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Regulation, Linkage, and Sequence of Mouse Metallothionein I and II Genes

PETER F. SEARLE, BARRY L. DAVISON, GARY W. STUART, THOMAS M. WILKIE, GUNNAR NORSTEDT,† AND RICHARD D. PALMITER*

Howard Hughes Medical Institute Laboratory, Department of Biochemistry, University of Washington, Seattle, Washington 98195

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The mouse metallothionein II (MT-II) gene is located ~6 kilobases upstream of the MT-I gene. A comparison of the sequences of mouse MT-I and MT-II genes (as well as those of other mammals) reveals that the coding regions are highly conserved even at "silent" positions but that the noncoding regions and introns are extremely divergent between primates and rodents. There are four blocks of conserved sequences in the promoters of mouse MT-I, mouse MT-II, and human MT-IIA genes; one includes the TATAAA sequence, and another has been implicated in regulation by heavy metals. Mouse MT-I and MT-II mRNAs are induced to approximately the same extent in vivo in response to cadmium, dexamethasone, or lipopolysaccharide. Mouse MT-I and MT-II genes are regulated by metals but not by glucocorticoids after transfection into HeLa cells.

Metallothioneins (MTs) are intracellular proteins produced by most organisms; in vertebrates there are two major forms, designated MT-I and MT-II. The cysteinyl residues (20 of 61 amino acids) of these proteins are perfectly conserved and function to coordinate heavy metals such as Zn, Cu, and Cd. MTs are thought to play an important role in heavy-metal detoxification and zinc and copper homeostasis (11, 22).

Mouse MT-I and human MT-II genes have been cloned (12, 24), as have been cDNAs corresponding to rat, Chinese hamster, and monkey MTs (1, 16, 36). Rodents appear to have one copy each of MT-I and MT-II genes, whereas primates have multiple copies (24, 36). Mouse MT-I can be detected in virtually all tissues and cell lines with the noteable exception of thymus and some thymoma cell lines (9, 28). Expression of the MT-I gene can be achieved in these thymic cell lines by selection for cadmium resistance (26), and the frequency of expression can be enhanced by treating the cells with 5-azacytidine or UV light before selection (6, 26). In all cases, expression of the MT-I gene is correlated with demethylation of DNA sequences in the vicinity of the gene (6, 26). Continued selection for cadmium resistance frequently results in amplification of the MT-I gene (3, 4, 11, 14, 29); in some cases, these amplified genes are located on double minutes, but in other cases, these small chromosomal fragments are not evident (3). The amplification of MT genes during selection for cadmium resistance provides genetic evidence that MTs are involved in metal detoxification.

The mouse MT-I gene is transcriptionally regulated by at least three different classes of inducers: heavy metals, glucocorticoid hormones, and an unidentified hormone made in response to inflammation. Each of these inducers acts rapidly; induction is apparent within 30 min and is maximal in 4 to 8 h (8, 9, 28).

Many heavy metals induce MT-I mRNA, including Cd, Zn, Cu, Co, Ni, Ag, Hg, and Bi, although in some cases the metals are not known to be bound to the resultant MT under physiological conditions (9, 11). Both intact mouse MT-I and human MT-II genes, as well as fusion genes composed of an MT promoter linked to various structural genes, have been introduced into foreign cells and shown to retain their metal inducibility (20, 23, 30, 33). By in vitro DNA mutagenesis and gene transfer techniques, a metal regulatory region has been shown to lie within the basic promoter of the mouse MT-I gene; it is centered at −50 base pairs (bp) relative to the transcription start site (Searle et al., manuscript in preparation). Localization of the glucocorticoid receptor binding site has been problematical for the mouse MT-I gene because glucocorticoid regulation is consistently lost upon either gene amplification or gene transfer (29, 30). However, Karin and collaborators (23) have been able to retain glucocorticoid regulation upon transfer of the human MT-II gene; this should allow rapid identification of the sequences involved in regulation by glucocorticoids. The response of MT genes during the inflammatory response has been studied only in vivo (32). One inducer of this type of response is the bacterial endotoxin, lipopolysaccharide (LPS), which is thought to activate macrophages to liberate a hormone that induces MT (8). We have shown that MT fusion genes in transgenic mice respond to LPS if they have MT-I sequences located between −180 and −350 bp (8).

It is apparent that MT gene expression is controlled at several different levels by a variety of environmental or hormonal signals. Although we have learned much about the mouse MT-I gene, further understanding of the regulation of these ubiquitous proteins may be derived by isolating all of the MT genes of an organism and characterizing their commitment, amplification, and expression in response to environmental signals. Perhaps this approach will provide insight into why there are two distinct MTs in all vertebrates. In this paper we show that the mouse MT-I and MT-II genes constitute a closely linked and coordinately regulated pair.

MATERIALS AND METHODS

Screening a lambda library. A partial *Hae*III-*Alu*I digest of mouse kidney DNA was inserted with *Eco*RI linkers into λ charon 4A. A library of ca. 10^6 phage (generously provided by Jim Miller) was transferred to nitrocellulose filters and screened with a unique, nick-translated *Kpn*I-*Bgl*II probe spanning the 5′ end of the mouse MT-I gene (see Fig. 1A). Several positive plaques were identified, and phage were purified by standard techniques. Restriction enzyme analy-

* Corresponding author.
† Present address: Department of Medical Nutrition, Huddinge University Hospital F69, 141 86 Huddinge, Sweden.

sis of DNA from three of these phage indicated that one contained DNA sequences that extended about 9 kilobases (kb) upstream of the MT-I gene. An 8-kb *Kpn*I fragment was isolated from this lambda DNA and cloned into the unique *Kpn*I site of a plasmid carrying the *Stu*I-*Hin*dIII fragment that encompasses the mouse MT-I gene to generate the plasmid designated K-H in Fig. 1. This plasmid was the starting point for the studies described here.

Nuclear transcription and hybridization conditions. Nuclei were isolated from control and zinc-induced Hepa cells (13 10-cm plates each) by disruption of the cells in buffer NA (31) with a tight Dounce homogenizer and centrifugation (10 min at 10,000 rpm) of the nuclei through an equal mixture of buffers NA and NB (31) in an HB-4 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). The nuclei (300 μg of DNA in 375 μl of buffer NC) were combined with 0.6 mCi of [α-^{32}P]UTP and the other components described previously (31) in a 0.5-ml total volume. After 45 min of incubation at 26°C, [^{32}P]RNA was isolated (31), and 5×10^7 cpm was hybridized to Southern blots of restricted plasmid DNA under our standard conditions (30). After the blots were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5× SET (30), the nitrocellulose sheets were incubated in 50 ml of 0.3 M NaCl–2 mM EDTA–10 mM Tris (pH 7.5) containing 100 μg of bovine serum albumin per ml, 10 μg of RNase A per ml, and 200 U of RNase T_1 per ml. The filters were then exposed to XAR-5 film for 10 h at −70°C with an intensifying screen.

