Response of *Drosophila* metallothionein promoters to metallic, heat shock and oxidative stresses

François Bonneton[a], Laurent Théodore[a,b], Philippe Silar[a], Gustavo Maroni[b], Maurice Wegnez[a,*]

[a]*Laboratoire d'Embryologie Moléculaire et Expérimentale, Unité de Recherche Associée 1134 du Centre National de la Recherche Scientifique, Université Paris XI, Bâtiment 445, 91405 Orsay Cedex, France*

[b]*Department of Biology, CB# 3280, Coker Hall, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, USA*

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Abstract **The metallothionein system in *Drosophila melanogaster* is composed of two genes, *Mtn* and *Mto*. In order to compare the induction properties of these genes, we transformed *D. melanogaster* with *P*-element vectors containing *Adh* and *lacZ* reporter genes under the control of *Mtn* and *Mto* promoters, respectively. *Mtn* and *Mto* transgenes are mainly expressed in the digestive tract. However, *Mtn* expression has been detected also in the fat body. *Mtn* and *Mto* transgenes respond differently to metallic, heat-shock and oxidative stresses. These data confirm that both genes are in part functionally different.**

Key words: Metallothionein; Metal intoxication; Heat-shock; Oxidative stress; *Drosophila melanogaster*

1. Introduction

Metallothioneins are small, cysteine-rich proteins that bind metal ions with high affinity. They are involved in metal detoxification [1] and in protection against free radicals [2]. Their function may be related also to absorption, transport and storage of metals [3]. These different aspects of metallothionein function have been studied using various experimental systems, ranging from yeast to man.

Drosophila melanogaster offers a broad spectrum of experimental opportunities to get an overall view on metallothionein role at the level of the whole organism. The two metallothionein genes of *Drosophila melanogaster*, *Mtn* and *Mto*, encode proteins displaying only 25% similarity [4]. Both genes are differently expressed during development [5,6], and their expression can be increased by the inductive effect of various metals [5,7,8]. Involvement of the *Mtn* gene in detoxification processes has been demonstrated by the characterization of Mtn^1 allele duplications in natural and laboratory strains. These strains synthesize *Mtn* mRNA at a higher level than those carrying one single Mtn^1 gene, and tolerate increased metal concentrations [9,10]. Duplications of the *Mto* gene or of the Mtn^3 allele have never been observed [5,11,12]. Here we report a study of the properties of the *Mto* and *Mtn* promoters in transgenic *Drosophila* strains using *lacZ* and *Adh* reporter genes, respectively.

2. Materials and methods

2.1. Transformation vectors

Two *Mto* promoter fragments, Po (*Dde*I fragment (−221 to +107), Fig. 1) and Go (*BamH*I–*Dde*I fragment spanning about 1.2 kb of *Mto* 5′ flanking region, Fig. 1), have been cloned upstream of the *lacZ* gene into the *Xba*I site of the *P*-element vector *pW–lac1* [13].

The *Mtn* promoter fragment, Pn (*Eco*RI–*Stu*I fragment (−373 to +54), Fig. 1), has been cloned upstream of the *Adh* gene as follows. A *Bgl*II site was first introduced by site directed mutagenesis in the *D. melanogaster Adh* genomic sequence at the +2 position (relative to the proximal transcription start) and the resulting construct was named pGA. The *EcoRI–StuI* (−373 to +54) fragment of the *Mtn* promoter was subcloned into pUC18 between *EcoR*I and *Sma*I yielding pMCR11. The *BglII–XbaI Adh* fragment was then cloned into pMCR11 between *BamH*I and *Xba*I to give pNPAG. The *EcoRI–XbaI* fragment of pNPAG was finally subcloned into *pCasper* [14] producing pCNPAG.

2.2. Fly transformation

Germ-line transformation was performed using standard protocols [15]. *Mto–lacZ* vectors were injected into *wΔ2–3* embryos [16]. The *Mtn–Adh* vector (pCNPAG) was co-injected with the *pHSΔ2–3* plasmid encoding transposase activity into *w*, Adh^{nul} embryos (SP55 stock). All inserts are maintained homozygous except for *Go22* in which insertion occurred on the TM6 balancer.

