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MT-III, a brain-specific member of the metallothionein gene family

(Alzheimer disease/chromosome assignment/gene expression/gene sequence)

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ABSTRACT **A third member of the metallothionein (MT) gene family, designated MT-III, was cloned by virtue of its homology to a human protein that was shown previously to inhibit neuronal survival in culture and to be deficient in the brains of people with Alzheimer disease. Human and mouse MT-IIIs have two insertions relative to all other known mammalian MTs: a threonine after the fourth amino acid and a block of six amino acids near the carboxyl terminus. The genes encoding MT-III resemble all other mammalian MT genes in their small size and exon/intron organization. The MT-III genes are closely linked to the other functional MT genes on human chromosome 16 and mouse chromosome 8. Mouse MT-III gene expression appears to be restricted to brain; in addition, it fails to respond to zinc, cadmium, dexamethasone, or bacterial endotoxin *in vivo*, thereby distinguishing MT-III from other known MTs.**

Neurofibrillary tangles, which are paired helical arrays of modified τ proteins, are one of the characteristic features observed in brains of individuals with Alzheimer disease (AD). Uchida *et al.* (1) hypothesized that these tangles, which occur in neurites, might result from inappropriate control of a neuronal growth factor. In pursuing this hypothesis, they identified, and then purified and sequenced, a protein that inhibits survival of cultured rat neurons. That protein, which they called growth inhibitory factor (GIF), resembles metallothioneins (MTs) in that it is short (68 amino acids), contains 20 cysteine residues, and binds zinc and copper. Immunocytochemistry revealed that GIF is present in glial (astrocyte) cells of normal aged individuals but is depleted in individuals with AD (1). These observations suggested that this MT-like protein normally plays a role in preventing neuronal sprouting and development of neurofibrillary tangles, but they raise the tantalizing question of how this might occur.

The MT-like protein that Uchida *et al.* (1) sequenced differs from all other mammalian MTs in having two insertions of amino acids. The structure of the traditional MTs has been solved by NMR and x-ray crystallographic methods, which confirmed that 20 cysteines coordinate 7 metal atoms in a tetrahedral configuration (reviewed in ref. 2). Thus, one can view the structure as a metal–cysteine core with several short loops of amino acids connecting the cysteines. In this view, one of the loops of GIF would be extended by one amino acid, while another would be extended by six amino acids. Considering that traditional MTs do not substitute for GIF in their bioassay, the modified loops may be responsible for its particular function.

To gain additional insight into the relationship of this MT-like protein to traditional MTs, we cloned the mouse and human genes encoding these proteins‡ and compared them with traditional MT genes. This analysis revealed that these genes retain all the characteristics of the traditional MT genes, suggesting that they be designated MT-III rather than GIF.

MATERIALS AND METHODS

Isolation of Mouse MT-III (mMT-III) cDNA Clones. cDNA was made from mouse cortex RNA by using reverse transcriptase and oligo(dT) as a primer. An aliquot of the sample was then amplified by PCR with 40 cycles (95°C for 1 min, 32°C for 1 min, and 72°C for 1 min) using degenerate oligonucleotides 323 and 324. The products were electrophoresed on 2% NuSieve (FMC BioProducts)/0.8% agarose, and a prominent band corresponding to about 180 base pairs (bp) was isolated and inserted into the *Hinc*II site of pUC18. A MT-like clone was identified by sequencing the inserts from several clones. A Stratagene λ ZAP mouse brain cDNA library was then screened with the cDNA insert described above. Five positive clones were plaque-purified, and the inserts were rescued as plasmids by using the Stratagene protocol and sequenced.

Screening Genomic Libraries. An amplified λ library (generously provided by Anton Berns, The Netherlands Cancer Institute, Amsterdam) was made by inserting a partial *Sau*3A digest of mouse strain 129 liver DNA into the *Xho* I site of LambdaGEM-12 (Promega) after partially filling in the ends with two nucleotides by using DNA polymerase. About 10^6 phage were screened with a radioactive probe (3×10^8 cpm) made from the PCR-amplified mMT-III cDNA. Positive plaques were purified and λ phage were isolated by CsCl banding. The entire 15-kilobase (kb) insert and several smaller fragments were subcloned in pBluescript (Stratagene) for restriction site mapping. A partial *Sau*3A digest of human DNA was inserted into the *Bam*HI site of pWE15 (Stratagene) under the conditions recommended by Little (3). About 260,000 cosmid clones were plated and duplicate lifts were screened as above. After colony purification of a number of positives, cosmid DNA was isolated by CsCl banding and various restriction fragments were subcloned in pBluescript for restriction site mapping.

