MOLECULAR AND CELLULAR BIOLOGY, Jan. 1986, p. 26–37
0270-7306/86/010026-12$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 6, No. 1

Structure, Organization, and Regulation of Human Metallothionein I_F Gene: Differential and Cell-Type-Specific Expression in Response to Heavy Metals and Glucocorticoids

UMESH VARSHNEY, NADIA JAHROUDI, RANDY FOSTER, AND LASHITEW GEDAMU*
Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Received 19 April 1985/Accepted 9 September 1985

We describe a human genomic clone containing the metallothionein (MT) I_F and MT I_G genes. Southern blot analysis and partial DNA sequence determinations show that these genes are organized in a head-to-head fashion and are located approximately 7.0 kilobases apart from each other. Sequence analysis shows that the MT I_F gene contains three exons separated by two introns. All of the intron-exon junctions are defined by the GT-AG rule. The 5′ flanking region shows the presence of a duplicated metal regulatory element (TGCGC CCGGCCC) important in heavy-metal induction of this gene and a sequence for its basal level expression (GCGGGGCGGGTGCAAAG). The 5′ flanking region is also highly G+C rich (~75%) and contains several GC boxes (GGGCGG), probably important in the binding of transcription factors. The TATAA box and the AATAAA sequence are represented by their variants, the TATCAA box and the AATTAA sequence, respectively. This gene is functional and inducible by heavy metals but not by dexamethasone in mouse LMTK$^-$ cells after its transfer on a plasmid containing the herpes simplex virus thymidine kinase gene. Further studies on various human cell lines show that this gene is not expressed in a splenic lymphoblastoid cell line (WI-L2) but is expressed in two hepatoma cell lines (Hep 3B2 and Hep G2) in response to cadmium, zinc, and copper. Dexamethasone appears to have no significant effect on its expression. The studies suggest that the MT I_F gene shows cell-type-specific expression and is differentially regulated by heavy metals and glucocorticoids.

Metallothioneins (MTs) are a family of low-molecular-weight, cysteine-rich proteins of about 6,000 to 7,000 molecular weight and are widely distributed in nature (13). They are expressed in many different tissues and cell types when these are exposed to heavy metals such as cadmium, zinc, copper, and mercury and to glucocorticoid hormones, for example, dexamethasone (for a review, see reference 12). The induction of these proteins is regulated mainly at the level of transcription (7, 15, 22). Further studies have suggested the involvement of both *cis*- and *trans*-acting factors in the control of expression of the mouse and some of the human MT genes (5, 16, 28, 31).

In the mouse two different MTs (MT I and MT II) are found, both of which are expressed to the same degree when induced by heavy metals or glucocorticoids (27). In the case of humans, however, the expression of the MTs is complicated by the occurrence of many MT isoforms. There are at least four to five different MT I and one MT II polypeptides (13). So far it has not been possible to detect any differences in the mode of metal binding by different MTs (12). Therefore, at present it is appropriate to suggest that the various MTs exist in humans possibly to provide fine control of gene expression. This situation has been further complicated at the gene level where they are encoded by a multigene family consisting of about 14 members (17, 34). It appears that about half of these members may be nonfunctional (14, 25, 33).

Studies by Richards et al. (25) show that the human MT II_A gene is maximally induced by heavy metals and by glucocorticoids but that one of the human MT I genes, MT I_A, is induced only by heavy metals. However, the mouse MT I and MT II genes are expressed to the same degree in the presence of metals or glucocorticoids (27). This difference in human MT gene regulation may explain the occurrence of many MT genes. To elucidate the molecular mechanisms that govern differential and possible tissue-specific regulation of the human MT gene family, we undertook studies involving the isolation, characterization, and expression of the MT genes. So far, only four members of the human MT multigene family have been fully sequenced, characterized, and published. Of these, one represents the processed pseudogene for MT I (33) and another represents that for MT II (17, 33), while the other two represent the unique MT II_A (17) and MT I_A functional genes (25). The MT II processed gene has been localized on chromosome 4 (14, 19a, 26). The MT functional genes and the intron-containing pseudogenes which probably resulted from the gene duplication events are located on chromosome 16 (14). Isolation of several MT clones from a human genomic library and Southern blot analysis studies suggest that the MT genes are clustered (14, 25; this report). However, the exact location of the MT II gene with respect to the MT I gene cluster is not yet known.

In this report we describe a λ clone isolated from the human genomic library. This clone consists of two tandemly arranged MT I genes, not described previously, designated as MT I_F and MT I_G. The MT I_F gene has been fully sequenced and contains two intervening sequences of sizes 582 and 331 base pairs (bp) that are spliced according to the GT-AG rule. This gene is shown to be functional from studies of Northern blot and S1 nuclease analyses of MT mRNA from the total nucleic acids isolated from the transfected mouse cells and induced human cell lines by using the 3′-untranslated region of this gene as a specific probe. Our studies show that the MT I_F gene is differentially regulated in response to various heavy metals and glucocorticoids and that its expression appears to be cell-type specific. This gene is inducible by cadmium, zinc, and

* Corresponding author.

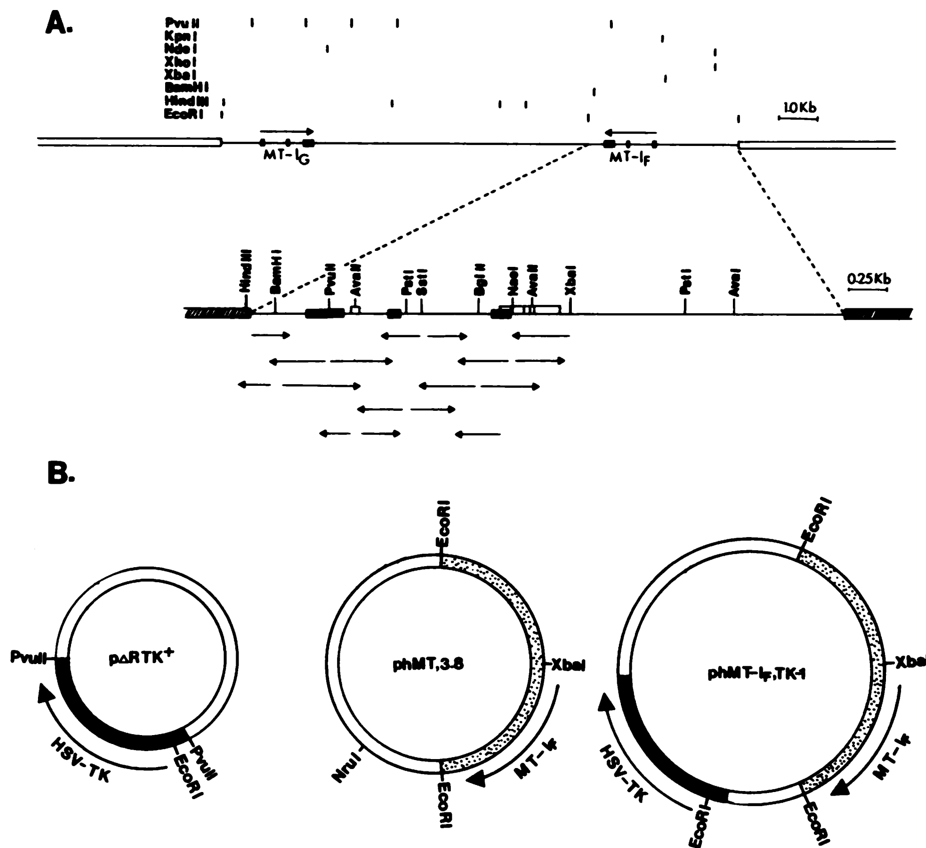

FIG. 1. Restriction mapping and plasmid constructions. (A) Restriction map of λ clone 14VS containing the MT I_G and MT I_F genes. The location and direction of the genes was established by Southern blot analyses and partial sequence analysis. The lambda arms are shown by open bars. The restriction sites are shown by vertical dashes. The 3.8-kb *Eco*RI fragment containing the MT I_F gene was subcloned into pBR322 (shown by hatched bars) and further mapped by restriction analysis. The arrows indicate the direction and extent of sequencing performed by the chemical degradation method. The *Ava*II sites are shown only for the region that was sequenced. (B) Plasmid constructs used in the sequence analysis and expression of the MT I_F gene. Details of the plasmid constructions are mentioned in the Materials and Methods section.

copper but not by glucocorticoids. The 5′ flanking region of the MT I_F gene was surveyed for regulatory sequences and compared with other human MT gene sequences in this region. It was shown to contain, like other MT genes, a duplicated heavy-metal regulatory element and a single copy of the sequences responsible for basal expression of the human MT genes. In addition, this region is highly G+C rich, and the sequence GGGCGG is repeated six times. The MT I_G gene also shows similar regulatory sequences.