2.3. Transgene expression

Digoxigenin-labeled RNA probes were used for in situ hybridization using a protocol described previously [6]. Detection of β-galactosidase and ADH activity in larval tissues was performed as described by Ashburner [17]. The β-galactosidase reaction was stopped after an overnight incubation. A 10 min incubation was sufficient for ADH detection.

2.4. Stress experiments

Metallic stresses were induced by rearing third-instar larvae during 24 h on *Instant Drosophila Medium* (Carolina Biological Supply Company) supplemented with various salts (0.2 g/ml salt solution).

For heat-shocking, adults were allowed to lay eggs during 24 h at 25°C. Progeny was then reared at 29°C and treated daily with 2 h, 37°C pulses. Gene expression was analyzed on third-instar larvae.

Oxidative stresses were induced by adding 10 mM paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, Sigma Chemical Co) to the medium throughout larval development. Paraquat generates O_2^- radicals in vivo [18].

3. Results and discussion

In order to compare the regulation of the two *Drosophila* metallothionein genes, fragments of the *Mto* and *Mtn* 5′ flanking regions (Fig. 1) were inserted upstream of the *lacZ* and *Adh* reporter genes, respectively. All transgenic lines constructed for this study are listed in Table 1. The junction between the promoter and reporter genes is localized in the leader sequences (+107 for *Mto*, +54 for *Mtn*). Two constructs extending either up to −221 (Po) or −1220 (Go) were tested in the case of *Mto*. For *Mtn*, a DNA fragment spanning 373 bp upstream of the transcription start (Pn) was chosen since it was previously shown to be sufficient to confer tissue-specificity and metal-

*Corresponding author. Fax: (33) (1) 69 85 35 38.

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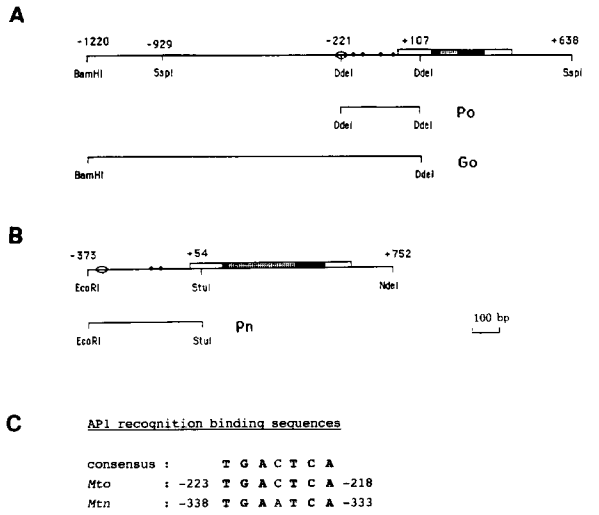

Fig. 1. *Drosophila* metallothionein gene structure. A: Structure of the *Mto* gene and of its promoter regions fused to the *lacZ* gene in Po and Go constructs. Position of the −1220 *BamHI* site is approximate, since the sequence has not been determined upstream of the −929 *SspI* site [5]. B: Structure of the *Mtn* gene and of its promoter region fused to the *Drosophila Adh* gene in the Pn construct. C: Putative AP1 recognition binding sequences located in *Mto* and *Mtn* promoters. Bold letters have been used for residues shared by *Mto*, *Mtn* and the AP1 core consensus sequence [19]. Symbols: open oval = AP1 site, black spots = MRE sites, open boxes = transcribed but not translated regions, black boxes = coding regions, dotted boxes = introns.

inducibility to a reporter gene in *Drosophila* transgenic lines [20,21].

3.1. *Basal expression of* Mto–lacZ *and* Mtn–Adh *transgenes*

The transcriptional patterns of the *Mto* and *Mtn* genes were previously analyzed by in situ hybridization on sections of third instar larvae [22]. These patterns were compared to those of the fusion genes in order to determine whether the sequences used to drive transgene expression are sufficient to mimic the endogenous expression of the metallothionein genes.