Primer Extension of mMT-III mRNA. Total nucleic acids (2.9 mg) were isolated from two mouse brains by SDS/proteinase K digestion followed by phenol/chloroform extraction. The nucleic acids were dissolved in 800 μl of DNase I buffer and digested with 40 units of RNase-free DNase for 5 min at 24°C. Then SDS and proteinase K were added and the mixture was incubated for 20 min at 45°C. This was followed by the addition of (*i*) 75 μg of single-stranded DNA from M13 phage carrying a genomic 950-bp *Eco*RI–*Bst*EII insert including the sequence complementary to exon 3 of mMT-III mRNA, (*ii*) 3.5×10^6 cpm of end-labeled oligonu-

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Abbreviations: MT, metallothionein; mMT and hMT, mouse and human MTs; GIF, growth inhibitory factor; AD, Alzheimer disease; MRE, metal regulatory element.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M93310 for mouse and M93311 for human).

cleotide 335 or 338 (complementary to exons 1 and 2 of mMT-III mRNA, respectively, and (*iii*) 200 μl of 3 M NaCl and 120 μl of formamide. The mixture was heated to 100°C for 10 min and then incubated at 45°C overnight. The sample was then passed through a Bio-Gel A-50m (Bio-Rad) column (1 × 40 cm) equilibrated in 0.15 M NaCl/0.1% SDS, and the fractions (void volume) that included the M13/mRNA/oligonucleotide hybrids were identified and precipitated with 2 vol of ethanol. The hybrids were dissolved in 10 mM Tris·HCl, pH 7.5/0.25 mM EDTA, and aliquots were added to reverse transcriptase buffer along with 10 units of reverse transcriptase from avian myeloblastosis virus and incubated for 30 min at 42°C. The products were electrophoresed on DNA sequencing gels.

MT-III mRNA Quantitation. Total nucleic acids were isolated and hybridized overnight with end-labeled oligonucleotide 338 and treated with S1 nuclease, and the S1-resistant oligonucleotides were collected on Whatman GF/C filters after precipitation with trichloroacetic acid as described (4). An M13 clone carrying the sense DNA strand of mMT-III served as a hybridization standard (4).

DNA Sequencing. Nucleotide sequences of genomic and cDNA subclones of mMT-III and human MT-III (hMT-III) were determined by the dideoxy chain-termination method using synthetic oligonucleotide primers and the Sequenase kit (United States Biochemical) according to the supplier's instructions. The sequence was obtained from both strands; regions of ambiguity were resolved by using 7-deaza-dITP instead of dGTP, or in some cases by tailing the reaction products with terminal deoxynucleotide transferase.

Oligonucleotides. The degenerate (R = both purine nucleotides, Y = both pyrimidine nucleotides, N = all four nucleotides) oligonucleotides 323 and 324 were used for PCR amplification of mMT-III from a brain cDNA library. Oligonucleotides 335 and 338 are complementary to exons 1 and 2, respectively; they were used for primer extension and mRNA quantitation; their locations are indicated in Fig. 1. Oligonucleotide 346 was used to measure MT-I mRNA. Sequences: no. 323, 5′-ATGGAYCCNGARAC; no. 324, 5′-CAYTTYTCNGCYTC; no. 335, 5′-ACAGGGGCAGGTCTC; no. 338, 5′-GCACTTGCATTTGTCCGAGCAG; and no. 346, 5′-CCATTCCGAGATCTGGTGAAG.