Isolation of an MT-II cDNA clone. Hepatic polyadenylic acid [poly(A)] containing RNA was isolated from the liver of mice treated for 4 h with $CdSO_4$ as described previously. The RNA was centrifuged on a 5 to 20% sucrose gradient, and the location of MT-I mRNA was determined by solution hybridization (10). cDNA was prepared from the peak fractions with reverse transcriptase and an oligodeoxythymidylic acid primer, and it was made double stranded with *Escherichia coli* DNA polymerase I (large fragment). The double-stranded DNA was inserted into the *Pst*I site of plasmid pBR322 by G-C tailing and then transfected into *E. coli* RR1. Tetracycline-resistant colonies containing MT-II sequences were identified by colony hybridization with a nick-translated MT-II gene probe (*Nco*I-*Sph*I; Fig. 1A). The insert from one potential MT-II cDNA clone was isolated by *Pst*I digestion and subcloned into plasmid M13 for sequence analysis. Sequencing revealed that this clone extends from nucleotide 123 of MT-II mRNA into the poly(A) tail (see below).

S_1 nuclease analysis. For MT-II, an *Apa*I-*Xba*I subclone in plasmid pBX322 (Fig. 1A) was linearized at the unique *Nco*I site that lies within exon 1, treated with calf intestinal alkaline phosphatase, and then digested with exonuclease III to remove about 150 nucleotides from each end. For MT-I we initially used the unique *Bgl*II site that lies in exon 1, but we found that the size of the fragment protected by RNA was too similar to that of the MT-II probe. Therefore, an intronless genomic clone (prepared by inserting an MT-I cDNA fragment between the first and last exons of a genomic MT-I clone) was digested with *Nar*I, which cuts in exon 3, and then treated with alkaline phosphatase and exonuclease III to remove about 300 nucleotides from each 3′ end. For quantitative S_1 analysis, equal molar amounts of the MT-I and MT-II probes described above were combined and labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase to a specific activity of ca. 10^7 cpm/pmol. The probes were separated from free nucleotides by passage through a Bio-Gel A-50 column.

To analyze MT mRNAs, 25 μg of total nucleic acids (TNA) was incubated with about 80,000 cpm of the combined probe in a 50-μl reaction mix containing 50% formamide, 0.3 M NaCl, and 30 mM HEPES (*N*-2-hydroxyethylpi-

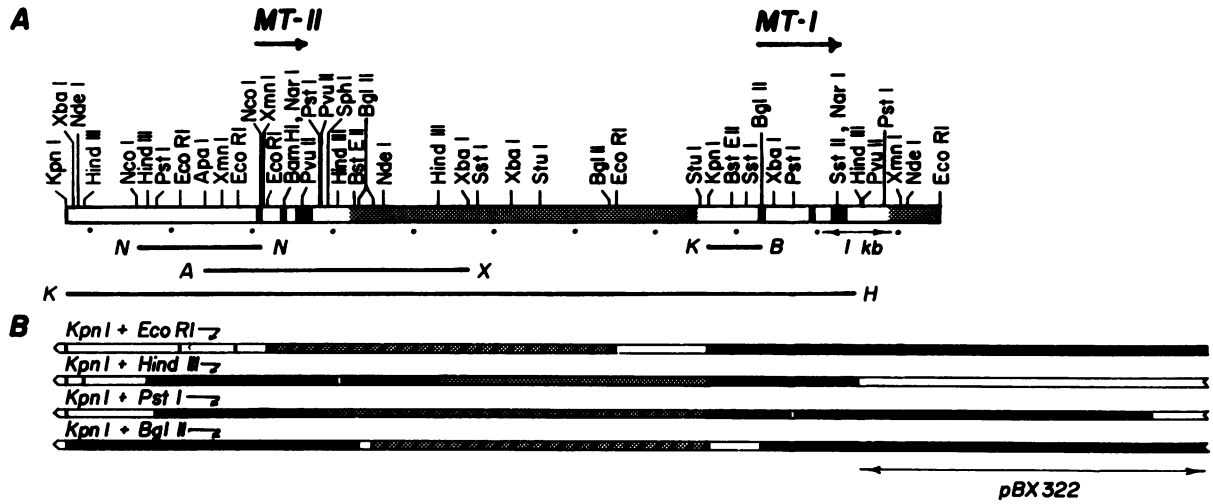

FIG. 1. Restriction map of the mouse MT gene locus. (A) Map showing the location of many 6-bp restriction sites. Sites are not shown for *Ava*I, *Bcl*I, *Hin*cII, *Mst*II, *Sma*I, and *Tth*111I. There are no sites for *Cla*I, *Eco*RV, *Hpa*I, *Mlu*I, *Nae*I, *Not*I, *Nru*I, *Pvu*I, *Sal*I, and *Xho*I. Solid bars represent exons of MT-I and MT-II genes as deduced by DNA sequencing. Stippled areas include repeat sequences that were detected by hybridization with nick-translated mouse DNA; the exact boundaries between unique and repeat sequences have not been established. The locations of several regions that were subcloned into plasmid pBX322 are shown below the restriction map; they are designated N-N, A-X, and K-H. The probe used to screen the lambda library is designated K-B. (B) The K-H subclone was inserted between the *Eco*RI and *Hin*dIII sites of plasmid pBX322; this vector is a high-copy version of plasmid pBR322 and has an *Xho*I linker at about position 3,000 on the plasmid pBR322 map. This plasmid was restricted with *Kpn*I and one other enzyme to generate the fragments shown here and in Fig. 2A. Hybridization of nuclear transcripts from control and zinc-treated cells to various restriction fragments is shown in Fig. 2B and C, and the results are summarized here. Solid bars reflect the greatest difference in hybridization when comparing control and zinc-induced RNA, cross-hatched and stippled bars reflect a moderate difference, bars that are only stippled reflect no difference, and open bars signify no hybridization.

perazine-*N'*-2-ethanesulfonic acid; pH 7.5). After hybridization for 14 h at 45°C, nucleic acids were diluted with 50 μl of water and precipitated with 200 μl of ethanol. The nucleic acids were dissolved in 45 μl of 10 mM NaCl, and then 5 μl of 3 M NaCl–0.3 M NaOAc–3 mM $Zn(OAc)_2$ (pH 4.5) was added, along with 4 μl of S_1 nuclease (equivalent to 8 U), and the mixture was incubated for 60 min at 40°C. The nucleic acids were precipitated with 200 μl of ethanol, dissolved in 20 μl of 8 M urea–0.1 M NaOH, and boiled for 2 min, and 10 μliters was electrophoresed on a 7.5% acrylamide–8 M urea gel. After being soaked in 10% HOAc, the gel was dried and autoradiographed.

Gene transfer. Several plates (100 mm) of HeLa cells were incubated with a calcium phosphate precipitate containing 20 μg of DNA (10 μg of MT plasmid, 2 μg of plasmid pKO-neo, and 8 μg of carrier herring sperm DNA) for about 4 h in Dulbecco modified Eagle medium containing 10% fetal calf serum and 100 μM chloroquine. Then the cells were shocked for 2 to 3 min with medium containing 15% glycerol. This medium was then replaced with normal Dulbecco modified Eagle medium plus 10% serum. Twenty-four hours later, neomycin analog G418 (GIBCO Laboratories, Grand Island, N.Y.) was added to 0.5 mg/ml to select for cells expressing the neomycin resistance gene in plasmid pKO-neo (38). Resistant clones were picked and expanded.