The Po and Go constructs (Fig. 1) behave differently. Under standard conditions, no transgenic β-galactosidase activity was detected in larvae of *Po* lines (Fig. 2A), while in the five *Go* lines, β-galactosidase activity was detected in the region of the middle midgut that corresponds to that of the iron cells (Fig. 2B). In all lines, no β-galactosidase activity was detected in other tissues. Differences between Po and Go constructs are likely due to regulatory sequences located between the positions −221 and −1220 of the *Mto* promoter. As shown in Fig. 2C,

Table 1
Transgenic lines

Constructs	Transgenic lines	Insertion sites
Po	*Po3*	X
(p*Mto*[−221; +107]-*lacZ*)	*Po7*	II
Go	*Go1*	II
(p*Mto*[−1220; +107]-*lacZ*)	*Go7*	II
	Go110	II
	Go123	X
	Go22	III (TM6)
Pn	*Pn4161*	2 insertions on III
(p*Mtn*[−373; +54]-*Adh*)	*Pn4162*	X and III

basal expression of the *Mtn–Adh* transgene was detected in Malpighian tubules and in two regions of the larval midgut : the gastric caeca and the iron cells region. The only site where both *Mto–lacZ* and *Mtn–Adh* transgenes are expressed in standard conditions thus is the iron cells region of the middle midgut, that is known to be implicated in metal metabolism [8,23].

The difference in β-galactosidase activity found between *Go* and *Po* lines may be due to the presence/absence of an AP1