Fluorescence *in Situ* Hybridization. Metaphase chromosome spreads and G_1 interphase cells were prepared by standard procedures (5, 6). MT-I and MT-III DNA probes were labeled by nick-translation with either biotin-11-dUTP (Sigma) or digoxigenin-11-dUTP (Boehringer Mannheim). A biotinylated centromere probe specific to chromosome 16 (16DZ2) was obtained from Oncor (Gaithersburg, MD). Denaturation of chromosomes, hybridization, and single-color detection were performed as described by Pinkel *et al.* (7) and modified by Kievits *et al.* (8), except that the hybridization was performed in 65% (vol/vol) formamide/10% dextran sulfate/2× SSC and the posthybridization washes were with 65% formamide/2× SSC at 42°C and then 0.1× SSC at 55°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Simultaneous detection of biotinylated and digoxigenated probes with Texas red- or fluorescein isothiocyanate (FITC)-labeled antibodies was performed essentially as described (9). Interphase mapping was done essentially as described (6) except that the images were viewed on a table-top projector and the distances between signals were measured with a 10× scale loupe.

RESULTS

Cloning Mouse and Human MT-III. On the basis of the protein sequence of human GIF (1), which we refer to as hMT-III, we designed two degenerate oligonucleotide 14-mers (see *Materials and Methods*) complementary to regions

A MOUSE

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AGGCTCACTGCTCAGCATCCCGTTTGGACCAAACTGATCAAGACAATCTGGGAGAAGAGG   60
GAGAAGAACCTACGGGAAGGGGCAATACTAATTTGTCTCTCTCAACTTGCAAAGATGGTA  120
CTGCGCAGGCACCTTCAGGGAGACGGCTGCAGTGGCAAGGAGTGGACAGCGGACAGGCTA  180
CTTTGGTCCTACACTCAGTGGAGACTCGGGACAGCAGTGCACACACACGGAGAGCAGGCG  240
CTGTGCGTGCATAGGGGCGGGCGCCAAGTCGCTGCTTGCGCGCCCCCGCCTGGGGCTATA  300
AAAGCCTTGCCACCTGCTGCCCTGGCTACGTAGCGCATCCGCTTGCCGGGAGGAACCAAG  360
                          ☞   ←——————————————335
                    323——————————————→
CTACGGCGGCTGCTGGACTGGATATGGACCCTGAGACCTGCCCCTGTCCTACTGGTGAGC  420
                         MetAspProGluThrCysProCysProThrG

CCCTTCCCCCTCCTGCAGCACTTTGCCCTTTCCTGGCAAAGAACCCACTCCTCTGTCTTC  480
ACTCAAGGACATTTGGGGGAGGAGTCCCTTCCCCCTACCCCCATCTTTAACCCTGTGATG  540
ATGATAATCTTCATTTAGGCATGGGGACGCCAGGTTTCCCTAGTATAATTCTTCGTGTGC  600
                     ←————————————————————————338
TCTCTTAGGTGGTTCCTGCACCTGCTCGGACAAATGCAAGTGCAAGGGCTGCAAATGCAC  660
        lyGlySerCysThrCysSerAspLysCysLysCysLysGlyCysLysCysTh

GAACTGCAAGAAGAGTGAGTGCACCCCCCCACCCCCAACCCCTGCCATAACCTCCCGCCG  720
rAsnCysLysLysS

CGCCCACCCCACCCCCACCAGACACTATGAAGCAAAGCTTCCTGCTGCAGACCTTCAGAT  780/
............................................................
AGCCTCCCATACTGCTTTCCTCTGCCCTTTGATGGAGACAGATGCCGACATCAGACTGGG  /1440

CACATATGTGCGCCCGCGCACACACACACACATTTTATCTACAGGCTGCTGCTCCTGCTG  1500
                                              erCysCysSerCysCy

CCCTGCCGGATGTGAGAAGTGTGCCAAGGACTGTGTGTGCAAAGGTGAAGAGGGGGCCAA  1560
sProAlaGlyCysGluLysCysAlaLysAspCysValCysLysGlyGluGluGlyAlaLy
     ←——————————324
GGCAGAGGCCGAGAAATGCAGCTGCTGCCAGTGAGGACCCAGACCCTCCCACACAGCCTA  1620
sAlaGluAlaGluLysCysSerCysCysGln---

