNA loading.

of $ZnCl_2$ ranging from 0.25 to 2.5 mM (Silar *et al.* 1990). Here, we tested whether higher zinc concentrations would induce metallothionein mRNA accumulation following a three day treatment.

Quantitative analyses revealed that high zinc concentrations can induce an increased accumulation of both *Drosophila* metallothionein mRNAs (Figure 6). Similar results were obtained with $ZnCl_2$ and $ZnSO_4$. The two genes exhibit the higher response to zinc during the third larval instar: there is a five times increase for *Mtn* mRNA and a 12 times increase for *Mto* mRNA following the exposure of larvae to 5 mM $ZnCl_2$ or $ZnSO_4$ for 16 h (Figure 6). In adults, induction is much weaker (1.6 relative increase for *Mtn* mRNA and 2.4 for *Mto*, respectively, for 3 day treatments; Figure 6). Differences between larvae and adults could be due to the fact that adults ingest less food than larvae. The weak effect of zinc on adults may explain why we did not detect any induction by this metal on metallothionein mRNA accumulation in our previous experiments, analyzing mRNAs from 1 day treated animals (Silar *et al.* 1990).

In situ hybridization demonstrated that zinc has specific effects on the localization of metallothionein transcripts when compared with copper and cadmium (Figure 7). In adults, zinc induces the expression of *Mtn* in the cardia.