MATERIALS AND METHODS

Isolation of MT clones and genomic blot analyses. Isolation of the MT-containing λ clones from a human genomic library (18), restriction mapping, and their cloning into pBR322 have been described previously (33). Hybridization conditions for genomic blots were slightly modified to increase the stringency of hybridizations. The hybridization buffer consisted of 3× SET (1× SET = 0.15 M NaCl, 0.03 M Tris, 2 mM disodium EDTA, pH 8.0), 10× Denhardt (1× Denhardt = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone 40, 0.02% Ficoll), 0.1% sodium dodecyl sulfate, 0.1% sodium PP_i, 50 μg of sheared and denatured *E. coli* DNA per ml, 125 μg of yeast total RNA per ml, and 20% formamide. The hybridizations were performed at 68°C for 16 to 20 h. The posthybridization washings were carried out at 68°C as described previously (33) with the addition of an extra wash with 0.1× SET–0.1% sodium dodecyl sulfate–0.1% sodium PP_i for 30 min. The filters were then air dried and subjected to autoradiography at −80°C by using preflashed Kodak XAR X-ray films and intensifying screens.

Nucleotide sequencing. DNA sequencing was performed from both strands of the DNA by the chemical degradation method (23). Sequencing strategies are given in Fig. 1A. Sequence analysis from the 5′ overhanging ends and the blunt ends of the DNA was done as described before (33). Sequencing from the 3′ overhanging ends was done after converting such ends into 5′ overhanging ends as follows. About 40 to 50 pmol of the 3′ overhanging ends of the DNA was treated with about 10 U of T_4 DNA polymerase (PL Biochemicals, Inc., Milwaukee, Wis.) in a 50-μl reaction buffer consisting of 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 50 μg of bovine serum albumin per ml at room temperature for 10 to 15 min. The reaction mixture was further treated with about 150 U of exonuclease III (New England BioLabs, Inc., Beverly, Mass.) for 10 s at 37°C to increase the 3′ to 5′ exonuclease activity. The reaction was stopped by quick extraction of the DNA with chloroform-phenol (50:50, vol/vol). The DNA was precipitated with ethanol and subjected to 5′ end labeling with [γ-^{32}P]ATP in the presence of polynucleotide kinase after dephosphorylating the generated 5′ overhanging ends (33).

```
          -400                                                         -350
TTGGCGCAAGAGACTGGGGTTGCACTGGGACTCTAGAAAGGCTTAGCAGTTGACGAAGGACCGGGCGGGGCCGGGGGGC
                            HinfI                             AvaII
                                 -300
GGGGCGAAGGCCAGGATCTCAGGTACCCGGAACCCCAAGGGGCGGGTGTAGCAGGCAATCTTGGCGAAACTGGGAAGGGC
                      KpnI
-250                                                   -200
GGGCAGGAGGGCAGGGAAGCCGCTCACCAGGCACAAAGCGCCTCCCGCTTGAGCGGACTCCAAAGGACGGTCCGCGGTG
                                                          HinfI       AvaII
                    -150                                                    -100
TGCAGCGAGCGCTGCGCTCAGGGGACCTTGCGCCCGGCCCTTCTGCTGCACACAGCCCACCCAGGACCTCCCGCAGCGCT
                      AvaII                                       AvaII
                                              -50
GACAGGCGGGGCGGGTGCAAAGACGGGGCGGGGTCTCTGCGCCCGGCCCCCTCCCCTGACTATCAAAGCAGCGGCCGGCT
                                                                             NaeI
        +1                                                     50
GTTGGGGTCCACCACGCCTTCCACCTGCCCCACTGCTTCTTCGCTTCTCTCTTGGAAAGTCCAGTCTCTCCTCGGCTTGC
      AvaII
                                    100
 MetAspProAsnCysSerCysAlaAlaG                                                 150
AATGGACCCCAACTGCTCCTGCGCCGCTGGTAAGGAACGCCGGGTTCCGTGCCTGGGGATGCTCGATTCCCAGACACCAT
   AvaII                                                          HinfI
                                                     200
AGAGAGTGTTCCTGGGTTTGAGAAGGTCGTATTTTGAGATCTCAACTGTAGGGGACTCCTTGACTTAGTCCAGTGCTTTC
                                      BglII             HinfI
                    250                                                 300
CTCTTGGCCAAGATCCTGAGAGCATTTCCTTCCTCTCTGTGCCTCTGTGTCAGCGTTGAGGGTACTGAGGCTCAAGGCTG

                                      350
TCCTGCTCCACGTCATGCGGTTTGTCCCAGGGCTGTTGGCTGAGCCCAGTGCACGACCAGGCTTGAGCAGCAGGATTAGA

         400                                                 450
TAGGAGGCAGGGGACATTGCCTCTCCGGGGTTCAGGACAGAAAGTCGAAGTCGCCGTCCTCCCAGGCTGTGCCTGGAGCC

                              500                                                550
TGGGACTTTCCTTTGGAGTGCAAACAGGAGGCTGCTTGGCCTTCCCAGCATGAAGGGAGAGGACATGGGGCTTCTCTTCC

                                                  600
TCTGCTCTGAGTGGGAAAGGAGCTCTGAGGGCTGACCCCGCACAGAGGAGGGGGCAATGGAGACTCATTAACTCACTGCT
                    SstI                                        HinfI
                                                                       ·700
                    650                                   lyValSerCysThrCysAlaGlySer
GTACCTCCTGCAGGTCACTCGCCGCTCACTGGCTTTTTTTTCTCTTTCTCGCAGGTGTCTCCTGCACCTGCGCTGGTTCC
          PstI
                                        750
CysLysCysLysGluCysLysCysThrSerCysLysLysS
TGCAAGTGCAAAGAGTGCAAATGCACCTCCTGCAAGAAGAGTGAGTGTGAGGCCATCTCCATGGTCTGGGGCTGTGGCTA

         800                                                 850
AGGTTGGGATGGAACCCAAGGCTGGCCCTGAGTGCATGCTTCTGGGGAACTGGCCTTCCTTTGTCCCCGTAGGTTGTCAC

                              900                                                950
TGCCTTTCTAGTCTTCTGCCCTGTGCAGGGCGCCTGGGCAGCTTTCTCATAGGAAGACCCACCCCAGATATTTCCCAGTT

                                                  1000
GTCTCCTGACAAAGCCATACCCTCCTGAACTGAGGGTCCTTTGTGGCTGGAGGCTCTGTTGGGGGCCTCTGTTGGGGAGG
                                    AvaII
                                                                    1100
                    1050                                erCysCysSerCysCysProValGlyCy
GAGGTCCCTGGGCAAGTTGGCTGTGACCTCTCATGCTCCTCTTCTTCCCCAGGCTGCTGCTCCTGCTGCCCCGTGGGCTG
  AvaII
                                      1150
sSerLysCysAlaGlnGlyCysValCysLysGlyAlaSerGluLysCysSerCysCysAspTer
TAGCAAGTGTGCCCAAGGCTGTGTTTGCAAAGGGGCGTCAGAGAAGTGCAGCTGCTGCGACTGATGCCAGGACAACCTTT
                                                  PvuII
      1200                                                1250
CTCCCAGATGTAAACAGAGAGACATGTACAAACCTGGATTTTTTTTTTATACCACCTTGACCCATTTGCTACATTCCTTT

                              1300                                              1350
TCCTGTGAAATATGTGAGTGATAATTAAACACTTTAGACCTGATTCTGACTTCAGTTTCCCTTATGTGCTTCAGAAATCA
                                          HinfI
                                              1400
GAGACTGGGGTGGGGGATCGAACTAGGGTTGCAGACTCCTAGGCTCTAAATGGAAATCTGAGTCCCTAACAATCAGAGTG
                                  HinfI                    HinfI
                    1450                                                1500
CATTAGGCAAGCCAAGCTGCCTCACTGTGCTTCCTCTTCTGTAGAATGGAATAACACTTCATCAGGTCATTGGTGGGGAT
                                                                             BamHI
                                        1550
CCAGAATACAGGATCACCTTCATTCTCAAATGTGGCACAGAAACTTCTCAATGCCTCCTGTTCCTTCTCTGATTTCTCTG

      1600                                                1650
CCCAACTTCAATTCCTCTTTGGATTTCAGGTTAGAAAGTGACTACACGGAATGGAATCAAACCCCTTAGTTATTTG
                                                        HinfI
```

The labeled DNA was either strand separated (20) or digested with appropriate restriction endonucleases, and the isolated fragments were sequenced. In some cases, e.g., from the *Pst*I site (Fig. 1A), sequencing was also performed after labeling the 3′ end of DNA fragments with [α-^{32}P]dCTP and T4 DNA polymerase (20).

Plasmid constructions. Plasmid constructions are shown in Fig. 1B. In brief, the 3.8-kilobase (kb) *Eco*RI fragment from the λ clone 14VS containing the MT I_F gene was cloned into the *Eco*RI site of pBR322. The recombinant plasmid was designated as phMT,3.8. A plasmid (pΔRTK$^+$) containing the herpes simplex virus thymidine kinase gene was obtained from D. I. Hoar (Department of the Medical Biochemistry, University of Calgary). The *Pvu*II fragment containing the herpes simplex virus thymidine kinase gene was excised out of pΔRTK$^+$ and subcloned into the *Nru*I site of phMT,3.8 through blunt-end ligation. The new plasmid containing the MT I_F and the herpes simplex virus thymidine kinase genes in the same orientation was designated as phMT-I_F,TK-1 and used in the transfection experiments.