TGTAAATAGTGCTGGGTGTCCCTGGTGGGGCACAACTGTTGTCTTCCCCCCCCCCCCCCC  1680
                                                    ✂
CCCCCCGCCGGCTGCCTGCTCCGGGGTGTGAATAAATCCCATGCACAACATGAACCCAAG  1740
ACTGGTCTCTTTTTCAAGTGCGAAGGATGTGGAAGGGTGGGGGAGGCCACTCAAGCCGGA  1800
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B HUMAN

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GCACGTCCCCTGCGGGACCCACGCGGGGAGTGGGCTGGCAGTGCGCGCATAGCGGCGGCG   60
AGTGGGTCGTGCACGCGGATGCGGGGTGGGAGTGGGGGCGCACGCGCGGGCGTGGGCGAG  120
CGGGCCCCGGCAGTGCACACACACGGCAGGGGCGGGCGACAGATGCAGTGCGTGCGCCGG  300
AGCCCAAGCGCACAAACGGAAAGAGCGGGCGCGGTGCGCAGGGGCGGGCGCCCAGCGGGC  240
TTGGCATGCGCGCCCCCGCCCGAGGCTATAAAAGCATCGCCACCTGCTGCCACTAGCCAA  300
                                                       ☞

GCCGCGCGTCCAGTTGCTTGGAGAAGCCCGTTCACCGCCTCCAGCTGCTGCTCTCCTCGA  360

CATGGACCCTGAGACCTGCCCCTGCCCTTCTGGTGAGCCCCCGCCCCCGCTCGCATCCTG  420
 MetAspProGluThrCysProCysProSerG

CGCACTGCGCGCCCTTGTACCTGCAAAGAAACCCACGCCCTGCGCCTTCGCTCAAGGACA  480
CTTGGGGGAAGGGCCCCTGATTCCCTATTCTTCACCTCGTGAAGGGCGGGCATGCCTGTG  540
TCGCGGAGAACAGGGAGACTTGGCACCCCATCTCCTCGTGACAGGCGTGGGGACCCGAGT  600

TCGTCCACATTAACCCTTCCTGTGGCGTCGCCCTCTCTAGGTGGCTCCTGCACCTGCGCG  660
                                              lyGlySerCysThrCysAla

GACTCCTGCAAGTGCGAGGGATGCAAATGCACCTCCTGCAAGAAGAGTGAGTGCGGGGAC  720
AspSerCysLysCysGluGlyCysLysCysThrSerCysLysLysS

CCTTCCCCTCTGCCGCCGCCCCTCTGCTCTGCGGAGTCGGTGTCTCACCACGCAGGATG  780/
............................................................
GGCTGAATGAACCAGGATGACTCCCCACCCAGCACCCTTCCCTCCCCTTTGATGGGGACG  /1560

AATTGGGGGAGTGTGCATCAGAGAGTGGTCATCTTCCATTTTATCTGCAGGCTGCTGCTC  1620
                                                     erCysCysSe

CTGCTGCCCTGCGGAGTGTGAGAAGTGTGCCAAGGACTGTGTGTGCAAAGGCGGAGAGGC  1680
rCysCysProAlaGluCysGluLysCysAlaLysAspCysValCysLysGlyGlyGluAl

AGCTGAGGCAGAAGCAGAGAAGTGCAGCTGCTGCCAGTGAGAAGGCACCCCTCCGTGTGG  1740
aAlaGluAlaGluAlaGluLysCysSerCysCysGln---

AGCACGTGGAGATAGTGCCAGGTGGCTCAGTGCCACCTATGCCTGTGGTGAAGTGTGGCT  1800
                                                          ✂
GGTGTCCCCTTCCCCTGCTGACCTTGGAGGAATGACAATAAATCCCATGAACAGCATGAG  1860
CCAAGGACTGGTCTCTTCTTAAAGGGGGGAAGGATGTGGAGCAGTGGGGGAGCCTATTCC  1920
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FIG. 1. Genomic sequences of mouse (*A*) and human (*B*) MT-III. For the mouse sequence the transcription start site is indicated by the hand, and the site of polyadenylylation is indicated by scissors. The TATAAA and AATAAA sequences are in underlined italics. The three-letter amino acid symbols are written below the coding region. The bold Gs in the 5′ untranslated and promoter regions of the mouse sequence indicate the 5′ ends of the cDNA clones. The metal regulatory elements (MREs) are underlined. The numbered arrows indicate the location of the oligonucleotides used for PCR, primer extension, and mRNA determination; they lie above the relevant sequence.

