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A DNA Segment Controlling Metal-Regulated Expression of the *Drosophila melanogaster* Metallothionein Gene *Mtn*

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Cloned fragments of DNA including the *Drosophila melanogaster* metallothionein gene *Mtn* and different amounts of 5′ flanking sequences were introduced into flies by P-element-mediated germ line transformation. Comparison of RNA levels in different transformants revealed that metal-regulated and tissue-specific expression of *Mtn* requires no more than 373 base pairs upstream of the initiation site of transcription. Transformants having an additional, transcribed copy of *Mtn* could tolerate increased concentrations of cadmium, indicating that *Mtn* expression is directly related to this phenotype. In separate experiments, these *D. melanogaster* promoter sequences were fused to the coding sequences of the herpes simplex virus thymidine kinase (TK) gene. After transfection of this fusion into baby hamster kidney cells, increases in TK activity and accumulation of TK RNA were inducible by metals. A series of 5′ and 3′ deletions showed that *D. melanogaster* sequences from −130 to −6 were sufficient to confer metal-regulated expression to the TK gene. The function of the *D. melanogaster* metallothionein promoter in mammalian cells indicates that the mechanism controlling metal regulation is evolutionarily conserved.

Metallothioneins (MTs) are small, cysteine-rich proteins that bind heavy metals. Although their complete physiological role is not yet known, they appear to protect cells against toxic metals and may be involved in the homeostatic regulation of essential elements such as zinc and copper (6, 8, 10, 20). In mammals, there are multiple MT genes, and exposure of cells or animals to heavy metals results in a rapid increase in their transcription (2, 5, 7, 12). Several of these genes have been cloned and found to retain their ability for metal-regulated expression following transfer into heterologous mammalian cells (7, 11, 18, 24).

Metal induction of mouse MT-1 gene transcription is controlled by metal-regulatory elements, 12-base-pair DNA segments that are repeated four times within the MT-1 promoter (28); similar metal-regulatory elements are present in other mammalian MT promoters (28). In *Drosophila melanogaster*, the MT gene *Mtn* is associated with DNA segments resembling mammalian metal-regulatory elements; seven such segments occur 300 base pairs or less upstream of the transcriptional initiation site, and each is identical to the consensus mammalian metal-regulatory element in at least 9 of 12 bases (15). To test whether the region containing these segments was responsible for metal-regulated expression of *Mtn* and whether this region would function in a heterologous mammalian system, we introduced portions of *Mtn* into flies and into baby hamster kidney cells. We show here that metal-regulated and tissue-specific expression of *Mtn* in *D. melanogaster* germ line transformants does not require more than 373 base pairs upstream of the transcriptional initiation site and that this region controls metal-regulated expression in hamster cells. In addition, we show that a shorter region extending from −130 to −6 is sufficient for metal regulation in these cells.

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MATERIALS AND METHODS

Vector constructions. A plasmid with P[−373*Mtn*L1*ry*$^+$] was constructed by ligating the 3.5-kilobase (kb) *Eco*RI fragment containing the *D. melanogaster* MT structural gene into the *Eco*RI site of pPSXΔ1 (obtained from J. Posakony, University of California, San Diego). This plasmid was then digested with *Bam*HI and ligated to a fragment (L1) derived from the mouse interspersed repeat L1Md (17). The resulting plasmid contained a transposon that was a precursor for all of those shown in Fig. 1A. For P[−3900*Mtn*L1*ry*$^+$], linear fragments obtained from a partial *Eco*RI digestion of this plasmid were ligated to a 3.5-kb *Eco*RI fragment that contained adjacent upstream sequences of *Mtn*; restriction endonuclease analysis was used to identify clones having this fragment inserted in such a way as to restore the natural organization of *Mtn*. For P[+54*Mtn*L1*ry*$^+$], these linear molecules were digested with *Stu*I and the products were fractionated on a 0.7% agarose gel. Molecules that contained the same DNA as those in the linear fragment but lacked a 427-base-pair *Eco*RI-*Stu*I fragment from the 5′ end of *Mtn* were extracted from the gel, treated with Klenow polymerase, and religated. The final step in the construction of each transposon was the insertion of an 8.1-kb *Sal*I fragment, containing *ry*$^+$, into the *Xho*I site within pPSXΔ1. Construction of P[−373*Mtnry*$^+$] was the same as that of P[–373*Mtn* L1*ry*$^+$], except that L1 was not added.

