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Analysis of the role of 5′ regulatory mutations in the activation of quiescent metallothionein genes after carcinogen treatment

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S49 are mouse thymic lymphoma cells which do not express the two closely linked mouse metallothionein (MT) genes; however, previous studies demonstrated that treatment of S49 cells with chemical carcinogens or ultraviolet irradiation can activate these quiescent genes. To determine if activation of MT-I or MT-II in these variants is a result of cis-acting mutations, we amplified and sequenced the immediate 5′ regions of 19 cadmium resistant S49 variants: MT-I$^+$/MT-II$^-$, MT-I$^-$/MT-II$^+$, MT-I$^+$/MT-II$^+$, and MT-I$^-$/MT-II$^-$. None of the variants contained mutations in the analyzed regions. Thus, the observed changes in MT expression must result from mutations at other sites or from non-mutational mechanisms.

S49 lymphoma cells do not express metallothionein genes; consequently they are exquisitely sensitive to the toxic effects of cadmium (Horibata and Harris, 1970; MacArthur et al., 1985). In previous studies, MT$^+$ S49 variants were recovered after treatment of S49 cells with chemical carcinogens or ultraviolet (uv) radiation and selection in Cd-containing medium (Lieberman et al., 1983; MacArthur et al., 1985). The changes in MT expression phenotype are heritable and provide a good system for the study of changes in cellular gene commitment and its relationship to carcinogen exposure.

Metallothioneins are small cysteine-rich proteins that play a role in homeostasis, detoxification of heavy metals, and free-hydroxyl radical scavenging (Beach and Palmiter, 1981; Durnam and Palmiter, 1981; Karin et al., 1985). Their expression is induced by many environmental agents, including Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, lipopolysacharides, free radicals, interferon, interleukin-1, glucocorticoids, and other stress factors (for review see Palmiter, 1987). Two major forms of metallothioneins are found in mammals (Karin and Richards, 1982), and the mouse haploid genome contains a single copy of each in a closely linked tandem arrangement (Searle et al., 1984). MT genes in mice are expressed and coordinately regulated in most tissues (Searle et al., 1984). The latter suggests that expression of both genes is regulated by common trans-acting regulatory factors.

Consensus metal regulatory elements and SP-1 binding sites are readily identified in the immediate 5′ region of mouse MT genes (E. Aguilar-Cordova et al., unpublished data; Mueller et al., 1988; Stuart et al., 1985; Searle et al., 1987). In transient expression studies, as little as 186 bp of the 5′ region of MT-I maintained com-

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plete metal-dependent regulation of an MT-TK fusion gene (Searle et al., 1985). Since small 5′ regions of the MT genes were sufficient for expression and metal regulation (Searle et al., 1985), and previous analysis of the methylation and hypersensitive site patterns of the MT genes in MT^+ S49 variants revealed no obvious patterns associated with expression (MacArthur et al., 1986; MacArthur and Lieberman, 1987), we investigated the possibility that cis-mutations might be involved in the activation of MT expression in these variants.

Materials and methods

DNA purification and primer preparation

Genomic DNA was isolated and purified from S49 cells and S49 variants as previously described (MacArthur et al., 1986). Metallothionein I and II primers EMT1-5 (TAAACTGCAGA GCAGCGATAGGCCGTA), EMT1-3 (CCAAGGA TCGGGAGTCTTACC), EMT1-3.2 (GAGCAGTT GGGGTCCATTCC), EMT2-5 (TCAGTCCCTGA GCCCAGAGAA), EMT2-3 (AACTGCAGTCCAT GGCGAGTGGAGGCG), and EMT2-3.2 (CGGT TGAAGATCGACGAGAG) were chemically synthesized (Applied Biosystems 381A DNA synthesizer), cleaved with 30% NH_4OH for 1 hour, and deprotected at 55°C overnight. The primers were lyophilized and purified by standard non-denaturing polyacrylamide gel electrophoresis, and their concentration determined by OD_{260} absorption. For direct sequencing primers were kinase-labeled in 20 µl final volume: 2 µl of 10X buffer (0.5M Tris-pH 9.5, 0.1M $MgCl_2$, 50mM DTT, 50% glycerol), 1–2 µl