DNA sequencing. Preliminary sequencing was by the rapid method of Guo and Wu (17). The A-X subclone (Fig. 1A) was linearized at the unique *Nco*I or *Bam*HI site, the 3' ends were digested to remove ca. 50 to 300 nucleotides with exonuclease III and elongated with *E. coli* DNA polymerase I (large fragment) in four dideoxynucleotide reactions (35), and then short fragments were released by secondary digestion with appropriate restriction enzymes and resolved on a sequencing gel. The majority of the MT-II gene was sequenced in plasmids containing deletions of the A-X fragment (Fig. 1A). The A-X fragment was subcloned in either orientation into the *Eco*RI site of plasmid pBX322, and deletions were constructed between various rare and unique restriction enzyme sites in the A-X fragment and the *Nde*I site of the plasmid vector, deleting the tetracycline resistance gene. These deletion plasmids were linearized with *Xho*I, boiled for 3 min, and annealed on ice with a heptadecamer oligonucleotide that hybridizes adjacent to the plasmid *Nde*I site (37, 39, 40). This primer was extended into the mouse sequences in four dideoxynucleotide reactions, and the products were separated on sequencing gels (35).

RESULTS

Location of zinc-inducible transcription units. We initially tried to clone MT-II genomic DNA based on cross-hybridization with MT-I probes; this approach failed because MT-I and MT-II genes are too dissimilar to cross-hybridize at moderate stringency, and there is a family of repeat sequences that cross-hybridizes with the MT-I gene at low stringency (D. Durnam and R. Palmiter, unpublished data). As an alternate approach, we reasoned that genes within families tend to be clustered within ca. 10 kb of each other; therefore, we cloned sequences upstream from the MT-I gene since the original lambda clones contained only 1.8 kb 5' of the MT-I gene and about 14 kb on the 3' side (12). By screening a partial *Hae*III-*Alu*I library of mouse DNA inserted with *Eco*RI linkers into λ charon 4A, a phage was isolated that included an additional 8 kb of 5' flanking DNA (D. Durnam and R. Palmiter, unpublished data). We subcloned an 8-kb *Kpn*I fragment into a plasmid containing the MT-I gene to generate plasmid K-H and then mapped the location of most 6-bp restriction sites (Fig. 1A).

To ascertain whether this 5' subclone contained any zinc-inducible transcription units in addition to the MT-I gene, we prepared two identical Southern blots of the K-H clone that had been restricted with various enzymes (Fig. 2A). These Southern blots were hybridized with ^{32}P-labeled nuclear RNA prepared by in vitro run-on transcription of nuclei isolated from either control or zinc-induced Hepa cells. We chose to use cadmium-resistant Hepa cells for these experiments because they have amplified the MT-I gene locus about 30-fold (11) and thus provide a stronger signal. Only some of the DNA bands on the Southern blot hybridized to nuclear RNA from control cells (Fig. 2B), whereas most of these bands as well as some additional bands hybridized intensely with nuclear RNA from zinc-treated cells (Fig. 2C). The hybridization data are summarized in Fig. 1B, which shows the location of the various bands. Some of the solid bars correspond to MT-I gene transcripts which thus serve as a convenient internal control. The other solid bars delineate another zinc-inducible transcription unit. An area between the two transcription units (exemplified by the 3.3-kb *Hin*dIII fragment) hybridizes weakly regardless of zinc treatment of the cells. This region includes repeat sequences (shown as stippled in Fig. 1A); therefore, this could represent cross-hybridization with transcripts from other parts of the genome. All of the bars that are cross-hatched and

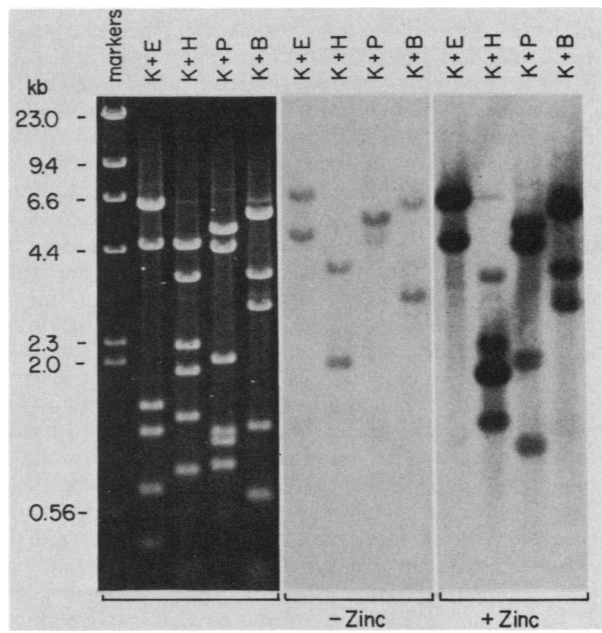

FIG. 2. Southern blots with nuclear transcripts from control or zinc-induced cells. Cadmium-resistant Hepa cells were withdrawn from cadmium treatment for 10 days; half of the cells served as controls (−zinc) and half were treated with 300 μM $ZnSO_4$ for 1 h (+zinc) before the nuclei were harvested. Nuclei (300 μg of DNA) were labeled with [^{32}P]UTP, and nuclear RNA transcripts were isolated. Meanwhile, subclone K-H was restricted with *Kpn*I (K) and either *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), or *Bgl*II (B), and 1.5 μg of DNA per lane was electrophoresed in duplicate on a 1% agarose gel. The left panel shows the ethidium bromide staining pattern. The DNA was transferred to nitrocellulose and hybridized with 5×10^7 cpm of [^{32}P]RNA from either control nuclei (center panel) or zinc-induced nuclei (right panel). See the text for details. The locations of the various restriction fragments and their relative hybridization to the two probes are depicted in Fig. 1B.