3′ deletions of the *Mtn* promoter were constructed by BAL 31 exonuclease digestion from the *Eco*RV site of a genomic subclone, pDm131 (15). This DNA was then digested with *Sma*I, ligated to yield circular plasmids, and subcloned. *Mtn* promoter fragments were obtained from each of these plasmids by digestion with *Eco*RI, treatment with Klenow polymerase, and then digestion with *Bam*HI. To create the *MtnTK* fusions, these fragments were used to replace the normal thymidine kinase (TK) promoter, be-

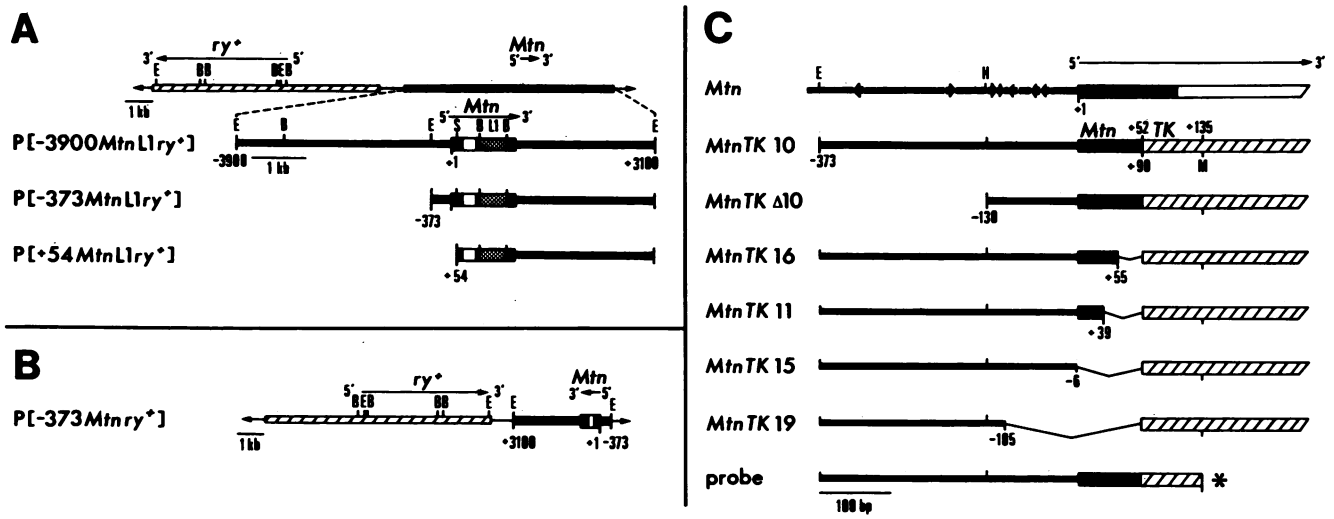

FIG. 1. *Mtn* vectors used in germ line transformation and transfection experiments. (A) Transposons carrying a *D. melanogaster* MT gene tagged with L1 DNA. Top line, restriction map of a transposon that contained one of the three different *Mtn* fragments shown below it. Symbols: ——, P-element sequences; →, ends of the transposon; ▬, L1 sequences; ▨, ry^+ sequence; ▬, transcribed portion of *Mtn* (exons); ▭, transcribed portion of *Mtn* (intron); ▬, flanking portions of *Mtn*. Sequence positions within *Mtn* are indicated below each line and are relative to the transcriptional initiation site at +1. Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Stu*I. (B) Transposon carrying a normal *D. melanogaster* MT gene. (C) Deletion mutations used in transfection experiments. Top line, wild-type restriction map of the 5′ region of *Mtn*. Regions of *Mtn* are represented as in panel A. Arrows represent 12-mers in which at least nine bases correspond to the consensus sequence identified as the metal-regulatory element in mammals (15, 28). Below, deletion mutations created by fusing portions of *Mtn* to the TK structural gene. ▨, TK sequences. The sizes of *Mtn* fragments were estimated from a polyacrylamide gel. Positions within *Mtn* are indicated below each line, and positions within *TK* are above; numbers are relative to their respective transcriptional initiation sites at +1. The end-labeled (*) probe that was used in S1 analysis (Fig. 3B) is shown on the bottom line. Restriction site: M, *Mlu*I.