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                          1    1       2                                       1    1  1          1    2
mMT-II   MDPN CSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKE         ASDKCSCCA 61
mMT-I    MDPN CSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG         AADKCTCCA 61
mMT-III  MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ 68
hMT-III  MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ 68
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FIG. 2. Alignment of mouse MT-I, MT-II, and MT-III proteins and comparison with hMT-III. The numbers indicate whether the mMT-III protein sequence is more like MT-II (2) or MT-I (1); bold letters indicate differences between MT-III and both MT-I and MT-II. Underlines indicate differences between human and mouse MT-III.

that were most disparate from MT-I or MT-II and tried to amplify the human and mouse genes directly from genomic DNA by PCR. When this approach failed to give expected products, we made cDNA from mouse brain by using reverse transcriptase with oligo(dT) as a primer and then amplified the cDNA by PCR using the same degenerate primers. Several visible bands were observed by ethidium bromide staining, one of which was the size expected for the mouse counterpart of hMT-III. DNA in this band was isolated and cloned in pBluescript. Three out of 10 colonies with inserts had a MT-like sequence similar to hMT-III. The insert from one of these positive clones was then used to screen about 10^6 clones of a commercial, amplified mouse brain cDNA library; this procedure resulted in about 250 positive clones, 5 of which were plaque purified. The sequences of these five clones were identical to the PCR product between the degenerate oligonucleotide primers. They all extended in the 3′ direction to a poly(A) tail of variable length and included a poly(C) tract in the 3′ untranslated region of 17–24 nucleotides. All five cDNAs included the presumptive translation start site and extended variable lengths further 5′.

The insert from the PCR-amplified clone was also used to screen mouse and human genomic libraries in Lambda-GEM-12 and pWE15, respectively. All five clones identified after screening 10^6 mouse λ phage were identical, whereas only one of five clones that was purified from the human cosmid library gave a strong hybridization signal and was pursued. Restriction fragments of about 2.5 kb that included all the sequence homologous to the cDNA were subcloned, restriction mapped, and then sequenced (Fig. 1).

Comparison of Mouse and Human MT-III. The hMT-III gene encodes a 68-amino acid protein identical to that sequenced by Uchida *et al.* (1), and the protein predicted from the mouse sequence is clearly homologous to the human protein (Fig. 2). The coding regions of the mouse and human MT-IIIs are 74% identical at the nucleotide level and 61 of 68 amino acids are identical. The coding regions of mouse and human MT-III are interrupted by two introns (Fig. 1) that are in the same positions relative to the conserved cysteines as they are in all other mammalian MTs (2). The 3′ end of mouse exon 3 can be deduced from the location of the poly(A) tract in the cDNAs; the 3′ end of hMT-III was assigned by homology to the mouse sequence. The 3′ untranslated regions of mouse and human MT-III bear little similarity except in the vicinity of the AATAAA polyadenylylation site. A poly(C) tract that ranges from 17 to 24 nucleotides in the five cDNAs that we sequenced is present in the 3′ untranslated region of the genomic sequence of mMT-III as 21 C residues, but it is absent from hMT-III.

To identify the transcription start site of mMT-III we used a reverse transcriptase primer extension assay. Because preliminary experiments indicated that this mRNA was relatively rare, MT-III mRNA was selectively enriched from total brain nucleic acid by annealing it with a single-stranded M13 DNA complementary to exon 3. Excess ^{32}P-labeled oligonucleotides complementary to exons 1 or 2 were also present. After the annealing reaction, the M13/mRNA/primer hybrids were separated from the bulk of the RNA and primer by agarose gel filtration and concentrated by ethanol precipitation. The primer was then extended by reverse transcription and the products were resolved on a sequencing gel with a DNA sequence ladder generated with the same primer. Fig. 3 shows that there was a strong band flanked by fainter bands when the primer from exon 1 was used. The strong band corresponds to a C located 32 nucleotides downstream of the first T of an obvious TATAAA element. We suspect that transcription initiates at the preceding A and that the cap structure of the mRNA accounts for the staggered ends. The same start site was predicted from reverse transcription using a primer in exon 2 (data not shown). No bands larger than the triplet shown in Fig. 3 were observed. Surprisingly, two of the five cDNA clones extended further 5′, while the other three ended 20 or 21 nucleotides downstream of the proposed transcription start site. No reverse transcriptase stops were noted in these regions with either primer. The 5′ end of hMT-III was assigned by alignment of the conserved TATA boxes. There is very little sequence identity in the 5′ untranslated regions of mouse and human MT-III (Fig. 1).