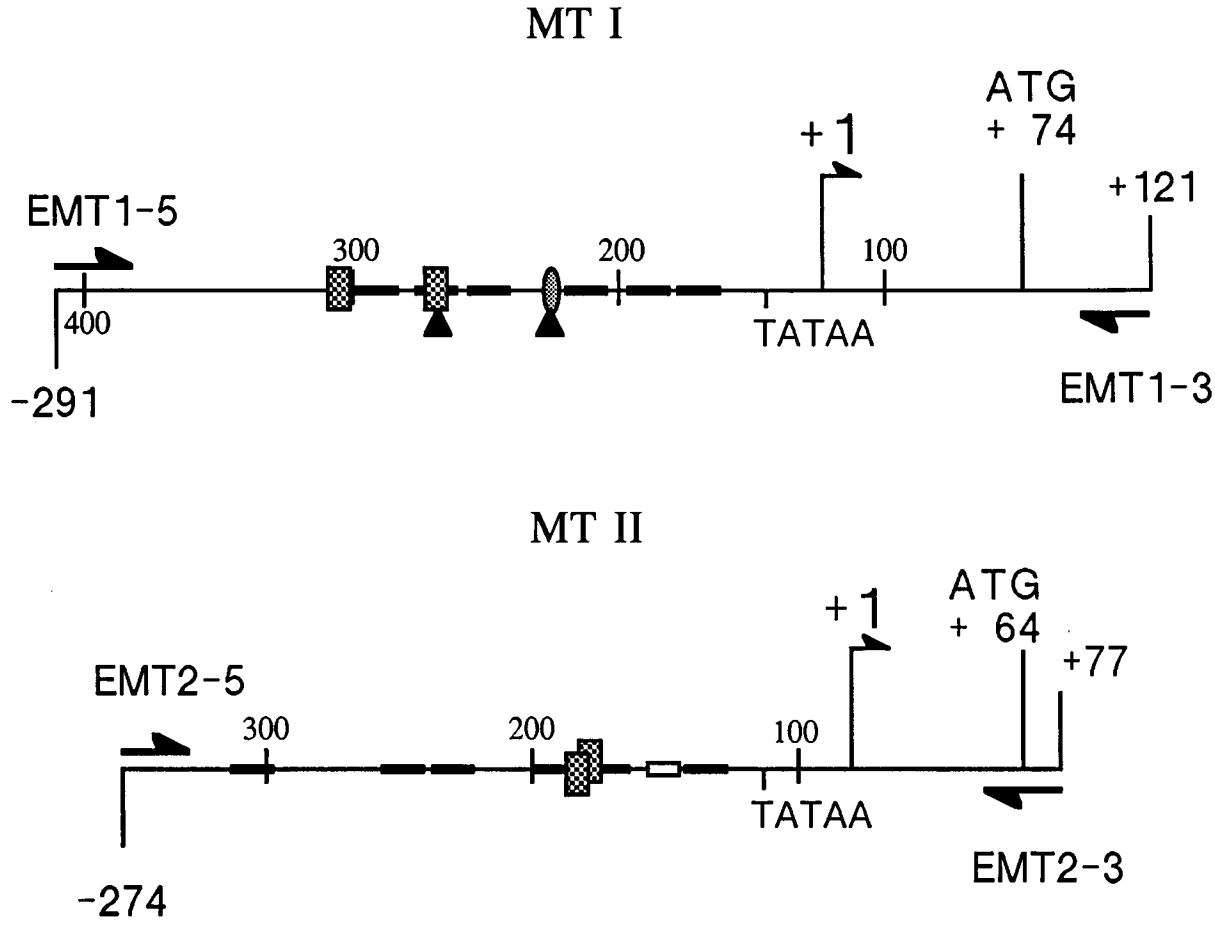

Figure 1. Amplified regions of MT-I and MT-II. ATG, translation start site; +1, transcription start site; TATAA, "tataa-box"; thick filled line, metal regulatory elements; thick open line, SV40 conserved sequence; checkered rectangles, SP-1 binding sites; oval, MLTF (major late transcription factor); black triangles, G-boxes. Regulatory regions for MT-I were defined according to Mueller et al. (1988). MT-II regions were defined by computer analysis and comparison with consensus sequences (Stuart et al., 1985).