tween the *Pvu*II and *Bgl*II sites, of a plasmid containing the *Pvu*II-*Hin*dIII fragment spanning the herpes simplex virus TK gene (type 1) (27). The 5′ deletion *MtnTK* Δ10 was created by subcloning the *Hin*dIII fusion fragment from *MtnTK* 10 into pUC18.

***D. melanogaster* transformation and hamster cell transfection.** Germ line transformation was carried out by the method of Rubin and Spradling (22). Adh^{fn6} *cn*; ry^{506} embryos were injected with mixtures containing a test plasmid at 300 μg/ml and pπ25.7wc (9) at 100 μg/ml. G1 transformants were crossed to flies of the host strain, and temporary lines were maintained by selecting ry^+ progeny. The linkage group for the transposon in each line was determined by scoring, after the appropriate genetic crosses, ry^+ segregation with respect to the balancers *CyO* (for the second chromosome) and *MKRS* (for the third). Permanent lines (a

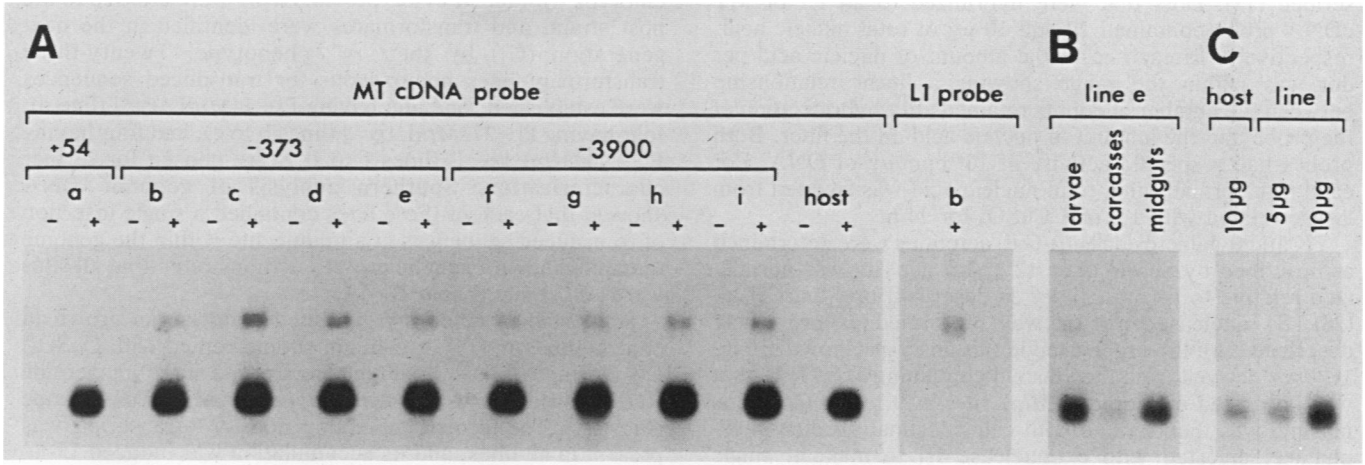

FIG. 2. Expression of transduced *Mtn* in germ line transformants. Autoradiographs of blots of total nucleic acid that was electrophoresed on denaturing agarose gels. (A) Each lane contained 5.0 μg of nucleic acid that was isolated from the host strain or from transformants containing P[+54*Mtn*L1ry^+] (line a), P[−373*Mtn*L1ry^+] (lines b to e), or P[−3900*Mtn*L1ry^+] (lines f to i). For each line, larvae were uninduced (−) or treated with 5.0 mM $CuSO_4$ for 48 h (+). Blots were hybridized to a *D. melanogaster* MT cDNA probe (13) or to an L1 probe. (B) Each lane contained nucleic acid from four larvae or from the dissected organs of four larvae. All larvae were from line e and were treated with 1.0 mM $CuSO_4$ for 24 h. The blot was hybridized to the *D. melanogaster* MT cDNA probe. Carcasses were all organs that remained after the midgut was removed. Similar results were obtained for lines b to d (data not shown). (C) Nucleic acid was isolated from larvae of the host strain or transformants having a transduced copy of a normal MT gene. All larvae were treated with 5.0 mM $CuSO_4$ for 24 h. The amount of total nucleic acid in each lane is indicated. The blot was hybridized to the same probe used in panel B.

TABLE 1. MT RNA levels in *D. melanogaster* germ line transformants

Probe	Transposon injected	Line	Chromosome of insertion	Mean cpm[a] ± SE	cpm relative to host
L1	P[+54*Mtn*L1*ry*$^+$]	a	3	12.4 ± 0.7	
	P[−373*Mtn*L1*ry*$^+$]	b	2	96.8 ± 8.2	
		c	2	176.8 ± 9.9	
		d	3	124.3 ± 8.7	
		e	3	102.2 ± 3.6	
	P[−3900*Mtn*L1*ry*$^+$]	f	2	96.7 ± 9.9	
		g	3	119.4 ± 7.4	
		h	3	80.1 ± 3.3	
		i	3	105.5 ± 5.8	
	None	Host		7.8 ± 0.3	
MT cDNA	P[−373*Mtnry*$^+$]	j	2	692.7 ± 66.4	1.6
		k	2	782.7 ± 41.6	1.8
		l	3	809.0 ± 80.8	1.9
		m	3	678.6 ± 24.2	1.6
	None	Host		430.3 ± 22.5	1.0