Tissue culture, transfection, and selection. The cell lines used in these studies were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) in the presence of CO_2. The medium was supplemented with 10% fetal bovine serum for the two human hepatoma cell lines (Hep G2 and Hep 3B2) and the mouse LMTK$^-$ cells. A human lymphoblastoid cell line (WI-L2) was grown under shaking conditions (29), and the medium was supplemented with 7.5% fetal bovine serum. The penicillin and streptomycin (Flow Laboratories, Inc., McLean, Va.) were used only with LMTK$^-$ cells. All the cell lines were grown at 37°C.

The mouse LMTK$^-$ cells were transfected with the plasmid phMT-I_F,TK-1 by the calcium phosphate precipitation technique. The preparation of DNA-calcium phosphate precipitate has been described earlier (19, 36). About 650-μl portions of the DNA-calcium phosphate suspension containing 6 μg of the plasmid were then added to each 100-mm plate of the mouse LMTK$^-$ cells. The cells were plated the night before at a density of about 10^6 cells per plate in 7.0 ml of medium. At 24 h after the addition of the DNA-calcium phosphate precipitate, the cells were subjected to selection in HAT (hypoxanthine-aminopterin-thymidine) medium (Flow Laboratories). The HAT medium was supplemented with 10% fetal bovine serum. The serum was dialyzed overnight under running cold tap water and for 6 h against 0.15 M NaCl with a Spectrapor-1 dialysis membrane (6,000 to 8,000 molecular weight cutoff value). The selection medium was replaced every 48 h. Isolated colonies were seen after about 3 weeks of growth in the selection medium. The isolated colonies were trypsinized, mixed together, and maintained in HAT medium.

Induction of MT mRNAs. The mouse LMTK$^-$ cells and the transformants were induced by supplementing the growth medium with one of the following inducers for 7 h: 5 μM $CdCl_2$ (Cd), 100 μM $ZnCl_2$ (Zn), 100 μM $CuCl_2$ (Cu), or 10 μM dexamethasone. The human hepatoma cell lines (Hep G2 and Hep 3B2) were induced as above by one of the following inducers for 7 h: 2 μM $CdCl_2$ (Cd), 100 μM $ZnCl_2$ (Zn), 100 μM $CuCl_2$ (Cu), or 10 μM dexamethasone. The lymphoblastoid cell line (WI-L2) was also induced in a way similar to that of the hepatoma cell lines except that the induction period was 12 h. After the induction of the cells for the periods mentioned above, the total nucleic acids were extracted and analyzed by Northern blot and S1 nuclease analyses.

Extraction of total nucleic acids and Northern blot and S1 nuclease analyses. The cells were lysed in extraction buffer consisting of 7 M urea, 2% sodium dodecyl sulfate, 10 mM Tris (pH 7.5), 0.135 M NaCl, and 1 mM disodium EDTA. Total nucleic acids were then extracted twice with water-saturated phenol followed by two to three extractions with chloroform-isoamyl alcohol (24:1, vol/vol), precipitated with 2.5 volumes of absolute ethanol, and further analyzed.

(i) Northern blot analyses. Total nucleic acids (10 μg) were electrophoresed on a 1.5% agarose horizontal gel in the presence of 10 mM methyl mercury hydroxide and transferred to diazobenzyloxymethyl paper (Transa-bind; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Alwine et al. (1). The filters were hybridized to nick-translated probes as described in the figure legends. Hybridization conditions were as described above. The hybridizing bands at the 9S positions are due to MT mRNAs.

(ii) S1 nuclease mapping. (*a*) *Transcription initiation site.* To determine the transcription initiation site (cap site), the 78-bp *Ava*II fragment containing the 5′ untranslated region of the MT I_F gene (Fig. 2) was 5′ end labeled and strand separated. The strand complimentary to the mRNA was purified and hybridized to the mRNA as described by Varshney and Gedamu (33) with some modifications. In brief, about 200,000 cpm of the probe was mixed with 50 μg of the total nucleic acids isolated from a 5 μM $CdCl_2$-induced or uninduced human hepatoma cell line (Hep G2) and suspended in 25 μl of a buffer consisting of 80% formamide, 400 mM NaCl, 40 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) (pH 6.5), and 1 mM disodium EDTA and overlaid with mineral oil. The nucleic acids were denatured at 85°C for 5 to 7 min and allowed to hybridize at 54°C for about 16 h. The hybridization reaction was diluted by the addition of 450 μl of ice-chilled S1 nuclease buffer (300 mM NaCl, 30 mM sodium acetate [pH 4.6], 1 mM $ZnSO_4$). Samples (100 μl) were treated with various S1 nuclease concentrations (as described in the figure legends) at 37°C for 40 min. The reaction was stopped by chloroform-phenol extraction, and the nucleic acids were recovered by ethanol precipitation. The samples were analyzed on a 6% acrylamide–urea gel (33).

(*b*) *Poly(A) addition site.* The 3′ terminus of the mRNA bearing the poly(A) sequence was determined by using a 3′-end-labeled probe. The *Pvu*II-*Hin*dIII fragment (Fig. 1A) was isolated and 3′ end labeled (20). After end labeling, a secondary digestion was performed with *Bam*HI. The 350-nucleotide fragment, 3′ end labeled at the *Pvu*II site, was isolated and used for S1 nuclease analysis of the MT mRNA from a human hepatoma cell line (Hep G2; see above). The nucleic acids were denatured as described above and hybridized at 45°C for about 16 h. Details of the S1 nuclease

FIG. 2. Nucleotide sequence of the MT I_F gene. Sequencing strategies are described in Fig. 1A. Sequence of the region between *Xba*I and *Eco*RI sites is shown. Sequences of exons and their translation product, TATCAA sequence, MRE1 (around −50), MRE2 (around −135), and basal level expression (around −75) are shown in boldface letters. The restriction sites used in the studies and the GC boxes (8) are underlined. The major transcription initiation site has been numbered as +1. Nucleotides downstream of the major cap site are numbered with positive numbers, whereas those upstream are numbered with negative numbers at an interval of 50 nucleotides.

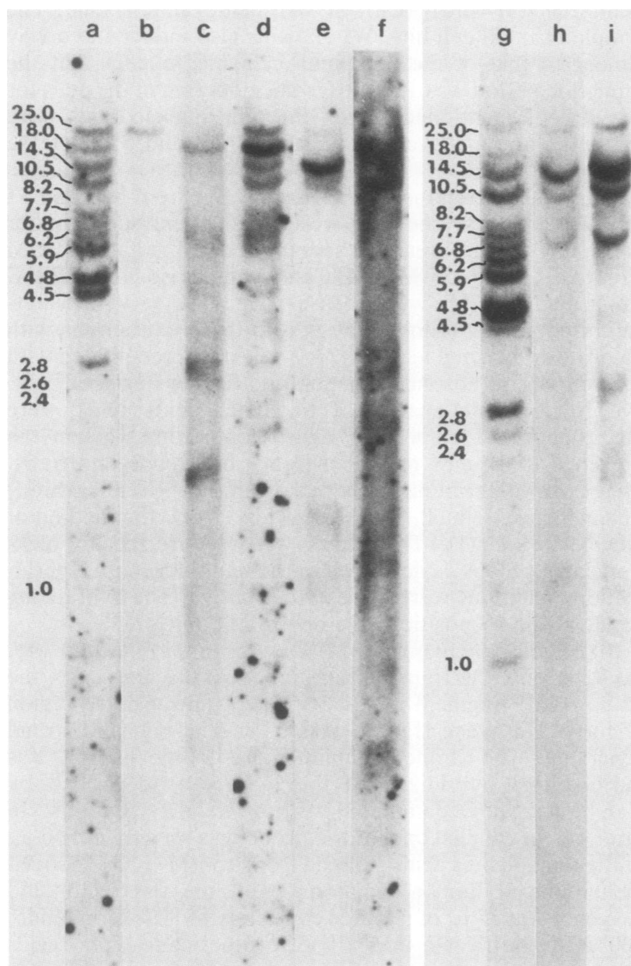

FIG. 3. Human MT gene organization. Human genomic DNA was digested with *Eco*RI and fractionated by electrophoresis on a 0.9% agarose horizontal gel. Each lane was loaded with 10 μg of the digested DNA. Samples in lanes a to f were from a heterozygote with respect to the MT II processed gene (34). The samples in lanes g, h, and i were from a 4.8-kb homozygote with respect to the MT II processed gene and were run on a separate gel. The DNA was transferred to a nitrocellulose paper, and parallel lanes were cut out. Each lane was hybridized to a different nick-translated probe as follows: lanes a and g to the human MT II processed gene contained in a 0.97-kb *Dde*I fragment (33, 34); lane b to the 9.7-kb *Eco*RI fragment containing the MT I_G gene (Fig. 1A); lane c to a 2.2-kb *Ava*I fragment containing the MT I processed gene (33); lane d to a 0.7-kb *Xba*I fragment, a subfragment of the 2.2-kb *Ava*I fragment in lane c above; lane e to the 3.8-kb *Eco*RI fragment of λ clone 14VS containing the MT I_F gene (Fig. 1A); lane f to a 3.3-kb *Hin*dIII fragment containing the MT sequences from λ clone 25S; lane h to the first intron of the MT I_F gene (*Bgl*II to *Pst*I [Fig. 1A]); lane i, longer exposure of lane h. The sizes of the MT bands that are seen to hybridize to MT probes are shown adjacent to lanes a and g. Bands at 2.6, 2.4, and 1.0 kb are seen in very long exposures only (as in lane g). In lanes b, e, and f the hybridization probes were very long. To avoid hybridization to the repetitive sequences, washings were performed under stringent conditions; hence only one band is seen to hybridize in each case.

