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Expression of the *Drosophila melanogaster* metallothionein genes in yeast

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The metallothionein system in *Drosophila melanogaster* is composed of two genes, *Mto* and *Mtn*, that code for distinctly different proteins. In order to compare the properties of *Mto* and *Mtn*, we transformed yeast with several fusion plasmids. The *Mto* and *Mtn* cDNAs, when placed under the control of *CUP1* or *PGK* promoters, can confer a copper-resistance phenotype to copper-hypersensitive cells. Both *Mto* and *Mtn* proteins can be characterized in extracts from transformed yeast cells.

Metallothionein; Metal-resistance; Transformation; *Drosophila melanogaster*

1. INTRODUCTION

Metallothioneins are small metal-binding proteins thought to be involved in both metal homeostasis and detoxification [1]. Two metallothionein genes, *Mtn* and *Mto*, have been cloned in *Drosophila melanogaster* [2,3]. These genes code for a 40 and a 43 amino acid protein, respectively. The two proteins have very different primary structures, since they share only 11 amino acids at identical positions when the best sequence alignment is considered [3]. The two genes are also differentially regulated during normal development. Transcription of *Mto* takes place in early developmental stages, while *Mtn* is mainly transcribed in larvae and adult flies [4]. All these properties suggest that *Mtn* and *Mto* most probably have different functions in *Drosophila*.

Transcription of the *Mto* and *Mtn* genes can be induced by various metal ions in larvae as well as in adult *Drosophila* [2-4]. There is no difficulty in discriminating between *Mto* and *Mtn* mRNA since the coding regions of both cDNA probes are only 50% isologous [3,5]. Although both genes are highly inducible by cadmium ions in flies, we were able to characterize only the *Mto* protein. All our attempts to detect the *Mtn* protein were unsuccessful [4,6]. One alternative way to characterize the *Mtn* protein was to express the gene in another species. The yeast *Saccharomyces cerevisiae* offers in this context several advantages. Its single metallothionein gene, *CUP1*, has been extensively studied, and strains with a disrupted *CUP1* gene can be used as recipients for transformation with exogenous metallothionein genes [7,8]. This allows a positive resistance screening for the expression of metallothionein genes.

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2. MATERIALS AND METHODS

2.1. *Yeast: growth conditions*

The yeast strain 55-6B [8] was grown at 30°C in complete medium (1% yeast extract, 1% bactopeptone, 2% glucose) or in minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with appropriate metabolites (histidine: 10 μg/ml, leucine: 60 μg/ml, tryptophan, adenine and uracil: 20 μg/ml).

Resistance tests were performed by streaking 5 μl of fresh stationary phase cultures onto minimal plates. Tests were read 3-5 days later.

2.2. *Plasmid constructions and transformation*

The plasmid RC4 [8] contains the *E. coli GalK* coding sequence inserted between the *CUP1* promoter and the *CYC1* termination sequence. The *GalK* sequence was replaced by *Mto* cDNA (pMtol: [4]) and *Mtn* cDNA (cDm51:[2]) in both orientations. The resulting inserts were then transferred into pEMBL Ye30 [9] to yield plasmids CUP-MTO and CUP-MTN with direct orientation (Fig. 1), and plasmids CUP-OMT and CUP-NMT with inverse orientation.

The plasmid pEMBL Ye30/2 contains a cassette formed by the *PGK* promoter and the *PGK* terminator [10]. The complete *Mto* cDNA and the *Eco*RI-*Bgl*II fragment of *Mtn* cDNA were inserted in both orientations into pEMBL Ye30/2 to give the plasmids PGK-MTO and PGK-MTN with direct orientation (Fig. 1), and the plasmids PGK-OMT and PGK-NMT with inverse orientation. The *Mtn* inserts in these constructs are shorter than in the CUP constructs.

Yeast cells were transformed using the LiCl method [11], and selected for leucine prototrophy. Transformations were checked by retransforming *E. coli* DH5a with yeast DNA.

2.3. *Metal induction and RNA analysis*

Yeast cells were inoculated into 5 ml of complete medium containing metals at various concentrations, and allowed to grow for 7 h. Cells were resuspended in 0.5 ml hot phenol and incubated for 5 min at 65°C. RNA was extracted by phenol-chloroform and precipitated overnight with 4 M LiCl at 4°C. Fly RNA was isolated according to Terracol [12]. Both *Mto* and *Mtn* cDNA inserts were purified by gel electrophoresis and labeled by random priming.