[a] For each line, mean of three determinations.

to m) that were homozygous for the chromosome containing the transposon were then obtained by using the same balancers. See reference 14 for descriptions of balancers and mutations.

Baby hamster kidney *TK*$^-$ cells were transfected as described by Searle et al. (25).

Molecular analyses. Isolation of total nucleic acid from *D. melanogaster* larvae and Northern blot analyses were performed as previously described (13). The relative expression of endogenous genes and transduced MT genes in lines b to i was determined by scintillation counting of portions of the blot that corresponded to individual bands on the autoradiograph (Fig. 2A).

Dot hybridizations were performed as previously described (13). Dots that were hybridized to an L1 or MT cDNA probe contained 20 and 10 μg of total nucleic acid, respectively. In each case, the amount of nucleic acid per dot was within the range showing a linear relationship between the number of counts retained after hybridization of the probe and the amount of nucleic acid on the filter. Both probes had a specific activity of 10^8 cpm/μg of DNA. For each transformant line, total nucleic acid was isolated from larvae treated with 1.0 mM $CuSO_4$ for 24 h.

TK and β-galactosidase (β-Gal) activities were determined as described by Searle et al. (25); TK activity was normalized relative to β-Gal activity as described by Stuart et al. (28). S1 nuclease analysis was performed as previously described (24); the probe used in this analysis (shown in Fig. 1C) was derived from the plasmid containing *MtnTK* 10 that was linearized at a unique *Mlu*I site, at +135 of *TK*. This plasmid was then treated with calf intestinal alkaline phosphatase, digested with exonuclease III to make it single stranded, and end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase.

RESULTS AND DISCUSSION

***D. melanogaster* transformants carrying a MT gene tagged with a fragment of heterologous DNA.** Germ line transformation vectors were constructed that contained, within a defective P-element, genomic restriction fragments derived from the *D. melanogaster* MT clone λDm13 (15) and an 8.1-kb rosy$^+$ (*ry*$^+$) fragment (4). Three different *Mtn* fragments, extending from −3900, −373, and +54 to +3100, were used to create transposons P[−3900*Mtn*L1*ry*$^+$], P[−373*Mtn*L1*ry*$^+$], and P[+54*Mtn*L1*ry*$^+$], respectively (Fig. 1A). To help distinguish the expression of transduced MT genes from that of endogenous ones, a 500-base-pair fragment of mouse DNA, L1, was inserted in exon 2 of *Mtn*. Probes derived from L1 failed to hybridize to genomic DNA from the host strain (data not shown); thus, this probe was used to determine directly the level of expression of transduced genes.