digestion and analysis of the S1-digested material were the same as described above. Because the 3′ untranslated region is very AT rich, the S1 nuclease digestion reaction was done at a slightly lower temperature (34°C) to minimize breaking of the hybrids.

RESULTS

MT gene family. Figure 3 (lanes a and g) shows a typical pattern of 12 to 14 bands when an *Eco*RI-digested genomic DNA is hybridized with a human MT-specific probe. In Fig. 3, many of these bands have been characterized. As seen in Fig. 1A, an *Eco*RI site divides the genomic insert of a λ clone (14VS) into 9.7- and 3.8-kb fragments containing the MT I_G and MT I_F genes, respectively. The left-hand 9.7-kb fragment and the right-hand 3.8-kb fragment were isolated, nick translated (21), and hybridized to the *Eco*RI-digested genomic Southern blot (30). The 9.7-kb fragment hybridized to the 25-kb band (Fig. 3, lane b), whereas the 3.8-kb fragment hybridized to the 14.5-kb band (Fig. 3, lane e). Based on these studies, it can be inferred that the 25- and 14.5-kb bands are contiguous in the genome. Earlier studies have shown that the MT I_A gene is also localized in the 14.5-kb band and that the 14.5-kb band is contiguous with the 4.5-kb (MT $I_{\psi D}$) and the 10.5-kb (MT $I_{\psi C}$ and MT I_B) bands (25).

We identified some of the other genomic bands by performing hybridization studies with isolated MT-containing sequences from some more λ clones. The 18.0-kb band is represented by an MT I processed pseudogene (33) (Fig. 3, lane c). Similarly, a clone (25S) hybridizing to the 10.5-kb band contains the MT I_B gene (Fig. 3, lane f; unpublished data). This band is also shown to possess an MT $I_{\psi C}$ gene (25). To further characterize these bands, we hybridized the *Eco*RI-digested genomic Southern blot with the MT I-specific intronic probe (*Bgl*II to *Pst*I; see Fig. 1A) encompassing most of the first intron of the MT I_F gene. This probe hybridized to the 25-, 14.5-, 10.5-, and 6.8-kb bands, suggesting that the intron-containing MT I genes are localized in these bands (Fig. 3, lanes h and i). It is obvious from this figure that this intronic probe is specific only to MT I genes because it does not hybridize to the 5.9-kb band containing the MT II_A gene (17). It should be noted that a 4.5-kb band containing the MT $I_{\psi D}$ gene (25) also did not hybridize to any significant level. It is quite possible that, being a pseudogene, its introns have sufficiently diverged so as to show no significant level of hybridization under our conditions of hybridization.

The 5.9- and 4.8/4.5-kb bands have been identified previously to contain the MT II_A gene (functional gene) and the MT II processed pseudogene (17, 34). The latter gene shows a restriction fragment length polymorphism that has been highly conserved among humans of different races (34). The nature of the 8.2-, 7.7-, 6.2-, 2.8-, 2.6-, 2.4-, and 1.0-kb bands is not clear.

Sequence analysis of MT I_F gene. The restriction map of the λ clone 14VS is shown in Fig. 1A. Southern blot analyses with specific probes established the presence of the MT I_G and MT I_F genes.

The MT I_F gene contained in the 3.8-kb *Eco*RI fragment of the λ clone was sequenced to its entirety, whereas the MT I_G gene contained in the 9.7-kb *Eco*RI fragment of the λ clone was only partially sequenced. Sequencing strategies of the MT I_F gene are shown in Fig. 1A. The sequence was confirmed by sequencing from both strands of the DNA. When the coding sequence of the MT I_F gene was transformed into an amino acid sequence, it differed in four positions from the published amino acid sequences of human MT I or MT II (12). Therefore, to classify this gene, we looked at the nucleotide sequence homologies. Both genomic hybridizations (Fig. 3) and the nucleotide sequence of this gene show that it is more homologous to the MT I

genes (25, 33). Therefore, this gene was classified as a gene belonging to the MT I family. Moreover, since the MT II protein is unique (12), we had no complications in classifying it as an MT I gene. Amino acid positions 11 (Val versus Gly), 42 (Ser versus Ala), 49 (Val versus Ile), and 61 (Asp versus Ala) were found to be different (Fig. 4). The amino acid sequence derived from the MT I_A gene (25) was also found to deviate from the MT I protein sequence published previously (12) in four positions (amino acid positions 17, 27, 39, and 40) (Fig. 4). These differences are probably due to ambiguity in the sequence at the protein level (J. H. R. Kagi, personal communication). This ambiguity in the MT I sequence probably resulted from the difficulties associated with the purification of various MT I isoforms (13).

The MT I_F gene contains two introns. The location of these introns is the same as in other MT genes (9, 17, 25, 27). However, the length of the introns is different than those of other MT genes. The first intron is 585 bp and the second one is 332 bp. These lengths in human MT II_A (17) are represented by 301 and 203 bp, respectively, while in MT I_A (27) they are 486 and 526 bp, respectively. Our Southern blot analysis indicated that the lengths of these introns in MT I_G are similar to those in MT I_F (unpublished data). It appears that the human MT I genes have evolved with bigger intron lengths. Both of the introns are spliced out according to the GT-AG rule (4).

Transcription initiation and polyadenylation sites. The putative major site of transcription initiation is shown by an A at position +1 in Fig. 2. Sequences in this region of the MT I_F gene are similar to those found in other human MT genes (17, 25). To confirm this transcription initiation site, an *Ava*II fragment containing the 5′ untranslated region of the gene was 5′ end labeled, and the anti-mRNA strand was isolated on a strand separation gel. The anti-mRNA strand was hybridized to the total nucleic acids isolated from $CdCl_2$-induced or uninduced Hep G2 cells and treated with S1 nuclease as described above. Unfortunately, the anti-mRNA strand is only one nucleotide longer than the actual sequence of the mRNA toward the 5′ end (*Ava*II generates a 5′ overhanging sequence). Therefore, we were expecting to shorten this probe by only one nucleotide in our S1 nuclease protection studies when the RNA from $CdCl_2$-induced cells was used. With increasing concentrations of S1 nuclease, the intensity of the lower band kept increasing while the upper band kept diminishing (Fig. 5). At lower concentrations of S1 nuclease, a significant amount of the upper band, i.e., the original *Ava*II fragment, was also seen and this could have been due to the inefficiency of S1 nuclease as a result of some stearic hindrance. On the other hand, it is also possible that it represented a minor transcription initiation site and that the intensity of this band decreased at higher concentrations due to the breaking of the hybrids at the end. We would have liked to use a probe which extended more toward the 5′ flanking sequences; however, we were unable to find any other restriction site in the first exon suitable for 5′ end labeling and thus for the definite determination of the transcription initiation site(s). Nevertheless, we are quite confident that the A shown at position +1 in Fig. 2 represents the major cap site because such a protection was not seen in similar experiments with the RNA from uninduced cells (Fig. 5). Our primer extension studies with the 3′ untranslated region (*Pvu*II-*Hin*fI) also supported this conclusion (data not shown).

The poly(A) addition site was determined by using a *Pvu*II-*Bam*HI fragment as a probe labeled at the 3′ end of the *Pvu*II site which cuts the gene in the third exon. The *Pvu*II-*Hin*dIII fragment (see Fig. 1A) was first isolated and 3′ end labeled with T4 DNA polymerase and [α-^{32}P]dCTP (20). The secondary digestion was performed with *Bam*HI, and the longer fragment (*Pvu*II-*Bam*HI) containing the 3′ untranslated and flanking regions was hybridized to MT mRNA as described above. The nucleotide G at position 1312 was found to be the putative poly(A) addition site and is 14 nucleotides away from the polyadenylation signal (24). Fortunately, the sequence at the poly(A) addition site also conforms to a *Hin*fI restriction endonuclease site (GANTC), and thus the *Hin*fI digest of the probe provided an accurate marker (Fig. 6C).