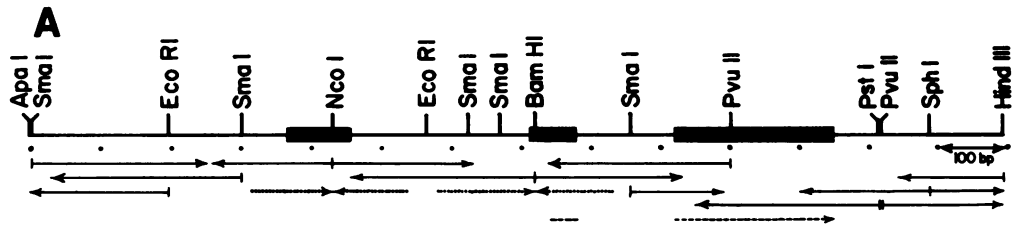

FIG. 3. Sequence of the mouse MT-II gene. (A) Sequencing strategy for the mouse MT-II gene. A map of relevant restriction enzyme sites between the unique *Apa*I site and the *Hin*dIII site 1.4 kb downstream is shown; the arrows indicate the direction and extent of sequencing runs. Solid arrows indicate sequences obtained by dideoxynucleotide sequencing in plasmids with the indicated restriction enzyme site ligated to the *Nde*I site of the vector. Dotted lines show the sequence obtained by the exonuclease III-dideoxynucleotide method (17); the dashed line shows the sequence obtained from a cDNA clone in plasmid M13. (B) The nucleotide sequence (5′ to 3′) of the complete mouse MT-II gene and flanking DNA. The three exons are shown in boldface type. The 5′ end of the gene was determined by S_1 nuclease mapping of the RNA, and the 3′ end was deduced from the position of the poly(A) tail in a cDNA clone.

stippled overlap the repeat region, which probably explains why the difference in hybridization intensity is not so marked with these fragments. Based on this transcription analysis, we conclude that another zinc-inducible transcription unit is located between the third *Eco*RI site and the fourth *Hin*dIII site from the left end of the map shown in Fig. 1A.

MT-II gene identified by DNA sequencing. The 3.3-kb *Apa*I-*Xba*I fragment (A-X in Fig. 1A) was subcloned in both orientations into the *Eco*RI site of plasmid pBX322, regenerating the *Eco*RI site at each end. An expanded map of restriction enzyme sites between the *Apa*I site and the *Hin*dIII site 1.4 kb downstream is shown in Fig. 3A. Initially, we sequenced in both directions from the *Bam*HI and *Nco*I sites by the rapid exonuclease III-dideoxynucleotide method (17). The decoded sequence matched the amino acid sequence of mouse MT-II (22), with the ATG initiation codon contained within the *Nco*I recognition sequence CCATGG, and the exon 2 spanned the *Bam*HI site. The transcriptional orientation was the same as that of the MT-I gene 6 kb downstream. To obtain the sequence of the presumptive promoter region and to complete the sequence of the structural gene, we used several approaches. Most of the sequence was obtained by constructing a series of deletions between convenient restriction enzyme sites in the A-X fragment which was cloned into the *Eco*RI site of plasmid pBX322 and the *Nde*I site of the plasmid vector, deleting the tetracycline resistance gene. These were sequenced by the dideoxynucleotide chain termination method (35) modified for double-stranded DNA (37, 39, 40). The 300-bp *Sma*I fragment of the promoter region was also sequenced in a pUC vector, and the *Eco*RI fragment spanning the *Nco*I site was also sequenced in plasmid M13. Finally, exon 3 and part of exon 2 were sequenced from a cDNA clone in phage M13. The sequencing strategy of the *Apa*I-*Hin*dIII region is shown in Fig. 3A, and the DNA sequence of this region is shown in Fig. 3B.

The 5′ end of exon 1 was deduced by S_1 nuclease mapping. Total RNA isolated from cadmium-resistant Friend erythroleukemia cells was hybridized with a probe generated by cutting a plasmid spanning the MT-II gene at its unique *Nco*I site (Fig. 3B), digesting away a few hundred nucleotides at the 3′ ends with exonuclease III, and labeling the 5′ ends with ^{32}P. After S_1 nuclease digestion, the sizes of the protected radioactive fragments were analyzed by comparing them with a sequencing ladder of the same region of DNA (27). As shown in Fig. 4, the smallest protected fragment migrated slightly more slowly than did the A in the sequence 5′-TCTCTCCATC-3′ shown on the left side of the figure; thus, we presume that this A corresponds to the mRNA cap site. A cluster of bands that are up to five nucleotides longer probably represent incomplete digestion of the probe due to steric hindrance by the cap structure. Consistent with this suggestion is the observation that the smallest bands are most resistant to increasing amounts of S_1 nuclease or increasing temperatures of S_1 nuclease digestion (Fig. 4, lanes 2 to 4). The A designated +1 is also consistent with the consensus mRNA start sequence (5) Y-C-A-Y (in which A is position +1 and Y is either pyrimidine).

The positions of the introns were assigned by comparison with the mouse MT-I and human MT-II genes (15, 24) and the protein sequence of mouse MT-II (21). The position of the second intron was confirmed by comparison with the sequences of the genomic and cDNA clones. Since our cDNA clone contains a portion of the poly(A) tail, we were able to assign the end of exon 3 to nucleotides +779 to +781, the ambiguity being due to the two A residues in the genomic sequence at this point. The 3′ end shown in Fig. 3B includes the first A, 15 nucleotides downstream of the AATAAA polyadenylation signal.

The protein encoded by this gene would differ from the published amino acid sequence of mouse MT-II (21) by having glutamic acid in place of glutamine at position 52; this difference probably reflects either an error in the protein sequence or an allelic difference. Because the gene we have sequenced is unique DNA and matches the published MT-II protein sequence in 60 of 61 amino acids, we consider it unlikely that this gene encodes a minor MT or is a pseudogene; therefore, we identify it as the mouse MT-II gene.