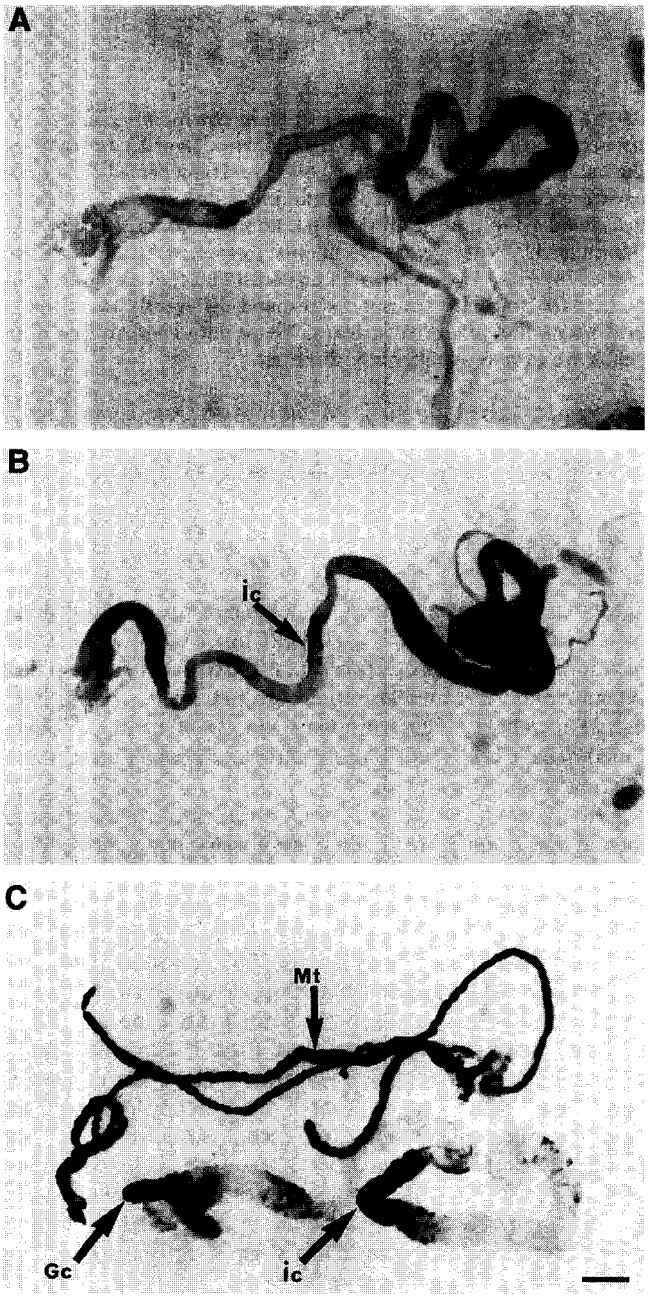

Fig. 2. Basal expression of the reporter genes in third instar larvae (cf. Table 1). The anterior part of the gut is on the left. A: Absence of *lacZ* expression in *Po7* larvae. The staining observed in the anterior midgut and in the middle of posterior midgut is similar to that due to the endogenous galactosidase activity. B: Detection of *lacZ* activity in the iron cells region of the *Go1* middle midgut. C: Distribution of ADH activity in the digestive tract of the *Pn4161* line. Symbols: Gc = gastric caeca, Ic = iron cells, Mt = Malpighian tubules. Scale bar = 0.25 mm.