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2.4. *Protein extraction and analysis*

Yeast cells were inoculated into 7.5 ml of minimum medium supplemented with appropriate metabolites and allowed to grow up to an optical density of 1 at 650 nm. They were then induced with 0.5 mM $CuSO_4$ for 30 min, after which time they were transferred to 2 ml fresh medium containing 0.5 mM $CuSO_4$ and 50 μCi [^{35}S]cysteine (Amersham, 1,000 Ci/mM), and incubated for 30 min. Cells were washed and total proteins extracted by incubating for 3 min in 200 μl of a boiling solution of 50 mM DTT, 2.5% SDS, 1 mM PMSF, 10 mM Tris-HCl, pH 8. Proteins were then carboxymethylated with 0.1 M iodoacetic acid and analysed on a 20% denaturing polyacrylamide gel.

Proteins of the *D. melanogaster* D cell line were labeled with [^{35}S]cysteine as previously described [13]. The yeast protocol was used for protein extraction and analysis.

3. RESULTS AND DISCUSSION

3.1. *Phenotypes of transformed yeast*

The yeast strain 55-6B is hypersensitive to copper intoxication due to disruption of the endogenous metallothionein gene *CUP1* [7,8]. To investigate both *Mto* and *Mtn Drosophila* metallothionein expression in yeast, we transformed 55-6B cells with the plasmids depicted in Fig. 1. The *CUP1* promoter is characterized by copper-inducible expression [14], while the *PGK* promoter is constitutively expressed at a high level [15]. Table I shows the resistance phenotypes associated with the various transformed strains. Clearly both metallothioneins are expressed in all these strains and confer a copper-resistance phenotype. *Mto* constructs confer higher resistance than *Mtn* constructs. The control strains obtained by transforming yeast cells with plasmids containing *Mto* or *Mtn* cDNA in inverted orientation with respect to promoters and terminators do not display any resistance. The sensitivity of strains CUP-MTO and CUP-MTN to cadmium and mercury (Table I) is not surprising since the *CUP1* promoter is not induced by these metals [14]. The *CUP1* and hamster *MT2* metallothioneins, when constitutively expressed in yeast using the *TDH* promoter, confer resistance not only to copper (up to 300 μM $CuSO_4$), but also to cadmium (up to 300 μM $CdCl_2$) [16,17]. As shown in Table I, *Drosophila* metallothioneins, expressed under the control of the constitutive *PGK* promoter, confer resistance to copper. However, both control and transformed cells are sensitive to low cadmium concentrations, the *Mto* protein nevertheless conferring a detectable resistance to cadmium. The *Mto* and *Mtn* proteins, thus, seem to be able to detoxify only copper in yeast. These results markedly differ from those obtained using the cadmium-resistant *Drosophila* CdR200 cell line, in which the endogenous metallothioneins efficiently protect cells against cadmium [3,13]. Duplications of the *Mtn* gene also confer higher resistance to cadmium. This protective effect most probably is due to the increased amount of metallothionein produced [18]. A particular cellular context thus appears necessary to endow metallothioneins with specific and functional metal-binding properties.

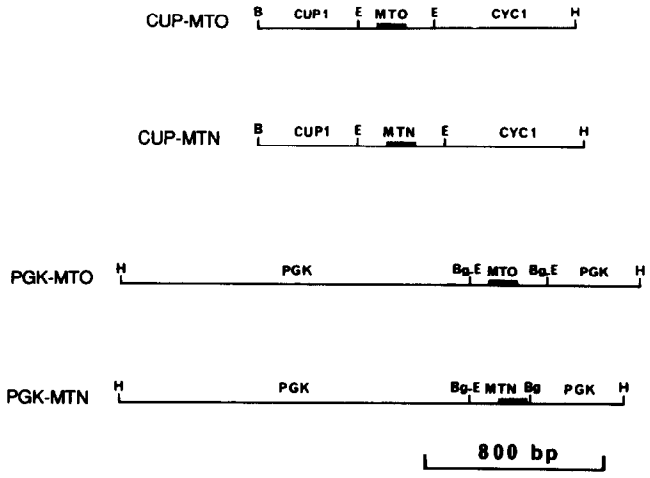

Fig. 1. Structure of the four fusion constructs in which the *Mto* and *Mtn* cDNA sequences are under the transcriptional control of *CUP1* and *PGK* promoters. Restriction sites: *Bam*HI (B), *Bgl*/II (Bg), *Eco*RI (E), *Hin*dIII (H).