Sequence comparisons. Figure 5 shows a comparison of the 5′ flanking DNA from the mouse MT-I and MT-II and human MT-IIA genes (15, 24). Four sequence elements appear to be conserved among these promoter regions. The conserved element closest to the site of transcription initiation (A in Fig. 5) contains the sequence TATAAA, which is found in most eucaryotic genes about 23 to 30 bp upstream of the transcribed region (5); it is thought to assist in the selection of the site at which transcription is initiated by RNA polymerase II. A second conserved region is centered between −49 to −54 bp (B in Fig. 5); this region has been implicated in the regulation of the MT-I gene by heavy metals (Searle et al., manuscript in preparation) and probably represents the binding site for a regulatory protein. Element B is also repeated further upstream in both MT-I and MT-II genes. The homology between the mouse and human MT-II genes continues immediately upstream of element B to give what may be regarded as a third element, C, between −60 and −70 bp. This includes the sequence

```
B                                   GGGC  CCCGGGGCAC CGCAAAGGGC
                                    Apa I Sma I

-350 GCGCGCGGGA GCAAGCAGGT GTCCTGGAAC CGGTTCCCAC CGGATCGCAG
     Bss HII

-300 ACCCTTTGCG CTCAGCTCCT TTGCTCTCAG TCCCTGAGCC CAGAGAAAGG

-250 GGGTGTGACT CAGCGCGGGG GGTGGGGGGA GGGCGTGTGC AGGCTCTGTC

-200 CCACGAGCAA AAAGAGGGCC GCTTGCAGAA TTCGGGTCGT GCGCAGGCCC
                                  Eco RI       Mst I

-150 AGGGGCGTGT GCTGGCCATA TCCCTTGAGC CAGAAAAAGG GCGTGTGCAG
                Bal I

-100 GCGGCGGGGG CGCGTGCATG GTGCCTTCCA CCCGGGCGGA GCTTTTGCGC
                                     Sma I

 -50 TCGACCCAAT ACTCTCCGCT ATAAAGGTCG CGTTCCGCGT GCTTCTCTCC

  +1 ATCACGCTCC TAGAACTCTT CAAACCGATC TCTCGTCGAT CTTCAACCGC +50

                   MetAsp ProAsnCysS erCysAlaSe rA
     CGCCTCCACT CGCCATGGAC CCCAACTGCT CCTGTGCCTC CGGTAAGGGG +100
                 Nco I

     GACTGCTGAC GGGATTTCTG GGAGAGCTAG ACAGGCTTTT TGGCCCCTCC +150

     TTTAGTAATT ACTTTAAGGG TACGACCGGC TACCCCTTCC GAATGAATTC +200
                                                  Eco RI

     TGAAGCACTC CTGCTCCTTT AAACTAGTCC TTGAGATAGT GGCTCGCCTA +250
                      Aha III

     CCCGGGTGAT TTGCCTCACC TTCCTAGGAG AACAGCGTTC AGGTACTCCC +300
     Sma I                                                 spGlySe
     GGGTCCCACT CAACCGCGCT CACTGACTGC CTTCTACTTT TAGATGGATC +350
     Sma I                                         Bam HI
     rCysSerCys AlaGlyAlaC ysLysCysLy sGlnCysLys CysThrSerC
     CTGCTCCTGC GCTGGCGCCT GCAAATGCAA ACAATGCAAA TGTACTTCCT +400
                    Nar I
     ysLysLysS
     GCAAGAAAAG TAAGTTGGAT CTTCTCTGCC ATTTCCCCGT CACTCTCCTG +450

     GGGTCCCTAG CCCGCCGCGC CGCGCCTTCC CTCCCGGGAG CGTTCAGGTG +500
                                      Sma I

     GTGTGCCTCT GACAAGGTTT CTCGCTCACG TTCAACTCTT CTCTCCCCAC +550

       erCysCys SerCysCysP roValGlyCy sAlaLysCys SerGlnGlyC
     AGGCTGCTGC TCCTGCTGCC CCGTGGGCTG TGCGAAGTGC TCCCAGGGCT +600

     ysIleCysLy sGluAlaSer AspLysCysS erCysCysAl aTer
     GCATCTGCAA AGAGGCTTCC GACAAGTGCA GCTGCTGTGC CTGAAGGGGG +650
                                    Pvu II

     GCGGAGGGGT CCCCACATCT GTGTAAATAG ACCATGTAGA AGCCTAGCCT +700

     TTTTTGTACA ACCCTGACTC GTTCTCCATA ACTTTTTCTA TAAAGCATGT +750

     AACTGACAAT AAAAGCCGTT GACTTGATTA ATTCAGCTTG TCTCTGTGCA +800

     TTGGTTAAGG GCTGGGCTGG CAGAGGCGTT GAGATGGGAC TGCAGCAGCT +850
                                               Pst I PvuII

     GAATTTGAGC TAGTCGGATT ATATTTCTCT GGACTTTGAC CGCCTAGAAA +900

     GTGGTCATGC AGAAGCATGC ATTGGTCACA GAAATGGGCC ATTCCTACTG +950
                  Sph I

     GGCGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG +1000

     TGTATTGGGG ATTAAACCGT AAGCTT
                           Hind III
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GGGCGG, or CCGCCC in the opposite strand; the latter sequence is also found in the mouse MT-I gene around −140 to −135 bp, and CCGCCT is present in the MT-I gene at −74 to −69 bp. The sequence CCGCCC is a conserved element in the 21-bp repeats of simian virus 40, which have been implicated in promoter function (13, 25), and this motif is also found in either orientation in the first and second distal promoter elements of the herpes simplex virus thymidine kinase gene. The fourth recognizable conserved sequence in the MT promoter regions, D, is centered between −84 and −101 bp upstream of the cap site. A similar sequence (D′ in Fig. 5) is repeated ca. 20 bp upstream of D in the mouse MT-II gene and at least twice further upstream, around −145 and −225 bp. We conclude that whereas most of the upstream sequences of the MT genes have diverged to a point from which no meaningful alignment can be made, certain sequence elements of between 7 and 13 bp have been conserved, which may indicate that they serve as recognition elements for components of the transcriptional machinery. The distance of the TATA box from the cap site varies by 3 bp in the MT genes, whereas the separation of the TATA box (element A) from element B varies by 2 bp. The separation of element D from B varies by 20 bp, suggesting

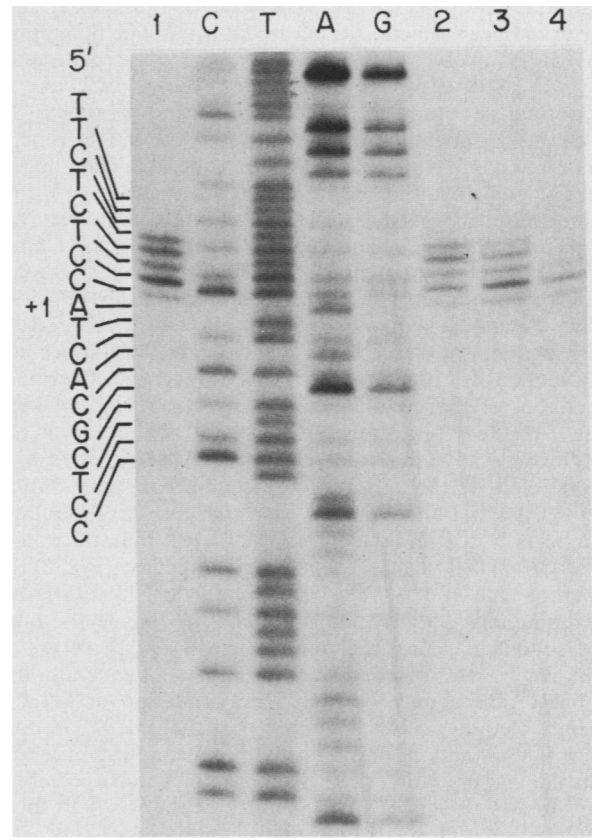

FIG. 4. Localization of the MT-II mRNA start site by S_1 nuclease mapping. Lanes C, T, A, and G correspond to a sequencing ladder generated by the Maxam-Gilbert techniques from an *Apa*I-*Nco*I fragment with the 5′-phosphate at the *Nco*I site being labeled; lettering of the lanes has been transposed so that the mRNA sequence can be read directly. Lanes 1 to 4 correspond to S_1 nuclease-resistant products obtained by digesting hybrids between the *Nco*I-labeled probe and MT-II mRNA as described in the text. Lanes 1 and 2, Digestion for 1 h at 37°C with 4 U of S_1 nuclease; lane 3, digestion with 8 U of S_1 nuclease for 1 h at 37°C; lane 4, digestion with 8 U of S_1 nuclease for 1 h at 45°C.