The common eucaryotic promoter sequence, the TATAA box, is represented by a variant called the TATCAA box in the −30 region. The polyadenylation signal, AATAAA (24), is also represented by a variant, the AATTAA sequence. Such variations in the TATAA box (3, 25, 32) and the AATAAA sequence (2, 6, 10, 11) have been reported in many eucaryotic genes. The MT I_F gene possessed a duplicate metal-responsive sequence (TGCGCCCGGCCC). These sequences are centered around the −50 and −135 regions. A sequence responsible for basal activity of the MT II_A gene (16) was also found in the MT I_F gene and is centered around −75 (GCGGGGCGGGTGCAAAG) and is compared with the basal level expression sequences of the other MT genes in Fig. 7.

Expression of MT I_F gene. To determine whether the MT I_F gene is functional, we analyzed the MT mRNA in mouse LMTK$^-$ cells after their transformation with the MT I_F gene. The expression of this gene was studied in the transformed mouse cells and in various human cell lines. To detect the MT I_F mRNA in the human cell lines under in vivo conditions, we first established the hybridization conditions in which we specifically hybridized the MT I_F sequences only, by using the probes from the 5′ and 3′ untranslated regions. We found that the 3′ untranslated region (*Pvu*II to *Bam*HI) of this gene hybridized specifically to the MT I_F gene (data not shown), and hence we used this DNA fragment as a specific hybridization probe in our studies.

(i) Expression in mouse cells. To demonstrate the functionality of the MT I_F gene and to study its expression without interference from other human genes, we transformed mouse LMTK$^-$ cells with the MT I_F gene as described above. The transformants after HAT selection were pooled together and treated as one cell line. The transformed cells were induced with various inducers of MT, namely $CdCl_2$, $ZnCl_2$, $CuCl_2$, and dexamethasone. Total nucleic acids were isolated, and the presence of MT I_F mRNA was assayed by Northern blot and S1 nuclease analyses by using an MT I_F (*Pvu*II-*Bam*HI)-specific probe. Northern blot analysis showed a hybridizable band in the 9S region (Fig. 6A and B), while a DNA fragment 152 nucleotides long was protected in S1 nuclease analyses (Fig. 6C). When total nucleic acids from transformed mouse cells were analyzed, both cadmium and zinc led to a clear induction of MT I_F gene (Fig. 6B and C). Because of the high basal levels of MT I_F mRNA in the transformants, induction by copper and dexamethasone could not be determined accurately. The MT I_F probe used in these studies showed no hybridization to the RNA samples isolated from uninduced or cadmium-induced mouse LMTK$^-$ cells (Fig. 6B and C). Therefore, the hybridization results are a true measure of the MT I_F gene expression in the transformed mouse cell line. The MT I_F mRNA from the transformed cells was also compared with the MT I_F mRNA from human cell lines (Fig. 6B and C). It is quite clear that both cells produce the same size MT I_F mRNA transcripts

```
                                             Thr
                                             Ala Gly     Ser     Thr
Human MT-I   Met Asp Pro Asn Cys Ser Cys Ala Pro Val Gly Val Cys Ala Cys Ala Gly Ser Cys Lys Cys Lys Glu Cys Lys Cys Thr Ser Cys Lys

                                             Ala Gly Val Ser     Thr     Ala Gly                                     Thr
Human MT-IF  ATG GAC CCC AAC TGC TCC TGC GCC GCT GGT GTC TCC TGC ACC TGC GCT GGT TCC TGC AAG TGC AAA GAG TGC AAA TGC ACC TCC TGC AAG

                                             Thr Gly Gly Ser     Thr     Thr Gly                                     Asn
Human MT-IA  --- --- --- --- --- --- --- --- A-- --- -G- --- --- --- --- A-- --C --- --- --A --- --- --- --- --- --- -A- --- --- ---

Human MT-IIA --- --T --- --- --- --- --- --- --C --- -A- --- --- --- --- --C --C --- --- --A --- --- --- --- --- --- --- --- --- ---

Human MT-II  Met Asp Pro Asn Cys Ser Cys Ala Ala Gly Asp Ser Cys Thr Cys Ala Gly Ser Cys Lys Cys Lys Glu Cys Lys Cys Thr Ser Cys Lys
                                                 10                                      20                                      30

                                                                                                     Thr     Asp
             Lys Ser Cys Cys Ser Cys Cys Pro Val Gly Cys Ala Lys Cys Ala Gln Gly Cys Ile Cys Lys Gly Ala Ser Glu Lys Cys Ser Cys Cys Ala

                                             Val Gly     Ser                     Val             Ala     Glu                     Asp
             AAG AGC TGC TGC TCC TGC TGC CCC GTG GGC TGT AGC AAG TGT GCC CAA GGC TGT GTT TGC AAA GGG GCG TCA GAG AAG TGC AGC TGC TGC GAC

                                             Met Ser     Ala                     Ile                     Glu                     Ala
             --- --- --- --- --- --- --- --- A-- A-- --- GC- --- --- --- --G --- --C A-A --- --- --- --A --- --- --- --- --- --- --T -C-

             --A --- --- --- --- --- --- --T --- --- --- GC- --- --- --- --G --- --C A-C --- --- --- --- --G --C --- --- --- --- --- -C-

             Lys Ser Cys Cys Ser Cys Cys Pro Val Gly Cys Ala Lys Cys Ala Gln Gly Cys Ile Cys Lys Gly Ala Ser Asp Lys Cys Ser Cys Cys Ala
                                                 40                                      50                                      60
```

FIG. 4. Comparison of nucleotide sequences and amino acid sequences encoded by the human MT I_F, MT I_A (25), and MT II_A genes with each other and with the amino acid sequences of the human MT I and MT II polypeptide sequences (M. M. Kissling and J. H. R. Kagi, as cited in reference 33). The human MT I gene is heterogeneous, and the amino acid positions that show heterogeneity are also shown in the main sequence. The alternative amino acids are shown above the amino acid sequence of the MT I polypeptide (33). The nucleotide sequences where shown by a dash (-) are the same as those of the MT I_F gene. The amino acid sequences where not shown are the same as those shown by the respective polypeptide sequences. The amino acid sequences of the MT I polypeptide show microheterogeneity. These positions and also the positions where the genes differ from each other are shown by that amino acid.

and show the same polyadenylation sites. We observed that in the transformed mouse cells, there were higher basal levels of the MT I_F mRNA. This is not unexpected since mouse LMTK$^-$ cells produce a higher basal level of MT (data not shown). We believe that the factors that lead to higher basal level expression of the internal genes may also lead to the higher basal level expression of the human MT I_F gene. At first, we considered this higher basal level expression to be due to a high copy number of the MT I_F gene in the transformants; however, from the Southern blot analysis this was found not to be the case. The gene copy number in the transformants is about 1.5 per mouse haploid genome size (data not shown). Moreover, the fact that dialyzed serum was used in the growth medium of these cells rules out the possible presence of heavy metals in the serum.

(ii) Expression in human cell lines. Figure 6A, B, and C show the results of the Northern blot and S1 nuclease analyses of two hepatoma cell lines, Hep G2 and Hep 3B2, and a lymphoblastoid cell line, WI-L2. The Hep G2 cells were derived from a hepatoblastoma, whereas the Hep 3B2 cells were derived from a hepatocarcinoma. Both of these cell lines (Hep G2 and Hep 3B2) have been defined as parenchymal cells (17a). The WI-L2 cells are of splenic origin (29). All of these cell lines produced total MT mRNA in response to various MT inducers (Fig. 6A). In a separate study the kinetics of total MT and MT I_F mRNA induction in response to the heavy metals (Cd, Zn, Cu) and dexamethasone were studied over a period of 48 h. The total MT mRNA and the MT I_F mRNA were analyzed by Northern blot analysis, using the coding region of the MT II processed gene (33) and the 3′ untranslated region of the MT I_F gene, respectively. The kinetics of the total MT mRNA and MT I_F mRNA induction in response to heavy metals was similar in the Hep G2 and Hep 3B2 cell lines, and the time of maximal induction was between 5 and 9 h (except for the copper induction of the Hep 3B2 cells, where it was 12 h). The time of maximal induction of the total MT mRNA in WI-L2 cells was about 12 h. These cells (WI-L2), however, did not lead to the induction of the MT I_F mRNA. Only one of the cell lines (i.e., Hep G2) responded to dexamethasone as shown by the induction of the total MT mRNA. The levels of total MT mRNA were found to be consistent after an initial lag period of about 5 h. MT I_F mRNA was not induced in response to dexamethasone (N. Jahroudi, U. Varshney, and L. Gedamu, unpublished data). In the studies presented here, we therefore analyzed the induction of MT mRNA at a common time point of 7 h in the Hep G2 and Hep 3B2 cells and at a common time point of 12 h in the WI-L2 cells. A 12-h time point for induction of MT mRNA by copper in the Hep 3B2 cells was also studied. Because these time points in each induction were not necessarily the times of maximal induction, in the following studies the conclusions are drawn only in qualitative terms.