γ^{32}ATP (DuPont, 6000 Ci/mmol, 0.149 Ci/ml), 1–2 µl T4 polynucleotide kinase (Promega, 8 µ/µl) to 20 µl with 20 pmoles of primer and H_2O at 37°C for 1 hour. Labeled primers were separated from unincorporated nucleotides by NICK columns (Pharmacia), dried in a Speed-Vac, and resuspended in 30 µl of H_2O. The primers used for sequencing were the same or slightly internal to those used for amplification (Fig. 1).

DNA amplification

The 5′ promoter regions of mouse metallothionein I and II were amplified by the polymerase chain reaction technique (Saiki et al., 1988) using primer sets EMT1-5/EMT1-3 and EMT2-5/EMT2-3, respectively (Fig. 1). Reactions were done in 100 µl final volume using 0.1–1.0 µg of genomic DNA, 50 pmoles of each primer in 10 mM Tris (pH 8.3), 5.5 mM $MgCl_2$, 0.001% gelatin, 100 µM each dNTP and 1 unit of AmpliTaq (Perkin Elmer-Cetus). A cold master mix (complete reaction mix minus genomic DNA) was prepared and aliquoted to tubes containing genomic DNA, covered with 50 µl of mineral oil, and immediately set at 94°C for 2 minutes followed by 35 cycles of 1 minute at 92°C, 1 minute at 60°C, and 1 minute at 72°C. Escherichia coli DNA was used as negative control for each amplification. Amplified products were analyzed in 2–3% 1:1 NuSieve: SeaKem TBE agarose gels.

Sequencing

Amplified products were cloned into pTZ-19R (Pharmacia) and sequenced by Sequenase (USB Corp.), using the manufacturer's suggested protocol. Alternatively, PCR products were sequenced directly (Aguilar-Cordova and Lieberman, 1991). In brief, PCR products were purified by size filtration using Ultrafree-MC 30,000 NMWL (Millipore Corp.). The final volume of the retentate was typically 20–30µl and represented 2–10 pmoles of template. A 1:20 mixture of Sequenase (USB Corp.) to each of the ddNTP termination mixtures was made and placed in ice just prior to use. A mixture (15 µl) of 1X Sequenase buffer with 1 pmole of template and 1 pmole of γ^{32}P-labeled sequencing primer (see above) was incubated at 95°C for 10 minutes; 3.5 µl of the mix were aliquoted to each of 4 tubes labeled T, C, A, G. Two µl of the appropriate Sequenase:ddNTP mixture were added to each of the corresponding tubes and incubated at 50°C for 10 minutes, after which 3.5 µl of STOP solution was added to each tube. The mixtures were denatured at 75°C for 2 minutes and loaded in 6% denaturing polyacrylamide gels.

Results

The polymerase chain reaction technique and primers described (Materials and Methods, Fig. 1) were used to amplify the regions between −291 to +121 of MT-I and −274 to +77 of MT-II from S49 cells (MT-I$^-$/MT-II$^-$) and Cd^r variants with four MT expression phenotypes (MT-I$^+$/MT-II$^-$, MT-I$^-$/MT-II$^+$, MT-I$^+$/MT-II$^+$ or MT-I$^-$/MT-II$^-$; see Table 1). The amplified regions contain all the described metal regulatory elements (MRE), SP-1 binding sites, G-boxes, MLTF and TATAA boxes of the mouse metallothionein genes (Fig. 1; see Searle et al., 1984; Searle et al., 1987; Mueller et al., 1988).

Direct sequencing of the MT 5′ regulatory region of S49 revealed an additional adenine nucleotide from the sequence published by Glanville et al. (1981) at position −112 of MT-I. This additional adenine was present in all the sequences examined (Table 1). No other sequence modifications were found.

Table 1. Metallothionein sequences analyzed from MT expression variants.

Expression phenotype was previously reported (MacArthur et al., 1986). "E" cell lines were exposed to ENU, "A" to NAAAF, and "U" to uv irradiation. Sequenced region indicates the range analyzed for each variant.