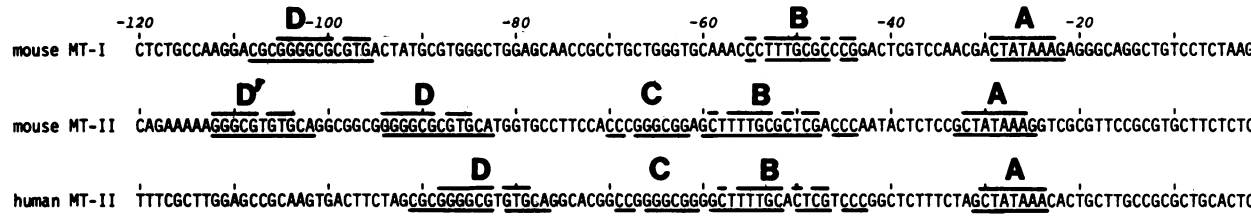

FIG. 5. Comparison of 120 bp of 5′ flanking DNA of the mouse MT-I and MT-II and the human MT-IIA genes. Conserved regions are indicated by bold letters above the sequences. In these regions, nucleotides underlined are conserved in two of the three promoter sequences; those also overlined are conserved in all three.

there may be less constraint on its positioning. Whereas both the mouse MT-I and human MT-IIA genes have dyad symmetries in the vicinity of the B element, the mouse MT-II sequence does not. Since the MT-II gene appears to be expressed at a level comparable to that of the MT-I gene, it seems that there is no universal requirement for a palindrome in this region.

The 5′ noncoding region of the mouse MT-II mRNA matches the Chinese hamster MT-II 5′ noncoding region in 24 of the 33 nucleotides for which the hamster sequence is available (16); less homology is seen with the human MT-IIA or mouse MT-I gene (15, 24). The sequence ACCACG that is conserved near the cap site of the mouse MT-I, human MT-IIA, and monkey MT-I and -II genes (36) has diverged to ATCACG in the mouse MT-II gene; the other conserved sequence recognized by Schmidt and Hamer (36), CTCPyAGC, does not appear in the mouse MT-II gene.

The mouse MT-II gene shows considerable homology to other MT genes in the coding region, having in the 183 bp of coding sequence only 38 mismatches with the mouse MT-I gene and only 15, 21, and 26 mismatches with the Chinese hamster, human, and monkey MT-II genes, respectively. The two introns are located at precisely the same positions within the coding sequence as in other MT genes (15, 24), but their sequences diverge immediately beyond the splice junction sequences. Figure 6 shows a comparison of the DNA sequences of the coding regions of eight MT-I and MT-II genes. By selecting the most frequent nucleotide at each position, we have derived a consensus MT sequence which might resemble the ancestral MT gene before gene duplication and speciation. We have used this consensus sequence as a basis for defining silent and nonsilent mutations in the MT-I and MT-II genes of the species indicated (Fig. 6). It is apparent that the number of silent base substitutions is approximately equal to the number that change the amino acid sequence. This fact could suggest that there are few selective constraints upon the protein sequence or, more probably, there may be significant selection acting upon silent nucleotides, also. As noted previously (12, 36), codon usage is markedly nonrandom, with a strong preference for C in the last position (38 of 61 codons). A third possibility, that there is selection for the ability of the RNA to form a preferred secondary structure (15), seems less likely, given the extent of divergence of the 5′ and 3′ noncoding regions.

In the 3′ untranslated region, the sequence TGTAAATA is conserved in mouse MT-II as in other MT genes (16), as is the oligothymidylate sequence about 60 nucleotides 3′ of the termination codon (36) and the AATAAA polyadenylation signal. In addition, the hamster, mouse, and human MT-II genes share several regions of homology between the oligothymidylate region and the AATAAA polyadenylation signal.

An interesting feature of the sequence 3′ of the mouse MT-II gene is a stretch of 50 alternating residues deoxyguanosine and thymidine commencing 174 bp downstream of the polyadenylation site. Poly(dG-dT) sequences have also been found in and around several other genes (34) and represent a family of repeated sequences in many eucaryotic genomes (19). Unlike some of the other occurrences of poly(dG-dT), that near the MT-II gene is not flanked by obvious direct repeats. Although this sequence is not found near the mouse MT-I gene and thus probably has no role in the normal regulation of MT genes, it will be of interest to determine whether it has any effect upon MT-II gene expression, given the potential of such alternating purine-pyrimidine sequences to adopt the Z-DNA conformation (2).

Regulation of mouse MT-I and MT-II genes in vivo. To ascertain whether mouse MT-I and MT-II genes might be differentially regulated with respect to any of the known inducing agents, we injected Swiss Webster mice with Cd, dexamethasone, or LPS and sacrificed the mice 4 h later, a time when MT-I would be maximally induced (8, 9, 18). Four mice were tested with each inducer, and a control group was not treated. We collected the liver, kidney, heart, and brain and pooled like tissues to prepare TNA for analysis. The relative abundance of MT-I mRNA and MT-II mRNA in each sample was assayed by solution hybridization to end-labeled MT-I and MT-II single-stranded DNA probes of identical specific activities. The RNA-DNA hybrids were then subjected to S_1 nuclease digestion and acrylamide gel electrophoresis. The two probes reacted specifically with MT-I and MT-II mRNAs to give unique protected bands of the expected sizes (Fig. 7A). Subsequently, the two probes were combined to provide a convenient means of comparing simultaneously the relative abundance of the two transcripts. The hybridization signal was proportional to the amount of mRNA added (Fig. 7B). In this experiment, the proportion of control and cadmium-induced kidney TNA was varied, but the total amount of TNA remained constant at 25 μg. Independent assays of the two TNA preparations by quantitative solution hybridization (10) revealed that 25 μg of control kidney TNA contained 6 pg of MT-I mRNA, whereas the cadmium-induced sample contained 145 pg; thus, with this exposure (14 h), ca. 20 pg of MT mRNA can be detected. Seven hours of hybridization was sufficient to achieve the maximum signal (Fig. 7B).