3.2. *Mto and Mtn RNA synthesis in transformed yeast*

The *CUP1* promoter, in the absence of any functional metallothionein, is constitutively expressed in yeast and thus no longer copper-inducible [7,19]. As shown in Fig. 2, the expression of both CUP-MTO and CUP-MTN constructs in 55-6B cells is greatly increased by copper, indicating that the expressed *Drosophila Mto* and *Mtn* metallothioneins act as retro-inhibitors of *CUP1* expression.

Two transcripts characterize the expression of the PGK-MTO and CUP-MTO constructs in the absence as well as in the presence of added copper (Fig. 2). The two transcripts of the CUP-MTO fusion are strongly induced by copper. The smaller transcript, for both PGK-MTO and CUP-MTO, has a size very similar to that of the *Drosophila Mto* mRNA. Probing with the promoter and terminator of the CUP-MTO plasmid shows that the longer transcript, but not the smaller, clearly hybridizes with both the *CUP1* promoter and the *CYC1* terminator (data not shown). The longer transcript of PGK-MTO hybridizes with the *PGK* terminator but not with the *PGK* promoter, while the smaller transcript does not hybridize either with the promoter or the terminator of *PGK* (data not shown). Two termination sites are thus used by yeast cells for both CUP-MTO and PGK-MTO constructs, and two transcription start sites for the CUP-MTO construct.

A single transcript is produced in all cases with constructs involving *Mtn* cDNA (Fig. 2). Synthesis of *Mtn* RNA is constitutive from the *PGK* promoter and copper-inducible from the *CUP1* promoter. Size differences between PGK-MTN and CUP-MTN transcripts are due to plasmid structure (see Materials and Methods).

Table I

Resistance phenotypes of transformed cells

	μM $CuSO_4$						μM $CdCl_2$			μM $HgCl_2$	
	0	125	250	500	1000	2000	1	2.5	5	12.5	25
55-6B	+	−	−	−	−	−	+	−	−	+	−
CUP-MTO	+	+	+	+	+	+	+	−	−	+	−
CUP-MTN	+	+	+	+	+	−	+	−	−	+	−
PGK-MTO	+	+	+	±	−	−	+	+	−	+	−
PGK-MTN	+	+	±	−	−	−	+	−	−	+	−

The phenotypes of the hypersensitive 55-6B yeast transformed with the fusion plasmids shown in Fig. 1, and treated with $CuSO_4$ $CdCl_2$ or $HgCl_2$, are shown: resistant cells (+), sensitive cells (−), intermediate phenotypes (±).

3.3. *Expression of Mto and Mtn protein in transformed yeast*

The *Mto* and *Mto* proteins differ markedly in their primary structure [3]. We have been able to purify and characterize the *Mto* protein [4,6]. Surprisingly, all attempts to do a similar analysis of the *Mtn* protein failed. Analysis of yeast transformed with CUP-MTO and CUP-MTN, and then copper-induced allowed detection of both [^{35}S]cysteine-labeled metallothioneins (Fig. 3, lanes 4 and 5). The position of the bands agrees with the expected molecular weights, i.e. 4500 and 3800 Da for *Mto* and *Mtn*, respectively. The *CUP1* protein has an expected molecular weight of 5600 D, and accordingly migrates slower than *Mto* (Fig. 3).

The [^{35}S]cysteine-labeled metallothioneins synthesized by cadmium- or copper-treated *Drosophila* cells migrate at the position of *Mto* (Fig. 3, lanes 1 and 2). It is to be noted that labeling is always significantly higher for cadmium-treated than for copper-treated cells [13,20]. There is no trace of *Mtn* protein in these conditions although the *Mtn* gene is efficiently transcribed (unpublished data). The most likely explanation for these unexpected results is that *Mtn* protein is sequestered in the lysosomal compartment very soon after its synthesis, where it would be converted into a highly