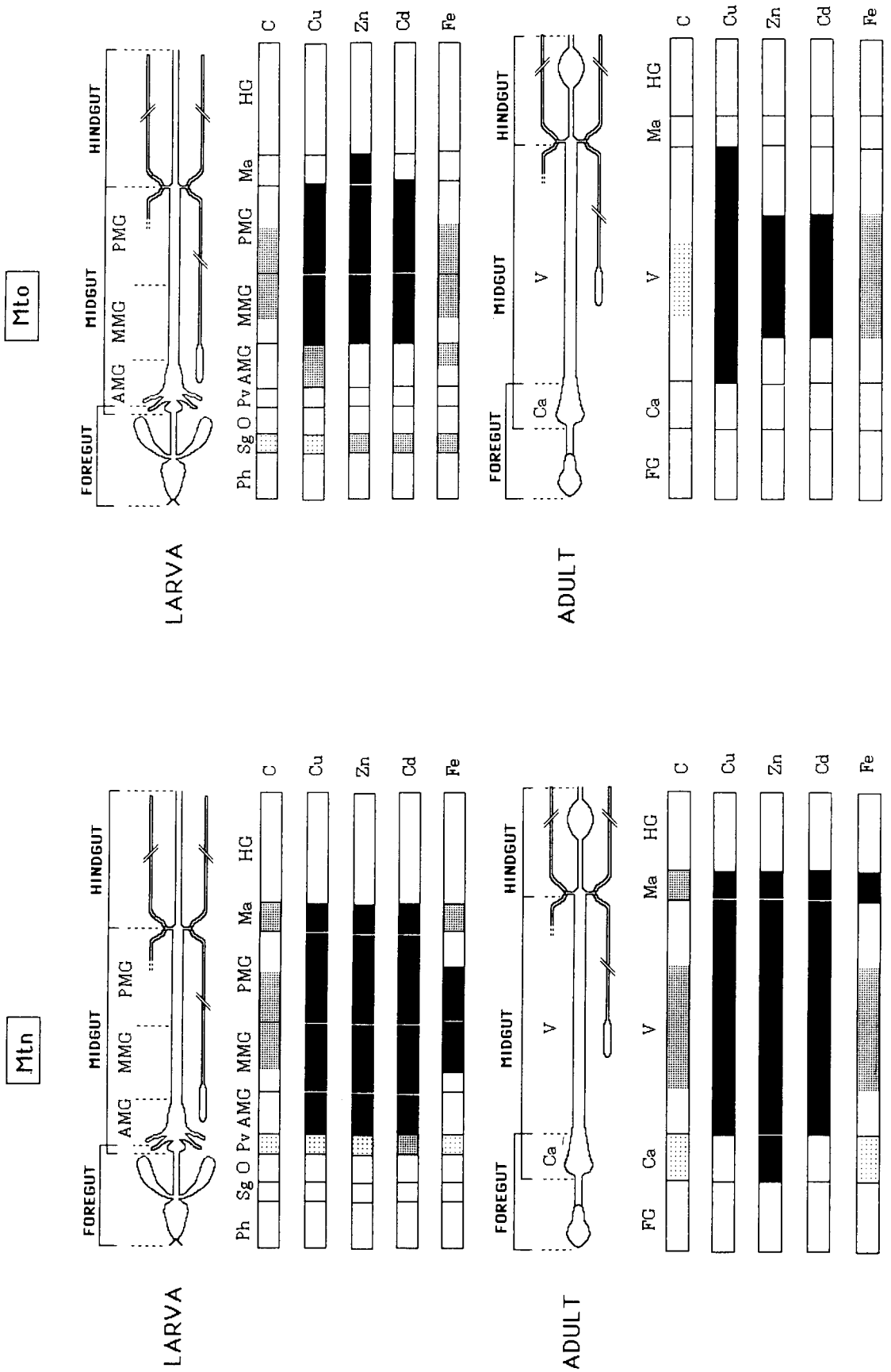

Figure 7. Distribution of *Mtn* and *Mto* transcripts in the digestive tract of *D. melanogaster* following metal treatment. Diagrammatic representation of the hybridization signals obtained for each type of metal treatment below the schematic drawing already described in Figure 5. A fourth grade, i.e. black, was used to symbolize very strong hybridization signals. Metal treatment: C = control (*cf.* Figure 5), Cu = 0.5 mM $CuSO_4$, Zn = 5 mM $ZnSO_4$, Cd = 0.1 mM $CdCl_2$, Fe = 0.5 mM $FeSO_4$.

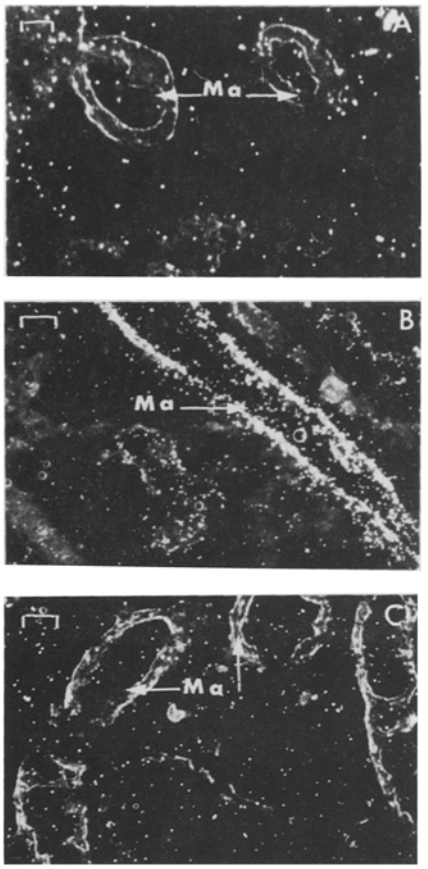

Figure 8. *In situ* localization of *Mto* mRNA transcripts in Malpighian tubules. Antisense and sense *Mto* [^{35}S]RNA probes were hybridized to RNA on sagittal sections of *D. melanogaster* third instar larvae. Panel (**A**): dark field picture showing the absence of hybridization signals with the antisense probe in the Malpighian tubules of control larvae. Panel (**B**): strong hybridization signals appear in Malpighian tubules of zinc-treated larvae (5 mM$ZnSO_4$). Panel (**C**): dark field picture showing the background signals obtained with the sense probe in the Malpighian tubules of zinc-treated larvae (5 mM$ZnSO_4$). Symbol: Ma = Malpighian tubules. Autoradiographic exposure: 7 days. Scale bar = 50 μm.

Accumulation of *Mto* mRNA, following zinc treatment, was observed in Malpighian tubules of larvae and pupae, but not in adults (Figure 8). The interest of this latter result is highlighted by the fact that Malpighian tubules are the main sites of zinc storage (Zierold & Wessing 1990). This organ contains concretions rich in sulfur and plays an important role in detoxification processes (Sohal & Lamb 1979). Thus, *Mto* may be involved in zinc metabolism of the Malpighian tubules.