From Fig. 6B and C it is clear that the heavy metals (cadmium, zinc, and copper) led to the induction of the MT I_F gene in the Hep G2 and Hep 3B2 cells. Induction of the MT I_F gene in the Hep 3B2 cells in response to copper was undetectable at 7 h (Fig. 6B and C). However, its induction was detectable at 12 h (Fig. 6C, lane Cu 1). Only one of the cell lines (Hep G2) responded to dexamethasone as shown by the induction of total MT mRNA (Fig. 6A). However, there was no induction of MT I_F mRNA, suggesting that this gene is not inducible by dexamethasone. This is not unexpected because the MT I_F gene does not contain the sequences responsible for glucocorticoid receptor binding (Fig. 2). To our surprise, the lymphoblastoid cell line WI-L2,

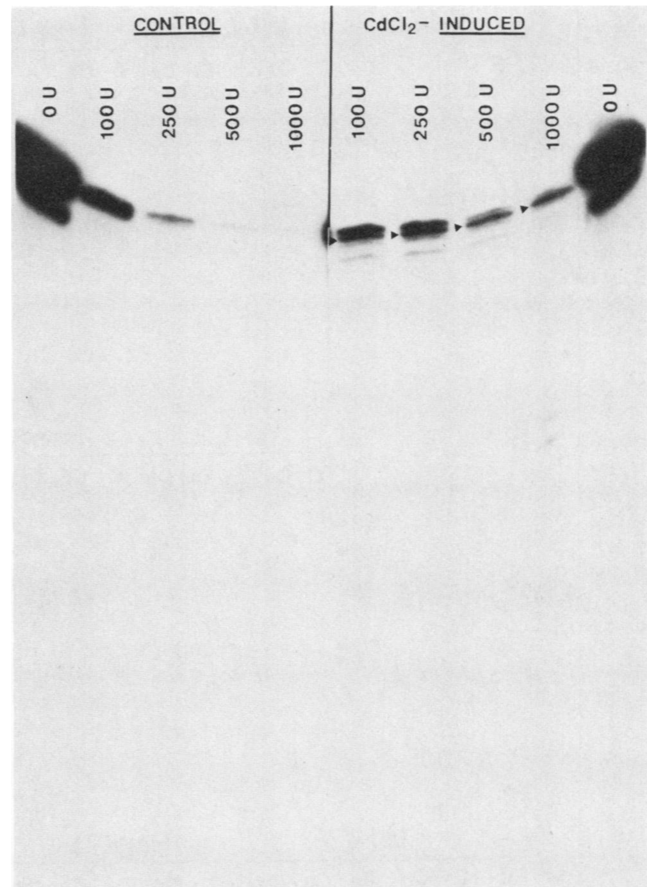

FIG. 5. Determination of the transcription initiation site. The 78-bp *Ava*II fragment containing the 5′ untranslated region of the MT I_F gene (Fig. 2) was labeled at the 5′ ends, and the anti-mRNA strand was isolated on a strand separation gel. The anti-mRNA strand was hybridized to the total nucleic acids extracted from the 5 μM $CdCl_2$-induced or uninduced (control) cell lines and digested with different concentrations of S1 nuclease (0, 100, 250, 500, or 1,000 U) as described in the Materials and Methods. The arrowheads in the $CdCl_2$-induced samples show the limit of protection and, therefore, the transcription initiation site. This band of protection corresponds to an A at the +1 position in the Fig. 2.

a cell line which produces total MT mRNA (Fig. 6A) and has earlier been shown to produce MT II mRNA and at least one MT I mRNA (33), did not express the MT I_F gene in response to any of the MT inducers studied (Fig. 6B and C). We reinvestigated this situation further by studying the expression of the MT I_F gene in these cells over a period of 48 h. The results show that there is no induction of MT I_F mRNA in these cells. From the Southern blot analysis of the WI-L2 genomic DNA, there are no apparent defects in the primary structure of the MT I_F gene (data not shown). The fact that the other MT genes (Fig. 6A) are expressed in response to heavy metals rules out the possibility that the WI-L2 cells are lacking in the regulatory proteins that mediate regulation by heavy metals. The concentrations of $CdCl_2$ (2 μM) and $ZnCl_2$ (100 μM) used to induce these cells were chosen based on the dose-response curves for these metals. These concentrations lead to about 50% inhibition of the growth rate of the WI-L2 cells (Jahroudi et al., unpublished data). In fact, the WI-L2 cells fail to express the MT I_F gene even in the presence of 15 μM $CdCl_2$ (in a resistant variant of WI-L2 cells [29]) or 200 μM $ZnCl_2$ (data not