The promoter regions of mouse and human MT-III bear little direct sequence identity, as is the case in comparison of MT-I and MT-II genes within or between species. However, a search for cis-acting MREs, with the consensus TGCRCNCR, that typically confer responsiveness to certain heavy metals reveals several in the 400 bp 5′ of the transcription start sites; see Fig. 1 (2, 10).

Comparison of MT-III with MT-II and MT-I. The coding regions of the three mouse MTs are identical at 118 of 183 nucleotide positions (Fig. 4). At 14 of 24 additional positions, mMT-III matches mMT-II, while it matches mMT-I at the other 10 positions. However, at the protein level, only two of eight informative amino acid comparisons reveal identity of MT-III with MT-II, whereas six of these eight are identical to MT-I (Figs. 2 and 4). In comparisons of hMT-III with other MTs, MT-III is more similar to MT-II at the protein level (1).

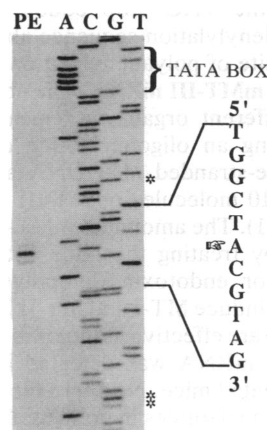

FIG. 3. Primer extension analysis of mMT-III mRNA. Oligonucleotide 335 was annealed with enriched mMT-III mRNA and extended with reverse transcriptase. The same oligonucleotide was used for dideoxy DNA sequencing from an M13 subclone. The primer extension (PE) products and sequencing reaction products (A, C, G, T) were electrophoresed on a 6% acrylamide gel, and the gel was fixed, dried, and exposed to Hyperfilm-MP (Amersham) for 2 days. The sequence at the transcription start site is shown and a hand indicates the most likely start site; the TATA box is indicated and asterisks mark the 5′ termini of three of the cDNA clones.

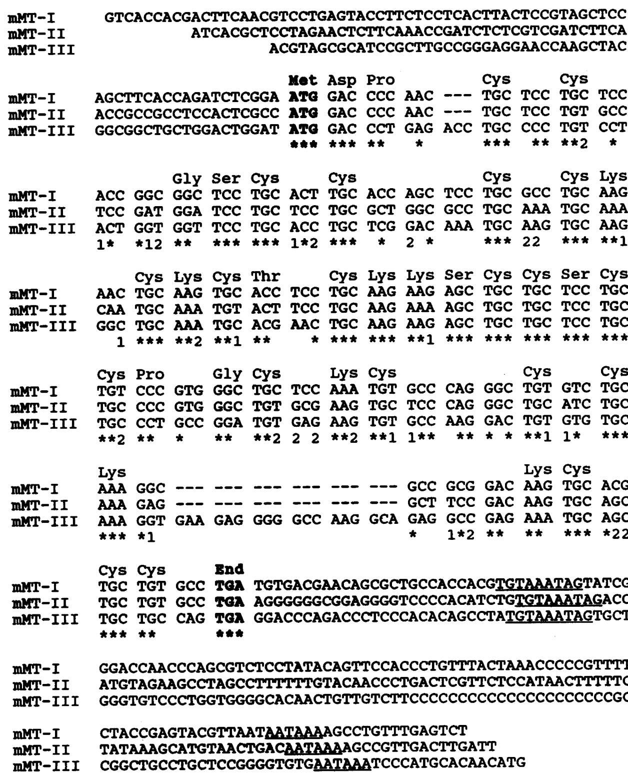

FIG. 4. Comparison of mMT-I, mMT-II, and mMT-III mRNAs and proteins. The DNA sequences corresponding to full-length mRNA deduced from cDNA clones and primer extension of mMT-III are compared with those previously published for mMT-I and mMT-II (11). *, Nucleotides in the coding region that are identical in all three sequences; 1, nucleotides in mMT-III that match mMT-I; 2, nucleotides in mMT-III that match mMT-II. Amino acids that are conserved among all three open reading frames are indicated in three-letter code. Two conserved sequences in the 3′ untranslated regions are underlined.