Iron effects. Iron, of the four metals tested, has the smallest inductive capacity on metallothionein mRNA accumulation (Figure 7). A slight, but reproducible extension of the *Mto* expression domain was observed in the midgut of iron-treated adults. Surprisingly, *Mto* mRNA accumulation is induced by iron in the proventriculus of pupae (Figure 9). This is also the case for the three other metals tested (not shown).

The cells responding to iron treatment are located within the region comprising the 'iron cells' described by Poulson & Bowen (1952) as iron-accumulating cells. Iron homeostasis in vertebrates is known to be controlled mainly by ferritin, a protein capable of binding iron as well as zinc, cadmium and copper (Price & Joshi 1982, 1983). The displacement of zinc and copper from ferritin, due to the higher affinity of ferritin for iron, could increase the amount of free zinc and copper ions. These ions would then be available to induce metallothionein genes. If this interpretation is correct, the inductive effect we observed for iron would be indirect. Iron effects on metallothionein regulation have been poorly investigated until now. It is known that, in the chick, parenteral injection of iron induces zinc metallothionein accumulation in the liver (McCormick 1984). Toxicity of free metallic ions in cells is probably controlled by both metallothioneins and ferritins. The study of the regulation of these two proteins in a single system may lead to a better understanding of cellular processes involved in metal homeostasis and detoxification.

Functional evolution of metallothionein genes in D. melanogaster

The duplication of genes is thought to provide a major source of functional novelty in evolution. Since two genes

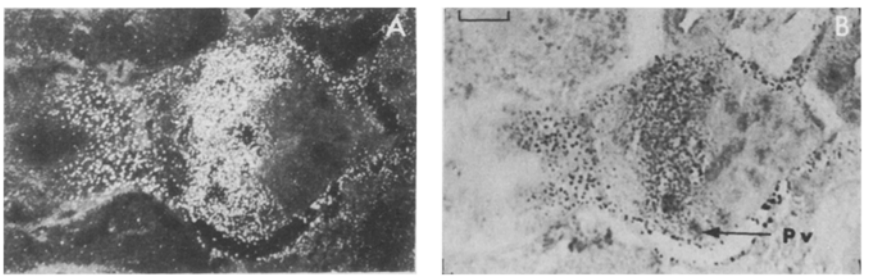

Figure 9. *In situ* localization of *Mto* mRNA transcripts in the proventriculus of iron-treated *D. melanogaster* pupae. An antisense *Mto* [^{35}S]RNA probe was hybridized to RNA on sagittal sections of *D. melanogaster* 3 day old iron-treated pupae. Panel (**A**): dark field picture showing hybridization signals in the proventriculus imaginal ring. This signal is detected only in metal-treated animals. Panel (**B**): bright field picture of the same section. Symbol: Pv – proventriculus. Autoradiographic exposure: 7 days. Scale bar = 50 μm.

derived from the duplication of a common ancestor gene may evolve independently, significant divergence is expected to occur with time at both the structural and functional levels. The metallothionein genes of *D. melanogaster* exemplify such a process. The *Jonah* genes constitute another family of duplicated genes in *Drosophila* that display specific expression patterns in the post-embryonic digestive tract (Carlson & Hogness 1985).

Available data suggest that the two *Drosophila* metallothioneins would be able to play similar roles, but also that they would have specific functions. Common functions would be related to metal detoxification. Both *Mtn* and *Mto* genes, clearly, are strongly induced in the midgut by metal treatments. Metal detoxification may be considered as a two-step process. In the first step, toxic effects of metals would be antagonized by their binding to high-affinity molecules such as metallothioneins or ferritins. In a second step, metals would be sequestered in special cytoplasmic structures (lysosomes, for example) and eliminated from the cytoplasm (Richards 1989). In insects, detoxification mainly occurs in the Malpighian tubules (Wessing & Eichelberg 1978). This organ, in *Drosophila*, expresses the *Mtn* gene at a high rate (Figures 5 and 7). The accumulation of *Mtn* and *Mto* mRNA in the midgut following metal intoxication suggests that the midgut also would participate in detoxification processes. It is significant, therefore, that Poulson & Bowen (1952), as well as Tapp & Hockaday (1977) and Lauverjat *et al.* (1989), have observed that the largest fraction of cytoplasmic copper is associated with lysosomes in the *D. melanogaster* middle midgut.