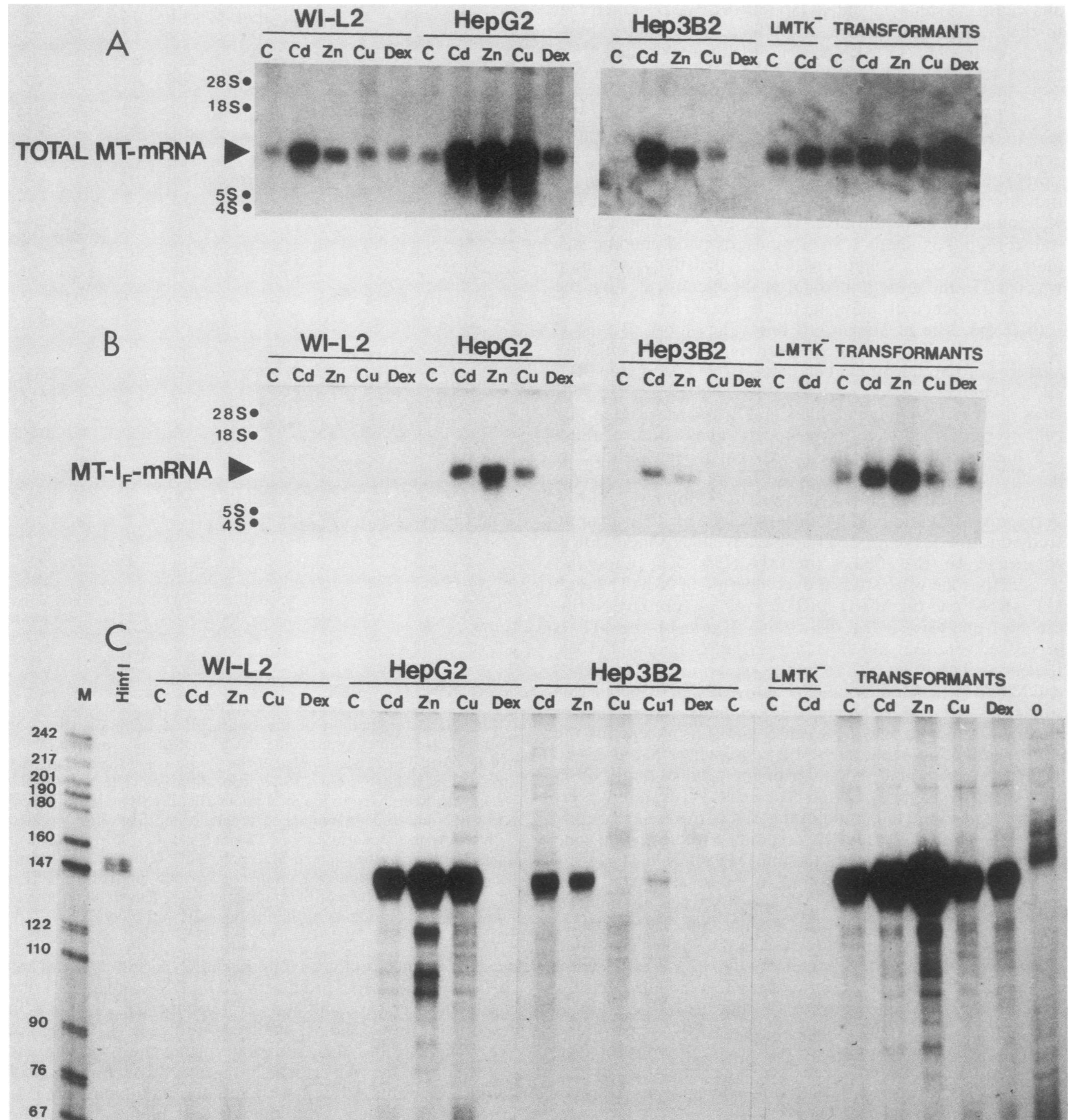

FIG. 6. Expression and determination of the polyadenylation site of the MT I_F gene. (A) Northern blot analysis of the total nucleic acids extracted from various uninduced, i.e., control (C), cell lines or cell lines induced with cadmium (Cd), zinc (Zn), copper (Cu), or dexamethasone (Dex). Total nucleic acids were electrophoresed, and the RNAs were transferred to diazobenzyloxymethyl paper as described in the Materials and Methods. The filters were hybridized to the MT II processed gene-coding region (*Bam*HI to *Pvu*II; see reference 33) to detect total MT mRNA. (B) Same as panel A but the filters were hybridized to the 3′ untranslated region of the MT I_F gene (*Pvu*II-*Hin*fI; Fig. 2) to specifically detect the MT I_F mRNA. (C) S1 nuclease analysis. For each analysis 10 μg of the total nucleic acids were suspended in 10 μl of the S1 hybridization buffer along with 60,000 cpm of the 3′-end-labeled probe and processed as described in Materials and Methods. Each reaction was then diluted with 100 μl of ice-chilled S1 nuclease buffer and digested with 250 U of S1 nuclease. The products were analyzed on a 6% polyacrylamide–urea gel. 3′-end-labeled *Hpa*II fragments of pBR322 were used as size markers (M). A *Hin*fI digest of the S1 nuclease probe that provided an accurate size marker for the band of protection is also run along with the markers (see Results). A sample untreated with S1 nuclease (0) but processed along with the samples treated with S1 nuclease is also run as a control. Most of the material in this slot runs at a 350-nucleotide-long DNA position (not shown). The definition of the various RNA samples is the same as described above for the Northern blot analysis except that the induction of the Hep 3B2 cells by copper was slightly modified in that one extra sample was analyzed. Cu1 represents induction of the Hep 3B2 cells by 100 μM $CuCl_2$ for 12 h.

shown). At present we are unable to explain why this gene is not expressed in the WI-L2 cells. One possibility may be that the MT I_F gene is hypermethylated in this cell line and is, therefore, not expressed (35). Our data, however, demonstrate that the MT I_F gene is a functional member of the MT multigene family. It is differentially regulated in response to metals and hormones and appears to be expressed in a cell-type-specific manner.

DISCUSSION

Differential induction of MT genes and possibility of tissue-specific gene expression. One of the most perplexing questions is the presence of several MT genes in humans. One possibility is that the different MT genes are induced by different inducers. Richards et al. (25) have performed such studies with human MT II_A and MT I_A genes. They found that the MT II_A gene is maximally induced by dexamethasone, zinc, and cadmium but that the MT I_A gene is maximally induced only by cadmium and is only weakly induced by glucocorticoids or zinc which are the physiological inducers. It has been suggested that the MT II_A gene is expressed for physiological reasons (e.g., zinc homeostasis), whereas the MT I's are expressed in response to heavy-metal toxicity (25).

Our studies also showed that the MT I_F gene is activated by cadmium and zinc in a way similar to that of MT I_A. However, unlike MT I_A, MT I_F is also induced by copper to a significant level at least in the human hepatoma cell lines, especially in Hep G2. Such differential expression could be attributed to the differences in the regulatory sequences in the 5′ flanking regions of the MT genes (25). In addition to this differential regulation, our studies also showed a cell-line-specific expression of the MT I_F gene. A lymphoblastoid cell line, WI-L2, of splenic origin did not appear to show any induction of MT I_F mRNA, yet it produced MT II and at least one MT I mRNA, different from MT I_F (33; Jahroudi et al., unpublished data). However, two hepatoma cell lines (Hep G2 and Hep 3B2) showed the induction of the MT I_F gene. Since these cell lines have been derived from different tissues (see Results), these studies suggest a tissue-specific gene expression of MT I_F. To address the question of the differential and tissue-specific gene expression of various MT I genes in detail, it is important that sequences of other MT I genes be determined and their expression studied. At present we are sequencing two more MT I genes. Our future work and that of others will be able to answer some of these questions.

Regulatory sequences and comparison of the 5′ flanking regions of the human MT genes. Karin et al. (16) have described the sequences responsible for metal regulation and glucocorticoid hormone regulation of the MT II_A gene. They have also described a sequence responsible for the basal activity of the MT II_A gene (GCGGGGCGTGTGCAGGG). Carter et al. (5) have also defined such sequences in the mouse MT I gene. These studies (5) have led to a consensus sequence responsible for metal regulation (5′-TGCGCCC GCT$^{C}_{T}$C-3′). On the basis of experiments with synthetic oligonucleotides, Stuart et al. (31) have also shown that this sequence is responsible for metal regulation of MT genes. The metal-responsive elements are highly conserved among the mouse and human MT genes. Based on this observation, it has been speculated that these regions may act as core recognition sites for the binding of regulatory factors, polymerase, or other transcription factors that recognize the sequence in the presence of metals (5). Although a single copy of such sequences is sufficient for mediating the response of metals, multiple copies are more efficient (31).

We found similar sequences for metal regulation and basal activity in the MT I_F gene (Fig. 7). Unlike the MT II_A gene, the MT I_F gene does not have any glucocorticoid receptor binding site, and therefore it is not inducible by dexamethasone. The metal regulatory element in MT I_F (TGCGCCCGGCCC) is duplicated (as in MT II_A) and is located at positions centered around −50 and −135 with respect to the major cap site. The basal level expression region (GCGGGGCGGGTGCAAAG) is located at around −75. Interestingly, the sequences in the 5′ flanking region are highly GC-rich. At several places they consist of stretches of Gs and Cs, especially between the two metal regulatory sequences. These sequences seem to be a common feature of all the MT genes described so far. Several of these sequences consist of a sequence GGGCGG, called the GC box (8), and are underlined in Fig. 2. This sequence has recently been shown to bind with a mammalian transcription factor, Sp1, in simian virus 40 and monkey genomes (8). This core sequence is also shown to bind to the Sp1 factor in the human MT I_A and MT II_A genes (R. Tjian, personal communication). These sequences may also be important in the regulation of expression of the MT I_F gene.

In addition, we also observed the presence of two putative stem and loop structures in the 5′ flanking region of the MT I_F gene. The first such structure starts at nucleotide position −37 and ends at −86. The first 17 and the last 15 nucleotides are involved in the stem structure, whereas the middle 18 nucleotides form the loop. The stem consists of 12 G · C and 1 A · T base pairs and four mismatches. The second such structure starts at nucleotide position −69 and ends at −145. The first 14 and the last 15 nucleotides form the stem, and the central 47 nucleotides form the loop. The stem consists of 10 G · C and 2 A · T bonds and three mismatches (structures not shown). It is interesting that both of these structures involve the metal regulatory and basal level expression sequences in the stem. Such structures may also be playing some role in the regulation of expression of the MT genes.

In Fig. 7 we compare the 5′ flanking sequences of four human MT genes. MT I_G has been partially sequenced in our laboratory (unpublished data). MT I_A and MT II_A sequences have been published earlier (17, 25). The 5′ flanking regions are highly G+C rich (about 75% GC) and quite homologous. A sequence with dyad symmetry (different from the stem and loop structures mentioned above) shown in mouse (27) and other human MT genes (17, 25) is also present in the MT I_F and MT I_G genes (shown with arrows in Fig. 7). In Fig. 7 the sequences are aligned so that the MRE1 (proximal to the TATAA box), MRE2 (distal to the TATAA box), and basal level expression sequences can be compared. MRE1 and MRE2 of MT I_F are completely homologous to themselves and to the MRE2 of MT II_A. Because the MRE2 of MT II_A has been shown to be functional (15, 16) and because exposure to heavy metals led to induction of the MT I_F gene (Fig. 6), we believe that these elements are also functional. Other metal regulatory elements shown in Fig. 7 have slight variations from the consensus sequence, yet the MRE1 of MT I_A and the MRE1 of MT II_A have been shown to be functional (16, 25).

In the absence of any consensus sequence for the basal level expression region, we compared the sequences of human MT I genes with those of human MT II_A. None of the MT I genes showed a perfect match with the MT II_A sequence. Our transfection studies on the expression of the MT I_F gene indicated that the two- to three-bp differences