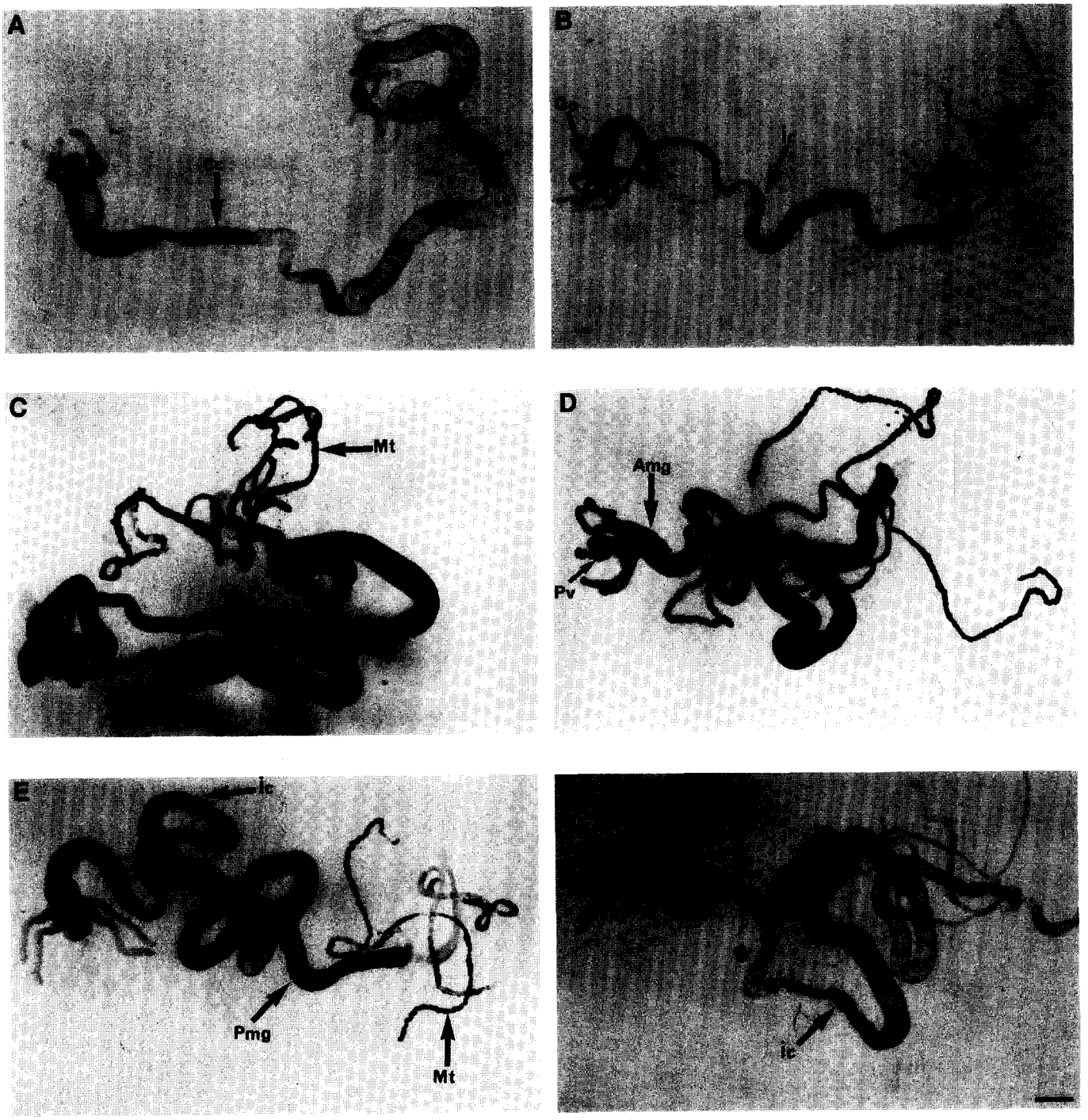

Fig. 3. Effects of various metal treatments on *Mto–lacZ* transgenes. The distribution of β-galactosidase activity is shown in the larval midgut of the *Po7* line after treatment with 0.5 mM $CuSO_4$ (A) and 0.5 mM $CdCl_2$ (B); and in the larval midgut of the *Go1* line after treatment with 0.5 mM $CuSO_4$ (C), 0.5 mM $CdCl_2$ (D), 0.5 mM $ZnSO_4$ (E) and 0.5 mM Fe(III)SO_4 (F). Symbols: Amg = anterior midgut, Cc = copper cells region, Gc = gastric caeca, Ic = iron cells region, Mt = Malpighian tubules, Pmg = posterior midgut, Pv = proventriculus. Scale bar = 0.25 mm.

regulatory element. In mammals, it has been shown that the AP1 transcription factor is an enhancer of metallothionein gene expression [24]. In invertebrates, consensus AP1-binding sites are present in the 5′ region of the two *Caenorhabditis elegans* metallothionein genes [25], as well as in the two *Drosophila* metallothionein genes (Fig. 1). A putative *Mto* AP1-binding site (positions −223 to −218, Fig. 1C) is present in the Go construct, but not in the Po construct since the *Dde*I site used to construct the short *Mto–lacZ* transgene interrupts the AP1 sequence (Fig. 1A). The *Mtn* promoter fragment used for the Pn construction includes an AP1 element (Fig. 1B,C), but the effect of its disruption has not been tested yet.

These results obtained with the transgenic lines (Fig. 1) are in good accordance with our previous analysis of *Mto* and *Mtn* transcription in larvae [22]. Furthermore, they allow a more precise identification of the structures expressing the metal-