The function of the MTN protein would be related essentially to detoxification processes. The progressive enhancement of *Mtn* gene transcription from late embryonic stages to the adult is compatible with this hypothesis. The MTO protein may have an additional function. This protein could control the storage of zinc and copper and their transfer to metalloenzymes or to zinc-dependent transcription factors. The fact that the *Mto* gene is expressed at a weak and constant level throughout developmental stages to the adult suggests a function in metal homeostasis. Firmer statements about the functional significance of the two very divergent metallothionein genes in *Drosophila* await more direct cellular and genetical evidences.

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References

Andrews GK, Huet-Hudson YM, Paria BC, McMaster MT, De SK, Dey SK. 1991 Metallothionein gene expression and metal regulation during preimplantation mouse embryo development. *Dev Biol* **145,** 13–27.

Beach LR, Palmiter RD. 1981 Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proc Natl Acad Sci USA* **78,** 2110–2114.

Bonneton F, Wegnez M. 1995 Developmental variability of metallothionein *Mtn* gene expression in the species of the *Drosophila melanogaster* subgroup. *Dev Genet* **16,** 253–263.

Bouquegneau JM, Ballan-Dufrançais C, Jeantet AY. 1985 Storage of Hg in the ileum of *Blatella germanica*: biochemical characterization of metallothionein. *Comp Biochem Physiol* **80C,** 95–98.

Bryant PJ, Levinson P. 1985 Intrinsic growth control in the imaginal primordia of *Drosophila*, and the autonomous action of the lethal mutation causing overgrowth. *Dev Biol* **107,** 355–363.

Carlson JR, Hogness DS. 1985 Developmental and functional analysis of *Jonah* gene expression. *Dev Biol* **108,** 355–368.

Debec A, Mokdad R, Wegnez M. 1985 Metallothioneins and resistance to cadmium poisoning in *Drosophila* cells. *Biochem Biophys Res Commun* **127,** 143–152.

Dimitriadis VK, Kastritsis CD. 1984 Ultrastructural analysis of the midgut of *Drosophila auraria* larvae. Morphological observations and their physiological implications. *Can J Zool* **62,** 659–669.

Erraïss NE, Silar P, Cadic-Jacquier A, Modkad-Gargouri R, Wegnez M. 1989 The metallothionein system of *Drosophila melanogaster*. In: Hamer DH, Winge DR, eds. *Metal Ion Homeostasis: Molecular Biology and Chemistry*. New York: Alan R Liss; 91–99.

Filshie BK, Poulson DF, Waterhouse DF. 1971 Ultrastructure of the copper-accumulating region of the *Drosophila* larval midgut. *Tissue Cell* **3,** 77–102.

Hamer DH. 1986 Metallothionein. *Annu Rev Biochem* **55,** 913–951.

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

Lange BW, Langley CH, Stephan W. 1990 Molecular evolution of *Drosophila* metallothionein genes. *Genetics* **126,** 921–932.

Lastowski-Perry D, Otto E, Maroni G. 1985 Nucleotide sequence and expression of a *Drosophila* metallothionein. *J Biol Chem* **260,** 1527–1530.

Lauverjat 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*. *Biol Metals* **2,** 97–107.

Marchal-Ségault D, Briançon C, Halpern S, Fragu P, Laugé G. 1990 Secondary ion mass spectrometry analysis of the copper distribution in *Drosophila melanogaster* chronically intoxicated with Bordeaux mixture. *Biol Cell* **70,** 129–132.

Maroni G. 1989 Animal metallothioneins. In: Shaw AJ, ed. *Heavy Metal Tolerance in Plants*. Boca Raton: CRC Press; 215–232.

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

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

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

Masters BA, Kelly EJ, Quaife CJ, Brinster RL,Palmiter RD. 1994 Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc Natl Acad Sci USA* **91,** 584–588.

Mastrippolito R, Bendali M, Charon Y, Leblanc M, Martin B, Tricoire H, Valentin L. 1991 SOFI: a bidimensional detector for fast direct on-line quantification of beta particles on blots. *BioTechniques* **11,** 778–783.