Cell	*Phenotype*		*Sequenced Region*	
Line	*MT-I*	*MT-II*	*MT-I*	*MT-II*
S49	−	−	−291 to +121	−274 to +77
E5	−	+	−158 to +61	
E6	−	+	−158 to +61	
E7	+	+	−158 to +61	
E10	+	+	−158 to +61	
E13	−	+	−158 to +61	
E14	+	+	−263 to +61	−253 to +26
E15	−	+	−291 to +121	−274 to +77
E16	+	−	−291 to +121	−274 to +77
E28	−	−	−263 to +61	−253 to +26
A8	+	−	−263 to +61	−253 to +26
A10	+	+	−263 to +61	−253 to +26
A14	−	−	−263 to +61	−253 to +26
A17	−	+	−263 to +61	−253 to +26
U15	+	−	−263 to +61	−253 to +26
U18	+	+	−263 to +61	
U19	+	+	−263 to +61	−253 to +26
U20	+	+	−263 to +61	−253 to +26
U23	−	−		−253 to +26

MT 5′ regulatory regions of metallothionein expression variants from clones treated with N-acetoxy-2-acetylaminofluorene (NAAF), N-ethylnitrosourea (ENU), or ultra-violet (uv) irradiation were amplified by PCR, sequenced, and compared to the sequences of S49. At least one representative of each expression pattern (MT-I only, MT-II only, MT-I and MT-II, or neither) from each treatment was included in the analysis (Table 1). No mutations were found in the promoter regions of any of the variants analyzed.

Sequences from two clones of amplified MT-I DNA from the E-15 cell variant DNA (E-15 expresses MT-II but not MT-I) showed G to A and G to C mutations at positions −88 and −111 respectively. Direct sequencing, without cloning, of PCR product from the same cells did not confirm the presence of these mutations in the original DNA. This finding underscores the danger of sequencing only a few cloned DNA fragments from PCR amplified products. If cloned products are to be sequenced, multiple sequences from more than one PCR reaction should be included.

Discussion

Murine cells contain two forms of metallothionein genes found within 6 kbp of each other and coordinately regulated (Searle et al., 1984). S49 cells do not express either MT gene. However, treatment with chemical carcinogens or uv irradiation and Cd selection led to MT^+ variants, some of which did not coordinately regulate the two genes (MacArthur et al., 1985; Lieberman et al., 1983). A possible explanation for the expression and differential regulation of MT genes in these variants is the occurrence of cis-mutations in the 5′ region of these genes. Although the target regions analyzed are small compared to the frequency of lesions expected from the DNA-damaging agents used (Tang et al., 1989; Bohr et al., 1985), positive selection on the large number of cells treated makes this an attractive hypothesis. S49 variants that express only one form of MT might reflect mutations sufficient for expression of only the expressed MT. Alternatively, expression of only one MT gene might reflect mutations sufficient for expression of both MT forms, with a subsequent mutation that quiesced the other MT gene. We tested the cis-mutation hypothesis by comparing the sequences of multiple S49 MT variants (Table 1) and found no mutations of the immediate 5′ regions of either MT-I or MT-II of these variants. Since the regions analyzed were larger than those shown to be necessary for accurate metal regulation of the MT promoters (Searle et al., 1985), the observed changes in MT gene activation in the S49 variants cannot be attributed to mutations in the cis-acting sequences of the immediate 5′ regions of the MT genes.

It is possible that mutations in other regions of the MT genes or in genes whose products regulate MT expression may be involved in the observed changes in MT phenotype. Alternatively, non-mutational mechanisms, such as changes in chromatin structure, may be responsible for the activation of these genes (MacArthur et al., 1986; MacArthur and Lieberman, 1987). It is also possible that temporary exposure to carcinogens leads to permanent changes in cellular gene expression by alteration of methylation patterns, chromatin structure, or trans-acting regulatory gene expression. Heritable changes in gene expression after carcinogen exposure without apparent sequence mutation indicate that the original insult may not need to be maintained and that sequence mutagenesis may not be necessary for carcinogenic potential. An extension of this suggestion would be that transient gene expression may also have heritable effects. These hypotheses will require further experimental analysis for verification.

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