Thus, it is difficult to decide whether MT-III is evolutionarily closer to MT-I or MT-II. Outside the coding region there is very little identity of these three mRNAs, with the exception of a conserved TGTAAATAG sequence about 25 nucleotides downstream of the TAG stop codon and a conserved AATAAA polyadenylylation sequence about 16 nucleotides upstream of the site of polyadenylylation (Fig. 4).

Quantitation of mMT-III mRNA. The abundance of mMT-III mRNA in different organs was measured by solution hybridization using an oligonucleotide complementary to exon 2 and single-stranded M13 DNA as a standard. We detected about 110 molecules of MT-III mRNA per cell in total brain (Table 1). The amount of mMT-III mRNA in brain was unaffected by treating the mice with zinc, cadmium, dexamethasone, or endotoxin (lipopolysaccaride). All of these compounds induce MT-I and MT-II in most organs, but only the latter two are effective inducers in brain (Table 1; ref. 11). No mMT-III mRNA was detected in other organs of control or zinc-treated mice. Northern blots revealed a weak band of about 500 nucleotides in brain total RNA samples but not in total RNA from several other organs (data not shown).

Chromosomal Location of MT-III Genes. Mouse MT-I and MT-II genes are about 6 kb apart on chromosome 8 (11). Likewise, 13 hMT-I genes and the hMT-II_A gene are clustered on human chromosome 16 (12). There is synteny with several other genetic loci in these regions of mouse chromosome 8 and human chromosome 16 as well (13). Preliminary fluorescence *in situ* hybridization to metaphase spreads suggested that the hMT-III gene was located on chromosome 16. This result was confirmed by cohybridization of hMT-III cosmid with a centromere probe specific to chromosome 16 (Fig. 5*A*). The location of hMT-III signal relative to the centromere was the same as for hMT-I_G (Fig. 5*B*). Two-color interphase nuclear mapping was performed using biotin-labeled hMT-III and digoxigenin-labeled hMT-I_G probes. From a random selection of 36 nuclei, the two signals were closely apposed in 21 cases, overlapping in 5, and superimposed in 10. Calculation of the mean distance between the signals as described by Trask *et al.* (6) suggests that the two genes are less than 85 kb apart. We have also shown that the MT-III genes are coamplified with the MT-I genes in cadmium-resistant mouse (S180 and Hepa 1A) or human (HeLa) cells (14), which would not be expected unless the genes were closely linked because the MT-III genes are not expressed in these cells (data not shown).

Table 1. MT mRNA levels in mouse brain and other organs

Organ	Treatment*	mRNA molecules per cell†	
		MT-III	MT-I
Brain	None	105 ± 3 (7)	141 ± 8 (2)
	Cd	106 ± 2 (4)	154 ± 24 (2)
	Zn	100 ± 8 (2)	116 ± 7 (2)
	Dex	119 ± 3 (4)	246 ± 16 (2)
	LPS	113 ± 3 (2)	364 ± 10 (2)
Other organs‡	Zn	0	up to 2,500

*Mice were given 25 mM $ZnSO_4$ in their drinking water and organs were collected 6 days later, or they were injected intraperitoneally with 1 mg of $CdSO_4$/kg body weight, 5 mg of dexamethasone (Dex)/kg, or 3 mg of lipopolysaccharide (LPS)/kg and organs were collected 4–6 hr later.

†MT-III and MT-I mRNAs were measured by solution hybridization using oligonucleotide 338 or 346, respectively, and M13 DNA standards (4). Values are mean ± SEM; the number of samples is shown in parenthesis. MT-I was measured on some of the same samples as were used for MT-III.

‡Other organs tested were adult liver (with each of the treatments listed above), fetal liver (day 16), and adult pancreas, intestine, kidney, heart, lung, testis, and ovary from mice with $ZnSO_4$ in their drinking water.