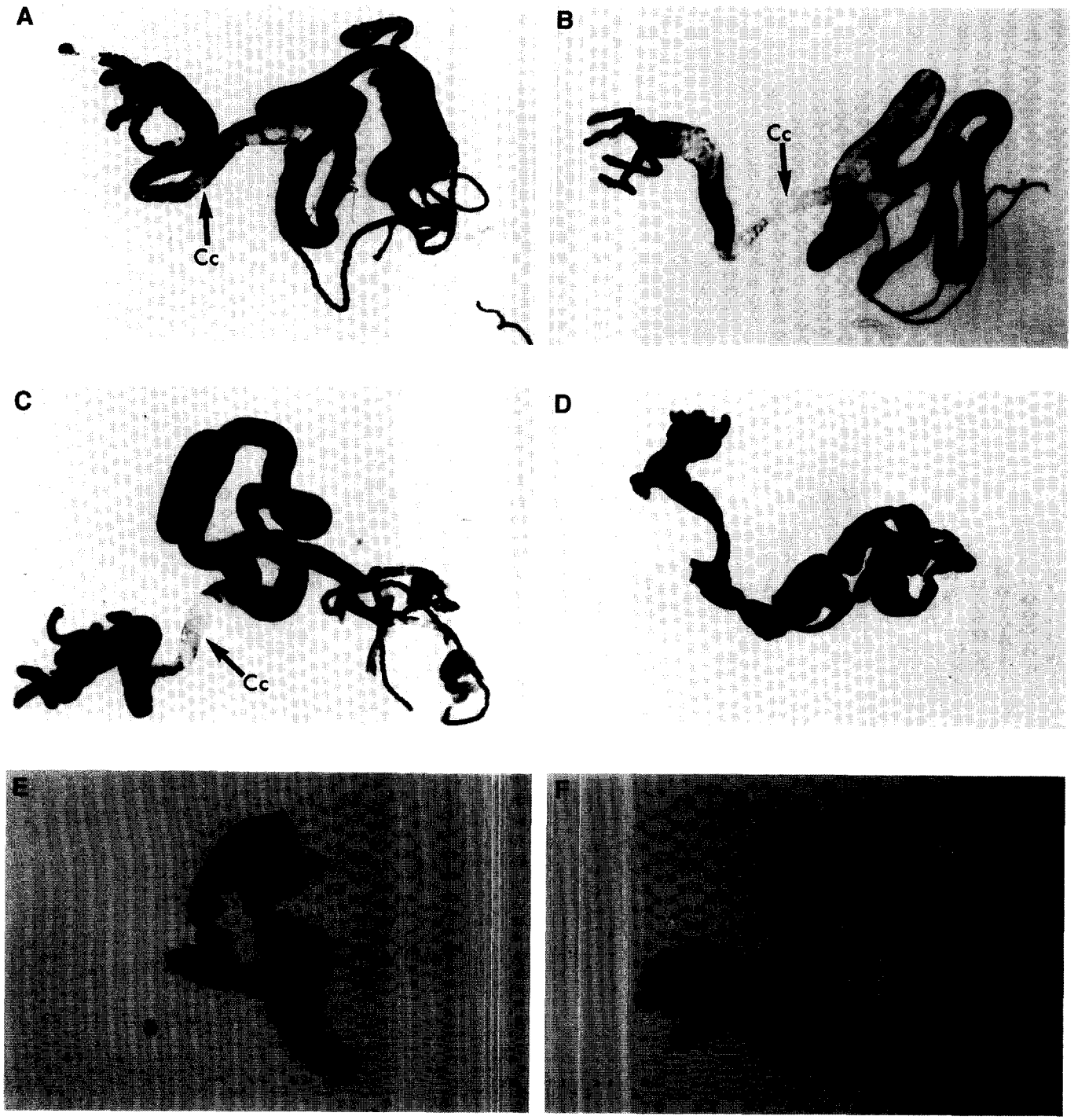

Fig. 4. Effects of various metal treatments on the *Mtn–Adh* transgene. The distribution of ADH activity is shown in the larval midgut of the *Pn4161* transgenic line after treatment with 0.5 mM $CuSO_4$ (A), 0.5 mM $CdCl_2$ (B) and 0.5 mM $ZnSO_4$ (C). Induction of *Adh* expression in the larval fat body after treatment with 0.5 mM $CuSO_4$ is shown in (D). As a control, *Mtn* transcripts were localized by in situ hybridization in the larval fat body of the wild-type Oregon R strain treated with 0.5 mM $CuSO_4$ (E: anti-sense probe, F: sense probe). Symbols: Cc = copper cells region. Scale bar = 0.25 mm.

lothionein genes. Therefore, we took advantage of *Go* and *Pn* transgenic lines as a tool to study the inducible regulation of *Drosophila* metallothionein genes.

3.2. Metal inducibility of the Mto promoter

Expression of the *Mto–lacZ* construct in *Po* lines was monitored under copper, cadmium and iron induction (Fig. 3). As shown in Fig. 3A, copper induces transgenic β-galactosidase expression in a sub-region of the middle midgut. This region contains interstitial cells and the so-called cuprophilic cells known to accumulate metal ions following copper or cadmium intoxication [26]. Cadmium effects are clearly different since β-galactosidase activity was observed in the iron cells region, the posterior midgut and the gastric caeca (Fig. 3B). No trans-