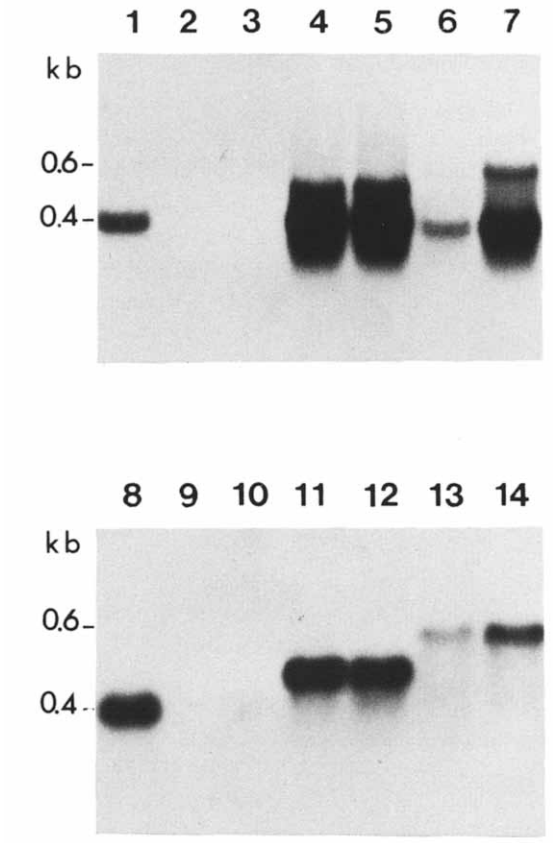

Fig. 2. Analysis of RNA complementary to *Mto* and *Mtn* coding sequences in 55-6B yeast transformed with the constructions shown in Fig. 1. Total RNA was probed with *Mto* cDNA (lanes 1–7) and *Mtn* cDNA (lanes 8–14). Origin of RNA samples: adult *Drosophila* flies treated with 100 μM $CdCl_2$ (lanes 1 and 8), untransformed control 55-6B cells (2 and 9), untransformed cells treated with 250 μM $CuSO_4$ (3 and 10), cells transformed with PGK-MTO without $CuSO_4$ treatment (4) and treated with 250 μM $CuSO_4$ (5), cells transformed with CUP-MTO without $CuSO_4$ treatment (6) and treated with 250 μM $CuSO_4$ (7), cells transformed with PGK-MTN without $CuSO_4$ treatment (11) and treated with 250 μM $CuSO_4$ (12), cells transformed with CUP-MTN without $CuSO_4$ treatment (13) and treated with 250 μM $CuSO_4$ (14).

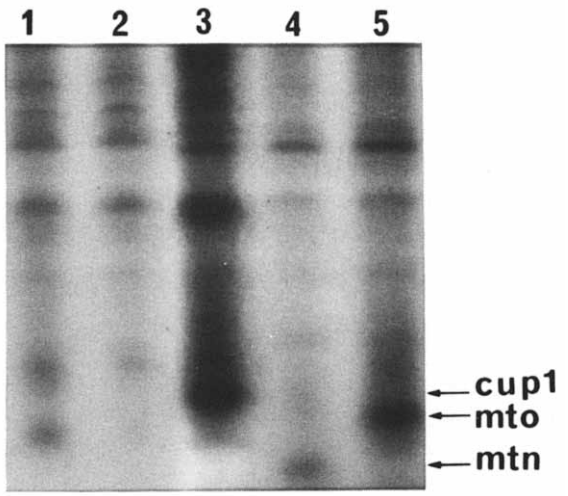

Fig. 3. Analysis of [^{35}S]cysteine-labeled proteins. Origin of protein samples: *Drosophila* D cells treated with 10 μM $CdCl_2$ (lane 1) and with 400 μM $CuSO_4$ (2), yeast strain W303-1b treated with 500 μM $CuSO_4$ (3), yeast strain 55-6B transformed with CUP-MTN (4) and with CUP-MTO (5), both treated with 500 μM $CuSO_4$. Arrows indicate the position of the *CUP1*, *Mto* and *Mtn* proteins. No labeled proteins were detected at the position of the metallothioneins in 55-6B control yeast as well as in control *Drosophila* cells (data not shown).

insoluble polymerized form. This hypothesis is supported by the ultrastructural study and electron-probe microanalysis of lysosomes in the midgut of copper-intoxicated *Drosophila* larvae [21]. Sequestering of metallothioneins inside lysosomes has already been demonstrated for copper-loaded metallothioneins in mammals [22]. All these data suggest that the *Mto* and *Mtn* proteins have different functional properties.

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