Each vector was coinjected with pπ25.7wc into *ry*^{506} host embryos with the M cytotype. Survivors were crossed to the host strain, and transformants were identified in the next generation (G_1) by their *ry*$^+$ phenotype. Twenty-three transformant lines homozygous for transduced sequences were established; one line having P[+54*Mtn*L1*ry*$^+$] (line a), four having P[−373*Mtn*L1*ry*$^+$] (lines b to e), and four having P[−3900*Mtn*L1*ry*$^+$], (lines f to i), were chosen for further characterization. Southern analysis of genomic DNA showed that each of these lines contained a single insertion of transduced sequences at a unique site within the genome (data not shown); genetic crosses demonstrated that all sites were autosomal (Table 1).

Total nucleic acid was extracted from larvae grown in control medium (16) or medium supplemented with $CuSO_4$. On Northern blots, a 400-nucleotide and a 900-nucleotide RNA hybridized to a *D. melanogaster* MT cDNA probe (Fig. 2A). The former, the endogenous MT transcript, was present in all lines, and its accumulation was induced 15- to 20-fold by metal treatment, as expected (13). The latter, present in lines b to i, was the transcript of the transduced genes; it was larger than the endogenous transcript by the expected amount and comigrated with the only RNA that hybridized to an L1 probe. It was also inducible, but the amount produced by the transduced genes was only a small fraction (10 to 15%) of the endogenous MT RNA. We were unable to detect the larger RNA in line a or the host strain.

TABLE 2. Tolerance of *D. melanogaster* germ line transformants to cadmium chloride

Transposon injected	Line	Mean LC_{50}[a] (μM) ± SE	Relative LC_{50}
P[+54*Mtn*L1*ry*$^+$][b]	a	14.6 ± 1.0	1.0
P[−373*Mtnry*$^+$][b]	l	24.3 ± 1.5	1.7
P[−373*Mtn*L1*ry*$^+$][c]	e	31.0 ± 1.0	1.0
P[−373*Mtnry*$^+$][c]	l	46.0 ± 4.0	1.5
	m	48.0 ± 4.0	1.5

[a] LC_{50} values represent the concentration of $CdCl_2$ that would be expected to yield 50% survival and were estimated by interpolation from the relative viability values described below.

[b] Average LC_{50} values were estimated from the results of three independent experiments. In each experiment, 10 male and 10 female flies of each line (a or l) laid eggs in a culture vial for 72 h and were then discarded (all flies were of the same age). Vials contained either control medium (16) or medium supplemented with various concentrations of $CdCl_2$ (20, 40, 80, or 160 μM). For each concentration, relative viability was calculated as the proportion of individuals (progeny) that reached pupariation under that treatment compared with individuals reared on unsupplemented food.

[c] Average LC_{50} values were estimated from the results of two independent experiments. These experiments were performed as described in footnote *b*, except that 30 first-instar larvae (from control medium), instead of the progeny of 10 pairs of flies, were cultured in each vial. All calculations were based on the numbers of these larvae that reached pupariation (16).

Dissections of metal-treated larvae showed that the transduced genes were expressed primarily within the midgut cells, as were the endogenous genes (Fig. 2B). We measured the level of expression of transduced genes by hybridization of an L1 probe to dot blots of total nucleic acid from treated larvae (Table 1). The amount of hybridization in lines f to i (−3900), as a group was not greater than that in lines b to e (−373). The low level of background hybridization in line a was similar to the host level; therefore, it appears that this transduced gene was not expressed. These results demonstrate that (i) sequences from −373 to +54 are required for the expression of *Mtn*; (ii) the presence of an additional 3.5 kb of 5′ flanking sequence does not increase this expression; and (iii) despite small differences observed among lines, the level of expression of functional transduced genes in each line is much lower than that of the endogenous genes.