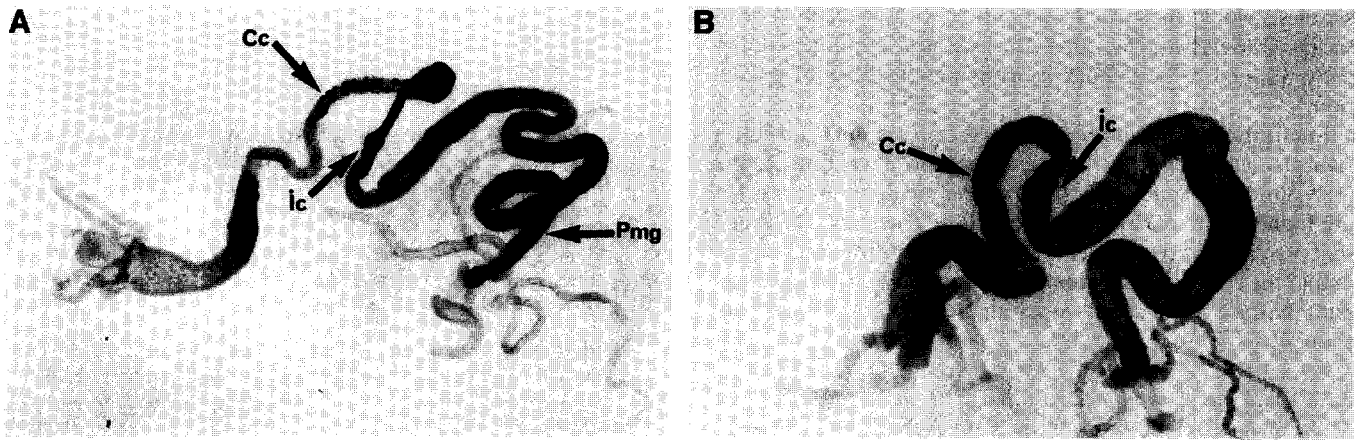

Fig. 5. Response of the *Mto* promoter to heat-shock and oxidative stresses. Distribution of β-galactosidase activity in *Go1* larval midgut is shown after heat-shock (A) and paraquat treatment (B). Symbols: Cc = copper cells region, Ic = iron cells region, Pmg = posterior midgut.

genic β-galactosidase activity was detected when larvae were grown on iron-containing medium (data not shown).

Inductions with several metals performed on *Go* lines demonstrate that other regulatory elements lie upstream the Po fragment (Fig. 3C–F). Copper, administered as $CuSO_4$, induces β-galactosidase expression all-over the midgut and in the Malpighian tubules (Fig. 3C). Silver ($AgNO_3$), an element sharing many chemical properties with copper, has identical effects (data not shown). Cadmium induces a strong expression in Malpighian tubules, but does not induce expression of the transgene in the anterior part of the midgut. However, a specific ring-shaped expression occurs in the proventriculus (Fig. 3D). The pattern of β-galactosidase staining extends in proportion with the concentration of the metal (data not shown). As shown in Fig. 3E, zinc induction is restricted to the iron cells region and to the posterior midgut. Iron, of all metals tested, has the smallest effect on *Mto* promoter, i.e. it induces β-galactosidase activity only in the iron cells region and, with a certain variability, in the copper cells region (Fig. 3F).

Taking into account the greater specificity of whole-mount analysis for the identification of gut regions, all these data are in good agreement with our previous results where endogenous expression was studied using in situ RNA hybridization on histological sections [22]. Therefore our results indicate that the metal regulatory elements involved during the larval stage are included within the 328 bp *Mto* gene fragment used in the Po constructs (−221 to +107; Fig. 1). This fragment comprises four MREs, which may be responsible for the metal inducibility [5]. The tissue-specific induction of the *Mto* promoter caused by the different metals suggests that unknown metal responsive transacting factors interact with various tissue-specific factors. However, the greater induction level obtained with the Go construct demonstrates the presence of some regulatory elements upstream of the −221 position in the *Mto* promoter.

3.3. *Metal inducibility of the Mtn promoter*

Inductive effects of metals on the *Mtn* promoter are illustrated in Fig. 4. Copper induces *Mtn*-driven *Adh* expression in the Malpighian tubules and all-over the midgut, however to a lesser extent in the copper cells region (Fig. 4A). Cadmium and zinc strongly induce *Adh* expression in the digestive tract of *Pn* larvae (Fig. 4B,C), while iron inducibility is significanlty lower (not shown). Interestingly, there is no induction of reporter expression in the copper cells region in cadmium and zinc intoxicated larvae.