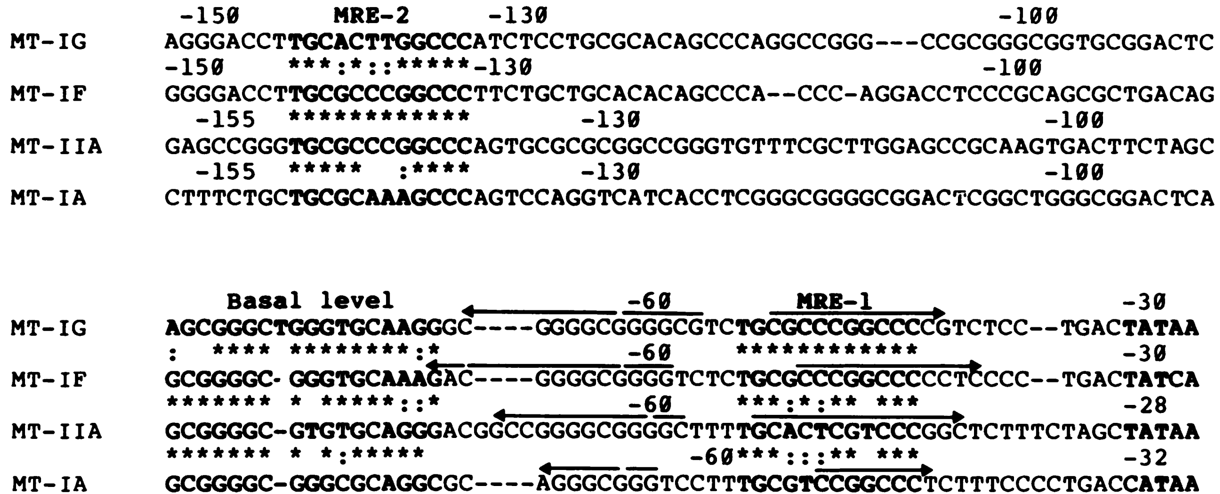

FIG. 7. Comparison of the 5′ flanking sequences of the MT I_F, MT I_G (R. Foster, U. Varshney, N. Jahroudi, and L. Gedamu, unpublished data), MT I_A (25), and MT II_A (17) genes. The sequences are arranged so that the MRE1, MRE2, and basal level expression sequences of the four genes can be compared. The dashes within the sequence are included to maximize the homology of the sequences. The MREs, basal level expression, and TATAA sequences are shown with boldface letters. An asterisk between the MRE1, MRE2, and basal level expression sequences shows a homologous nucleotide between the two genes, whereas a colon indicates a purine-to-purine or a pyrimidine-to-pyrimidine substitution. A gap indicates a mismatched base.

that are observed do not alter the basal level of expression of the MT I_F genes.

In conclusion, we described the organization of two of the MT I genes (MT I_F and MT I_G) and further analyzed the structure and function of the MT I_F gene. Our data show that this gene is regulated by cadmium, zinc, and copper but not by dexamethasone and possibly shows cell-type-specific expression.

ACKNOWLEDGMENTS

This work was generously supported by the grants from the Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research, (AHFMR) and the Provincial Cancer Board to L.G. U.V., N.J., and R.F. were supported by the studentships from AHFMR.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. USA **74**:5350–5354.
2. **Amara, S. G., R. M. Evans, and M. G. Rosenfeld.** 1984. Calcitonin-calcitonin gene-related peptide transcription unit: tissue-specific expression involves selective use of alternative polyadenylation sites. Mol. Cell. Biol. **4**:2151–2160.
3. **Borras, T., J. M. Nickerson, A. B. Chapelinsky, and J. Piatigorsky.** 1985. Structural and functional evidence for differential promoter activity of the two linked δ-crystallin genes in the chicken. EMBO J. **4**:445–452.
4. **Breathnach, R., and P. Chambon.** 1981. Organisation and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
5. **Carter, A. D., B. K. Felber, M. J. Walling, M. F. Jubier, C. J. Schmidt, and D. H. Hamer.** 1984. Duplicated heavy metal control sequences of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA **81**:7392–7396.
6. **Cheng, H. L., F. R. Blattner, L. Fitzmaurice, J. F. Mushinski, and P. W. Tucker.** 1982. Structure of genes for membrane and secreted murine IgD heavy chains. Nature (London) **296**:410–415.
7. **Durnam, D. M., and R. D. Palmiter.** 1981. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. Proc. Natl. Acad. Sci. USA **256**:5712–5716.
8. **Gidoni, D., W. S. Dynam, and R. Tjian.** 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature (London) **312**:409–413.
9. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature (London) **292**:267–269.
10. **Hagenbuchle, O., R. Bovey, and R. A. Young.** 1980. Tissue specific expression of mouse α-amylase genes: nucleotide sequence of isoenzyme mRNAs from pancrease and salivary glands. Cell **21**:179–187.
11. **Jung, A., A. E. Sippel, M. Grez, and G. Schutz.** 1980. Exons encode functional and structural unit of chicken lysozome. Proc. Natl. Acad. Sci. USA **77**:5759–5763.
12. **Kagi, J. H. R., and M. Nordberg (ed.).** 1979. Metallothionein. Birkhauser, Basel.
13. **Kagi, J. H. R., M. Vasak, R. Lerch, D. E. O. Gilg, P. Huziker, N. R. Bernhard, and M. Good.** 1984. Structure of mammalian metallothionein. Environ. Health Perspect. **54**:93–103.
14. **Karin, M., R. L. Eddy, W. M. Henry, L. L. Haley, M. G. Byers, and T. B. Shaws.** 1984. Human metallothionein genes are clustered on chromosome 16. Proc. Natl. Acad. Sci. USA **81**:5494–5498.
15. **Karin, M., A. Haslinger, H. Holtgreve, G. Cathala, E. Slater, and J. D. Baxter.** 1984. Activation of a heterologous promoter in response to dexamethasone and cadmium by metallothionein gene 5′-flanking DNA. Cell **36**:371–379.
16. **Karin, M., A. Haslinger, H. Holtgreve, R. I. Richards, P. Krauter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature (London) **308**:513–519.
17. **Karin, M., and R. I. Richards.** 1982. Human metallothionein genes—primary structure of the metallothionein-II gene and a related processed gene. Nature (London) **299**:797–802.

17a. **Knowles, B. B., D. B. Searles, and D. P. Aden.** 1984. Human hepatoma-derived cell lines, p. 196–202. *In* F. V. Chisari (ed.), Advances in hepatitis research. Masson Publishing Co. U.S.A., Inc., Chicago.

18. **Lawn, R. M., E. F. Fritsch, R. C. Parker, G. Blake, and T. Maniatis.** 1978. The isolation and characterization of linked δ- and β-globin genes from cloned library of human DNA. Cell **15**:1157–1174.
19. **Lewis, N. H., P. R. Srinivasan, N. Stokoe, and L. Siminovitch.**

1980. Parameters governing the transfer of the genes for thymidine kinase and dihydrofolate reductase into mouse cells using metaphase chromosomes or DNA. Somatic Cell Genet. **6**:333–347.

19a. **Lieberman, H. B., M. Rabin, P. E. Barker, F. H. Ruddle, U. Varshney, and L. Gedamu.** 1985. Human metallothionein-II processed gene is located in region p11–q21 of chromosome 4. Cytogenet. Cell Genet. **39**:109–115.

20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

21. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. USA **72**:1184–1188.

22. **Mayo, K. E., R. Warren, and R. D. Palmiter.** 1982. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. Cell **29**:99–108.

23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.

24. **Proudfoot, N. J., and G. G. Brownlee.** 1976. 3′ non-coding region sequences in eucaryotic messenger RNA. Nature (London) **263**:211–214.

25. **Richards, R. I., A. Heguy, and M. Karin.** 1984. Structural and functional analysis of the human metallothionein-I_A gene: differential induction by metal ions and glucocorticoids. Cell **37**:263–272.

26. **Schmidt, C. J., D. H. Hamer, and O. W. McBride.** 1984. Chromosomal localization of human metallothionein genes: implications for Menke's disease. Science **224**:1104–1106.

27. **Searle, P. F., B. L. Davidson, G. W. Stuart, T. M. Wilkie, G. Norstedt, and R. D. Palmiter.** 1984. Regulation, linkage and sequence of mouse metallothionein-I and II genes. Mol. Cell. Biol. **4**:1221–1230.

28. **Sequin, C., B. K. Felber, A. D. Carter, and D. H. Hamer.** 1984. Competition for cellular factors that activate metallothionein gene transcription. Nature (London) **312**:781–785.

29. **Shworak, N. W., F. F. Snyder, and L. Gedamu.** 1983. Identification of a cadmium binding protein from a cadmium resistant variant of human lymphoblastoid cell (WI-L2). Biochim. Biophys. Acta **763**:322–338.

30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

31. **Stuart, G. W., P. F. Searle, H. Y. Chen, R. L. Brinster, and R. D. Palmiter.** 1984. A 12-base pair DNA motif that is repeated several times in metallothionein gene promoter confers metal regulation to heterologous gene. Proc. Natl. Acad. Sci. USA **81**:7318–7322.

32. **Valerio, D., M. G. C. Duyvesteyn, B. M. M. Dekkar, G. Weeda, T. M. Berkvens, L. van der Voorn, H. van Ormondt, and A. J. van der Eb.** 1985. Adenosine deaminase: characterization and expression of a gene with remarkable promoter. EMBO J. **4**:437–443.

33. **Varshney, U., and L. Gedamu.** 1984. Human metallothionein MT-I and MT-II processed genes. Gene **31**:135–145.

34. **Varshney, U., D. I. Hoar, D. Starozik, and L. Gedamu.** 1984. A frequent restriction fragment length polymorphism in the human metallothionein-II processed gene region is evolutionarily conserved. Mol. Biol. Med. **2**:193–206.

35. **Waalwijk, C., and R. A. Flavell.** 1978. MspI, an isoschizomer of HpaII which leaves both methylated and unmethylated HpaII sites. Nucleic Acids Res. **5**:3231–3236.

36. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase focus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.