***D. melanogaster* transformants carrying a transduced, normal MT gene.** To determine whether the low level of expression of transduced genes was due to the presence of the heterologous L1 sequence, a normal MT gene was introduced into flies. A segment of *Mtn* similar to the one in P[−373*Mtn*L1*ry*$^+$], but lacking the L1 segment, was used to create the transposon P[−373*Mtnry*$^+$] (Fig. 1B). Four lines (j to m) homozygous for the chromosome containing this transposon were analyzed. Southern blots showed that each line contained a single insertion of the transposon at a unique site (data not shown), and genetic analyses revealed that each site was on an autosome (Table 1). Thus, each line had four copies of *Mtn*; if the transduced genes were functioning normally, the levels of MT RNA in these lines should be twice as high as those in the host strain.

MT RNA levels in the host strain and in these transformants were compared by hybridization of the MT cDNA probe to total nucleic acid from treated larvae. Northern blots showed that some transformants appeared to have approximately twice as much of the 400-nucleotide MT transcript as the host strain (Fig. 2C). Dot hybridizations revealed that the transduced genes were expressed at a level equivalent to 60 to 90% of the endogenous gene (Table 1). Differences in the expression of transduced genes are attributed to the influence of chromosomal position and have been observed for other genes (1, 23, 26). These results suggest that the low level of expression observed for genes carrying the L1 segment is due either to interference of this segment with transcription or, more likely, to instability of L1-MT RNA. Thus it appears that the region of *Mtn* from −373 to +3100 contains most, if not all, of the sequences required for normal expression of this gene. These limits are consistent with those suggested by a tandem duplication of this gene, *Dp(3;3)Mtn*^{H22}; larvae with this duplication had an extra copy of sequences from −228 to +2000 and contained twice as much MT RNA as strains with a single copy (20).

Transformants carrying this transposon were 1.5- to 1.7-fold more resistant to cadmium chloride than were transformants carrying P[+54*Mtn*L1*ry*$^+$] or P[−372*Mtn*L1*ry*$^+$] (Table 2). These results indicate that these transduced genes are functional and that *Mtn* is directly responsible for resistance to cadmium.