A strong expression of *Mtn*-driven *Adh* in the fat body was also observed in copper-treated larvae (Fig. 4D). Since this site had not been detected earlier using in situ hybridization on histological sections [22], we performed whole-mount *Mtn* in situ hybridization on dissected fat bodies to test whether the transgenic expression reflected the endogenous *Mtn* expression. This experiment (Fig. 4E,F) showed that indeed *Mtn* transcripts are detected in fat bodies of copper-fed larvae, which confirms the results obtained with the transgenic approach (Fig. 4D) and earlier observations [27]. Therefore it is likely that expression of *Mtn* in the fat body was not detected previously for technical reasons, this structure being altered by the histological procedures. Among the other metal tested, only cadmium increases *Mtn* expression in the fat bodies, however to a weaker extent than copper does (data not shown).

3.4. *Response of* Mtn *and* Mto *transgenes to heat-shock and oxidative stresses*

Numerous agents and stress-producing conditions induce metallothionein synthesis [28]. We tested the effects of both heat-shock and oxidative treatments on *Mto* and *Mtn* promoters using *Go* and *Pn* transgenic lines. Expression of the *lacZ* reporter gene is induced by heat-shock in the iron cells region and in the posterior midgut of *Go* larvae, and to a lesser extent in the copper cells region (Fig. 5A). In heat-shocked *Pn* larvae, the *Adh* expression is slightly enhanced, but only in regions where the *Mtn* promoter is active in standard conditions (not shown). The enhanced expression of *Mto* and *Mtn* transgenes after heat-shock could depend upon the metal content of the cells prior to the stress. Metals, in differentiated cells of the digestive tract, are likely bound to different kinds of metallomolecules. It was proposed for *Caenorhabditis elegans* transgenic lines carrying metallothionein promoter–*lacZ* fusion genes that one of the effects of heat-shock might be disruption of metal binding to endogenous metallomolecules [25]. Metal ions would thus be free to interact with the metal regulatory system of metallothionein genes. Induction of metallothionein

synthesis would therefore be an indirect consequence of heat-shock. However, the fact that *Mtn* and *Mto* promoters differently respond to heat-shock suggests another level of regulation.

Implication of metallothioneins in the protection against free radicals has been the subject of numerous recent studies [2,29,30]. We tested whether *Drosophila* metallothionein genes are inducible by paraquat, a free radical generator. We found that β-galactosidase activity is enhanced in the iron cells region and in the copper cells region of *Go* larvae (Fig. 5B). Effects of paraquat are significantly lower on *Pn* transgenic lines (not shown).

3.5. *Metal metabolism in* Drosophila

Drosophila metallothionein genes are expressed mostly in the digestive tract. However, expression of the *Mtn* promoter is detected also in the fat body (Fig. 4E). This result suggests that ingested metals can bypass the intestinal barrier and reach other tissues. Expression of the *Mtn–Adh* transgene in the Malpighian tubules (Figs. 2C and 4A–C) could also be due to the uptake by this organ of metal ions present in the surrounding hemolymph. The absence of metal-induced *Mtn* expression in other tissues suggests either that all metal ions bypassing the digestive tract are taken up by the fat body and the Malpighian tubules, or that another level of tissue-specificity prevents *Mtn* expression in the other organs of the larvae.

Expression patterns of *Mtn* and *Mto* genes in *Drosophila* larvae and adults clearly differ, but nevertheless display some overlapping [22, this study]. The tissue-specificity could be correlated with distinct cellular properties. We previously demonstrated that the MTO protein can be easily purified from *Drosophila* while the MTN protein cannot be extracted by usual methods [5]. In contrast the MTN protein can be purified without any problem when it is produced in a yeast heterologous system [31]. Therefore it is likely that failure to purify MTN from *Drosophila* would be due to polymerization of the protein in the lysosomes of midgut cells [32]. These data suggest that the MTO function would be mainly related to active metal metabolism in the cell, while the MTN function would be more directly related to metal detoxification. The problem now is to uncover the mechanisms allowing differential expression of the *Mtn* and *Mto* genes.

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