With these conditions, we then assayed tissue samples from the four test groups of mice. We found that induction of MT-I mRNA and induction of MT-II mRNA were qualitatively very similar (Fig. 7C and D). As with the MT-I gene, the MT-II gene was highly inducible by cadmium in liver and kidney and less so in heart and brain. Interestingly, LPS was the most potent inducer of both MT mRNAs in liver, heart, and brain. Although the signal for MT-II transcripts is stronger than that for MT-I in most samples, it is possible that the RNAs are slightly degraded, which would arti-

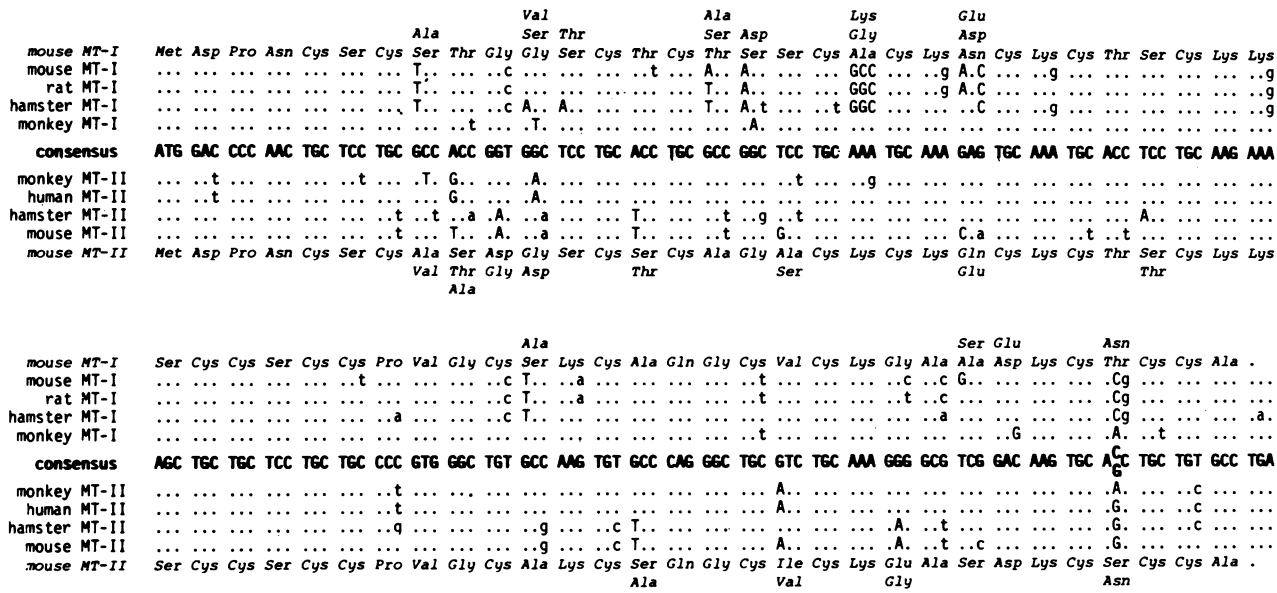

FIG. 6. Comparison of the nucleotide sequences of the coding regions of MT genes (1, 12, 16, 24, 36). The consensus sequence (bold type) represents the most frequent nucleotide at each position. For each of the MT genes, identity with the consensus sequence is indicated by a dot. Lowercase letters indicate mismatches with the consensus sequence which are silent; uppercase letters indicate differences that change the encoded amino acid. The amino acid sequences of mouse MT-I and MT-II genes are also shown; alternative amino acids found in the other MT genes are shown above and below the mouse sequences.

factually decrease the relative intensity of the larger MT-I protected fragment. Quantitative solution hybridization assays with uniformly labeled probes (10) revealed that the ratio of MT-I mRNA to MT-II mRNA was 2 to 3 in all samples except cadmium-induced liver and kidney, where the ratio was 5 to 7 (data not shown). Note that both endogenous MT mRNAs were highly inducible by dexamethasone in liver, heart, and brain (Fig. 7C and D).

We also tested the responsiveness of MT-I and MT-II genes in mouse L cells to different concentrations of dexamethasone and found that their responses were virtually identical. Half-maximal induction was achieved with 15 nM

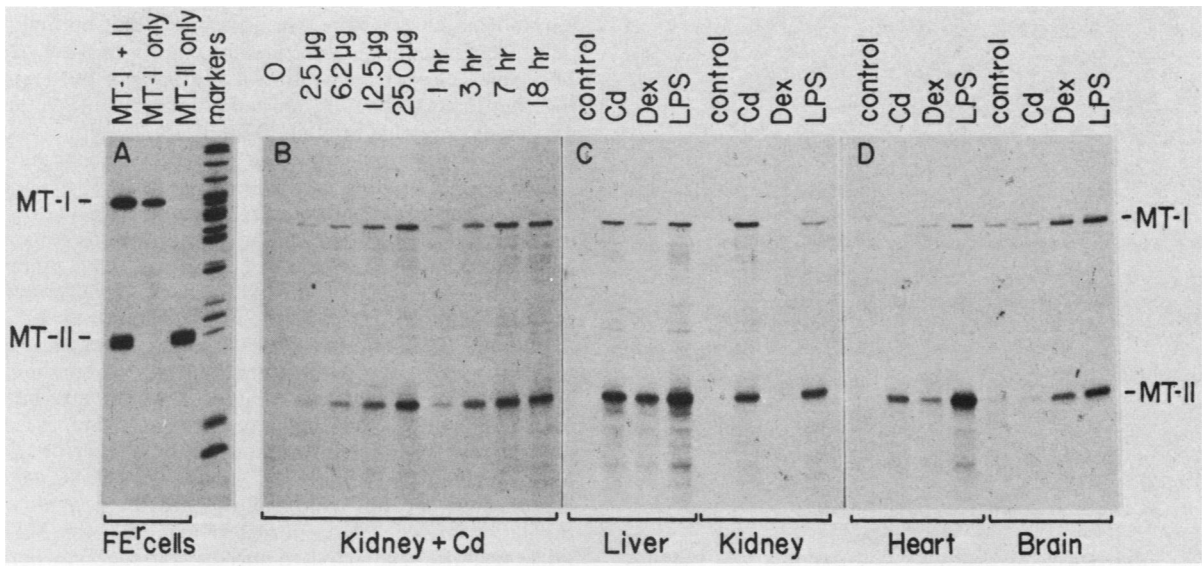

FIG. 7. Regulation of MT-I and MT-II mRNA abundance by cadmium (Cd), dexamethasone (Dex), and lipopolysaccharide (LPS). (A) Specificity of probes for MT-I and MT-II mRNAs. TNA from Cd-resistant Friend erythroleukemia cells was incubated either with kinased probes corresponding to the MT-I or MT-II gene or a mixture of both probes. After 14 h of hybridization, the single-stranded regions were digested with S_1 nuclease, and the resistant products were separated on a 7.5% acrylamide gel and visualized by autoradiography. See the text for details. (B) Optimization of hybridization conditions. Various amounts of TNA from Cd-induced kidney were mixed with TNA from control kidney such that the total amount of TNA was always 25 µg; these samples were then hybridized for 14 h with a mixture of both probes. TNA (25 µg) from Cd-induced kidney was also hybridized for various times with both probes before S_1 treatment. (C) and (D) Groups of four mice either served as controls (untreated) or were injected with Cd, dexamethasone, or LPS 5 h before the isolation and pooling of their livers, kidneys, hearts, or brains. TNA (25 µg) was incubated with both probes for 14 h, and the S_1-resistant products were separated on a 7.5% acrylamide gel and visualized by autoradiography.

dexamethasone, and maximal induction (4.5- to 6-fold) was obtained with 75 nM dexamethasone. The ratio of MT-I mRNA to MT-II mRNA in these cells was 1.5 (data not shown).

Mouse MT-I and MT-II genes are regulated by zinc and cadmium but not by glucocorticoids after transfection into human cells. Endogenous mouse MT-I and MT-II genes are transcriptionally regulated by heavy metals and glucocorticoids (9, 18, 28–30). However, the glucocorticoid response of the MT-I gene is selectively lost after transfection or amplification of the gene (29, 30). To determine whether this loss of hormone response applies to the mouse MT-II gene as well, we transfected plasmids pAX, carrying the MT-II gene, and pKH, carrying both MT-II and MT-I genes, into human HeLa cells. These plasmids were cotransfected along with pKO-neo, a plasmid carrying the neomycin resistance gene of Tn*5* with a simian virus 40 promoter, and cells resistant to the neomycin analog G418 were selected (38).

The accumulation of MT-I and MT-II mRNAs in two clonal and one multiclonal cell population in response to either dexamethasone or heavy metals is presented in Fig. 8. In each case, the amount of MT-I or MT-II mRNA increased markedly after exposure to zinc or cadmium. The magnitudes of these inductions, obtained by counting appropriately excised gel slides, ranged from 8- to 14-fold, indicating that the transfected MT genes remained fully responsive to heavy metals. The ratio of MT-I mRNA to MT-II mRNA produced in these cells was 0.2 to 0.25, opposite that observed for endogenous MT genes. We assume that the accumulation of MT mRNA in response to heavy metals reflects an increase in transcriptional initiation events, since heavy metals do not significantly enhance MT-I mRNA stability in human cells (30). In contrast, the relatively small

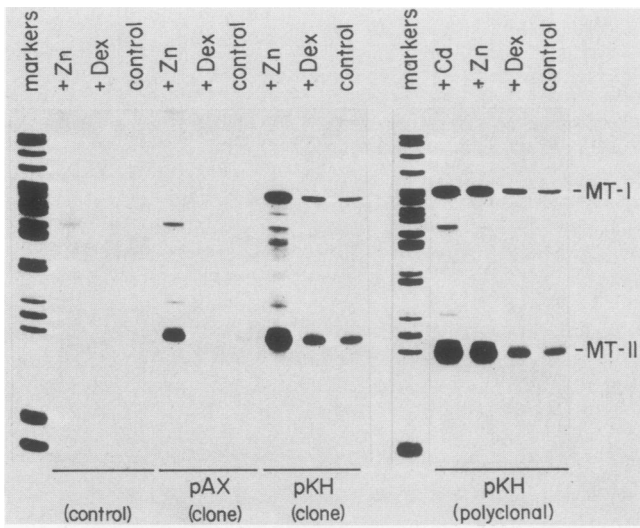