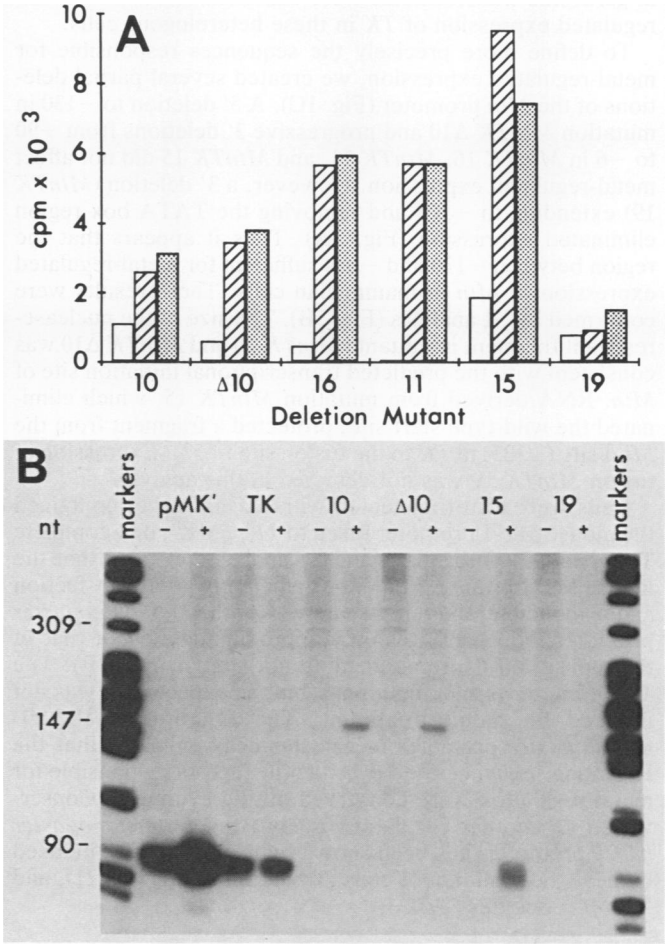

FIG. 3. Relative expression of deletion mutations after transfection into baby hamster kidney cells. (A) For each mutation, we assayed TK activity in untreated cells (▭) and in cells treated with 0.1 mM $ZnCl_2$ for 12 h (▨, ▩; results of two independent experiments). TK values are relative to β-Gal activities. Uninduced cells containing the mutation *MtnTK* 11 had a β-Gal activity level that was below background; therefore, no TK value is given. (B) Autoradiograph showing the S1 nuclease-resistant products of a digestion of hybrids between the probe shown in Fig. 1C and RNA that was isolated from transfected cells. Cells were grown for 8 h in the absence (−) or presence (+) of 0.1 mM $ZnCl_2$. Markers are end-labeled *Hpa*II fragments of pBR322.

Function of the *Mtn* promoter in transfected hamster cells. The 5′ flanking region required for *Mtn* expression in germ line transformants had been previously shown to contain DNA sequences that are strikingly similar to those responsible for metal regulation of the mouse MT-1 gene (15, 28). To determine whether the function of this region was conserved, we examined the activity of the *D. melanogaster* promoter in mammalian cells. *Mtn* sequences from −373 to +90 were fused to a viral TK gene to create *MtnTK* 10 (Fig. 1C). The plasmid with this fusion and a reference plasmid containing the mouse MT-1 promoter fused to a β-Gal gene, pMT-βgal (27), were cotransfected into TK^- baby hamster kidney cells. TK and β-Gal activities in cells grown in the presence or absence of metal were assayed; β-Gal served as a control for variation in transfection efficiency and thus provided an internal standard for comparison of *Mtn* promoter activity in each experiment. TK activity was inducible in cells containing *MtnTK* 10 (Fig. 3A); therefore, the same 5′ flanking sequences that were required for *Mtn* expression in germ line transformants were sufficient to control metal-regulated expression of *TK* in these heterologous cells.

To define more precisely the sequences responsible for metal-regulated expression, we created several partial deletions of the *Mtn* promoter (Fig. 1C). A 5′ deletion to −130 in mutation *MtnTK* Δ10 and progressive 3′ deletions from +90 to −6 in *MtnTK* 16, *MtnTK* 11, and *MtnTK* 15 did not affect metal-regulated expression. However, a 3′ deletion (*MtnTK* 19) extending to −105 and removing the TATA box region eliminated expression (Fig. 3A). Thus it appears that the region between −130 and −6 is sufficient for metal-regulated expression of *Mtn* in mammalian cells. These results were confirmed by S1 analysis (Fig. 3B). The size of the nuclease-resistant fragment in mutants *MtnTK* 10 and *MtnTK* Δ10 was consistent with the predicted transcriptional initiation site of *Mtn*. RNA derived from mutation *MtnTK* 15, which eliminated the wild-type start site, protected a fragment from the *Mlu*I site (+135) in *TK* to the fusion site (+52). Expression of fusion *MtnTK* 19 was not detected in this analysis.

Cells were also transfected with plasmids that contained the mouse MT-1 promoter fused to *TK*, pMK′, or a complete TK gene (27). TK assays and S1 analysis revealed that the mouse MT-1 promoter functions efficiently after transfection and is inducible; the relative activity of the *D. melanogaster* promoter, however, was only approximately 5% of that of the mouse MT-1 promoter (data not shown; Fig. 3B). The TK promoter is also functional, but, as expected, it was not induced by metal treatment. The function of the *D. melanogaster* promoter in hamster cells indicates that the *cis*-acting sequences and *trans*-acting factors responsible for metal regulation were conserved during evolution. Conservation of another regulatory region, the *D. melanogaster* *hsp70* promoter, has been shown by its ability to be induced by heat shock in mouse cells (3), monkey cells (19, 21), and *Xenopus* oocytes (29).

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