McCormick CC. 1984 The tissue-specific accumulation of hepatic zinc metallothionein following parenteral iron loading. *Proc Soc Exp Biol Med* **176,** 392–402.

Michalska AE, Choo KHA. 1993 Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc Natl Acad Sci USA* **90,** 8088–8092.

Miller A. 1965 The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: Demerec M, ed. *Biology of Drosophila*. New York: Hafner; 420–534.

Mokdad R, Debec A, Wegnez M. 1987 Metallothionein genes in *Drosophila melanogaster* constitute a dual system. *Proc Natl Acad Sci USA* **84,** 2658–2662.

Nemer M, Thornton RD, Stuebing EW, Harlow P. 1991 Structure, spatial, and temporal expression of two sea urchin metallothionein genes, $SpMTB_1$ and SpMTA. *J Biol Chem* **266,** 6586–6593.

Otto E, Young JE, Maroni G. 1986 Structure and expression of a tandem duplication of the *Drosophila* metallothionein gene. *Proc Natl Acad Sci USA* **83,** 6025–6029.

Poulson DF, Bowen VT. 1952 Organization and function of the inorganic constituents of nuclei. *Exp Cell Res, Suppl* **2,** 161–179.

Poulson DF, Bowen VT, Hilse RM, Rubinson AC. 1952 The copper metabolism of *Drosophila*. *Proc Natl Acad Sci USA* **38,** 912–921.

Price D, Joshi JG. 1982 Ferritin: a zinc detoxicant and a zinc ion donor. *Proc Natl Acad Sci USA* **79,** 3116–3119.

Price DJ, Joshi JG. 1983 Ferritin-binding of beryllium and other divalent metal ions. *J Biol Chem* **258,** 10873–10880.

Richards MP. 1989 Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism. *J Nutr* **119,** 1062–1070.

Sato M, Bremner I. 1993 Oxygen free radicals and metallothionein. *Free Rad Biol Med* **14,** 325–337.

Silar P, Théodore L, Mokdad R, Erraïss NE, Cadic A, Wegnez M. 1990 Metallothionein *Mto* gene of *Drosophila melanogaster*: Structure and regulation *J Mol Biol* **215,** 217–224.

Sohal RS, Lamb RE, 1979 Storage-excretion of metallic cations in the adult housefly, *Musca domestica*. *J Insect Physiol* **25,** 119–124.

Sohal RS, Peters PD, Hall TA. 1976 Fine structure and X-ray microanalysis of mineralized concretions in the Malpighian tubules of the housefly, *Musca domestica*. *Tissue Cell* **8,** 447–458.

Tamai KT, Gralla EB, Ellerby LM, Valentine JS, Thiele DJ. 1993 Yeast and mammalian metallothioneins functionally substitute for yeast copper–zinc superoxide dismutase. *Proc Natl Acad Sci USA* **90,** 8013–8017.

Tapp RL, Hockaday A. 1977 Combined histochemical and X-ray microanalytical studies on the copper-accumulating granules in the mid-gut of larval *Drosophila*. *J Cell Sci* **26,** 201–215.

Terra WR, Espinoza-Fuentes FP, Ribeiro AF, Ferreira C. 1988 The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion. *J Insect Physiol* **34,** 463–472.

Théodore L, Ho AS, Maroni G. 1991 Recent evolutionary history of the metallothionein gene *Mtn* in *Drosophila*. *Genet Res Camb* **58,** 203–210.

Tsuji S, Kobayashi H, Uchida Y, Ihara Y, Miyatake T. 1992 Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. *EMBO J* **11,** 4843–4850.

Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M. 1991 The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron* **7,** 337–347.

Wessing A, Eichelberg D. 1978 Malpighian tubules, rectal papillae and excretion. In: Ashburner M, Wright TRF, eds. *The Genetics and Biology of Drosophila*. London: Academic Press; **2C,** 1–42.

Zierold K, Wessing A. 1990 Mass dense vacuoles in *Drosophila* Malpighian tubules contain zinc, not sodium. A reinvestigation by X-ray microanalysis of cryosections. *Eur J Cell Biol* **53,** 222–226.