FIG. 8. Expression of MT-I and MT-II genes after transfection into HeLa cells. HeLa cells were transfected by the calcium phosphate method with plasmid pAX or pKH (see Fig. 1A) plus plasmid pKO-neo, and cells resistant to 0.5 mg of the neomycin analog G418 per ml were selected. Individual clones as well as polyclonal populations were expanded for analysis. Cells were treated with 100 μM $ZnSO_4$, 30 μM $CdSO_4$, or 100 nM dexamethasone (Dex) for 10 h before TNA was harvested. TNA (25 μg) was hybridized with end-labeled MT-I and MT-II probes and digested with S_1 nuclease; then the resistant fragments were separated by acrylamide gel electrophoresis, and the gel was autoradiographed. The migration of MT-I and MT-II-protected fragments is indicated. The markers are end-labeled *Hpa*II-cut plasmid pBR322.

increase (1.2- to 2.3-fold) in MT-I and MT-II mRNA accumulation observed after administration of dexamethasone was much lower than the response of the endogenous MT genes in vivo and is comparable to the previously noted effect of the hormone on MT-I mRNA stability (29, 30). It thus appears that mouse MT-I and MT-II genes, when transfected either individually or together in the naturally occurring configuration, are not transcriptionally regulated by glucocorticoids.

DISCUSSION

Only two mouse MT proteins have been identified (21), and they are encoded by the MT-I and MT-II genes described here. Although we cannot rule out the possible existence of other MT genes in mice, any that exist must have diverged sufficiently from the MT-I and MT-II genes to be undetectable by Southern blot analysis, and they do not contribute a significant amount of MT protein. These observations in mice contrast with the abundance of MT genes detected in Southern blots of primate DNA (24). One of these multiple copies has been identified as a processed gene lacking both promoter and introns (24).

As a technical note, we point out the utility of mapping transcription units by Southern blotting with nuclear run-on transcripts. This method allowed us to identify MT-II gene clones when all previous approaches had failed. The size of the transcription unit can also be estimated by blotting a judicious choice of restriction fragments. Note, for example, that the MT-II transcription unit extends into the next *Hin*dIII fragment beyond the structural gene, suggesting that the RNA polymerase II transcribes well beyond the poly(A) addition signal and through the 50 bp of dG-dT. However, it is important to be wary of hybridization to repeat sequences because this hybridization might be due to transcripts arising from other loci.

The mouse MT-I gene has previously been assigned to chromosome 8 (7); thus, we now know that both genes are on this chromosome. The close proximity of the two mouse MT genes explains why they have always been amplified together during selection for cadmium-resistant cell lines (3, 4). Considering that the two MT genes lie within 6 kb of each other, it is perhaps significant that they can be activated, i.e., committed to be expressed, independently of each other during selection for cadmium-resistant S49 cells (26). Knowing that there are two linked transcription units should facilitate the interpretation of changes in DNA methylation that always correlate with MT gene activation in these thymoma cell lines (6, 26; unpublished data).

Why are there two MT genes? One might suggest that the two proteins have different functions; however, the number and position of cysteinyl residues, which are intimately involved in metal binding, are invariant, and there is no physical evidence for a difference in the affinity of MT-I and MT-II for various metals (22). Another possibility is that the presence of two MT genes allows greater flexibility in the regulation of MT expression. For example, the MT-I gene might be more responsive to one set of inducers, whereas the MT-II gene might respond better to others. Considering the develpmental regulation of MT gene expression and the response of these genes to signals arising from inflammation and stress, as well as their regulation by both essential and toxic metals, this proposal seems quite likely. Our initial studies of regulation in vivo by three different inducers suggest that the two genes respond similarly to an optimal dose of each inducer, and in L cells they show identical dose-response relationships for dexamethasone. Further ex-

periments may reveal significant differences in the relative sensitivities of these two genes to heavy metals or other signals. We have looked at only four adult organs, so perhaps more extreme differences in regulation will be observed in other organs or during development.

The similar regulation of MT-I and II genes would lead one to expect them to share common promoter elements and, indeed, several blocks of conserved sequence have been identified in the 5′ flanking DNA. One of these regions (B in Fig. 5) is probably involved in regulation by heavy metals, but the precise location of promoter elements involved in regulation by LPS and dexamethasone is uncertain.

We were particularly surprised that the mouse MT-II gene, like the MT-I gene, does not appear to be transcriptionally regulated by dexamethasone after transfection of HeLa cells. This result is especially perplexing since Karin and collaborators have achieved regulation of human MT-II genes after transfection into rat cells (23). We initially thought that retention of glucocorticoid regulation might be a property of MT-II genes, but we now suspect that both mouse MT genes require some *cis*-acting modification that does not occur under the experimental conditions tried thus far.

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