DISCUSSION

MT-I, MT-II, and MT-III genes are of similar size, share identical exon/intron boundaries, and are clustered on the same chromosome. The coding regions are 65% identical at the nucleic acid level, but the 5′ and 3′ untranslated regions, introns, and promoters bear little similarity. The proteins

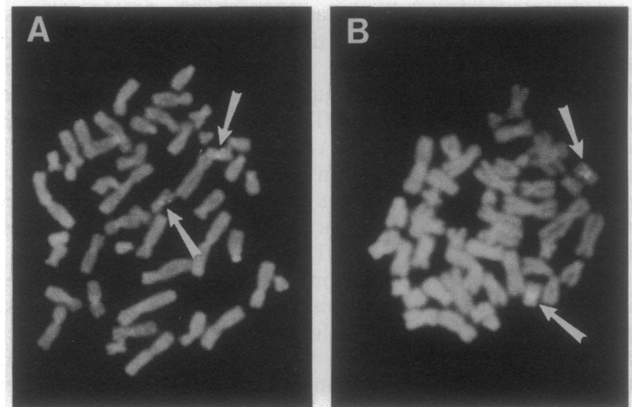

FIG. 5. Fluorescence *in situ* hybridization mapping of hMT-III. (*A*) Cohybridization of hMT-III cosmid and D16Z2 centromere probe to a metaphase spread from primary human embryo lung cells. The centromere signals are close together; the MT-III signals are on the long arms of chromosome 16 (arrows). (*B*) Cohybridization of hMT-I_G and D16Z2.

have characteristic spacing of cysteines, which coordinate seven metal atoms, and they lack aromatic amino acids. These data suggest that a triplication of an ancestral MT gene gave rise to precursors of contemporary MT-I, MT-II, and MT-III genes. After triplication, the MT-III gene acquired the two insertions to generate a polypeptide of 68 amino acids rather than 61. At the protein level, mMT-III is more like mMT-I than mMT-II, whereas hMT-III is more like hMT-II. In comparing the coding regions at the nucleic acid level, they are all approximately equally diverged from each other, which supports the triplication hypothesis. The 6-amino acid insertion in the third exon of MT-III is not conserved between mouse and human; only 3 of the 6 residues are identical, which suggests that if this loop is critical for MT-III function, then its structure may be more important than its exact sequence.

Chromosome mapping indicates that all the known functional MT genes are closely linked on mouse chromosome 8 and human chromosome 16. No physical linkage of mouse or human MT-III to other members of the family has been demonstrated yet, but the proximity of the fluorescent labels and the fact that the genes are coamplified in cadmium-resistant cells suggest that they are within 85 kb of each other. Restriction mapping of large DNA fragments separated by pulsed-field gel electrophoresis or analysis of yeast artificial chromosome clones should clarify the chromosomal arrangement of these genes.

We have recently shown that DNA flanking the MT-I and MT-II genes confers copy-number-dependent and position-independent gene expression to a marked MT-I gene in transgenic mice and has led us to conclude that these DNA regions create a functional chromosomal domain. The existence of a linked MT-III gene raises the question of whether it exists in an adjacent chromosomal domain or whether there is one large MT chromosomal domain that is more complex than we initially envisioned. Further mapping studies should help resolve this issue.

Mouse MT-III expression appears to be restricted to brain, whereas MT-I and MT-II are expressed ubiquitously. Immunocytochemical data indicate that MT-III is expressed in astrocytes (1). It will be interesting to determine whether glial cells in the periphery also express MT-III. The restricted expression of MT-III may reflect its organization into a separate chromosomal domain. In rodents, the MT-I and MT-II genes are regulated coordinately in many organs by a variety of metals and mediators of physiological stress, including glucocorticoids and interleukins (11, 15). Some of the cis-acting elements responsible for transcriptional regulation have been defined genetically and biochemically (2, 16). All mammalian MT genes have several MREs in the promoter region, although their position and orientation vary from one gene to another. Likewise, the MT-III genes have several MREs that agree with the consensus sequence. The fact that none of the MT genes responds to zinc or cadmium in brain may reflect physiological mechanisms that protect the brain from elevated levels of metals in the blood. MT-III gene expression was also unaffected by hormones that do induce MT-I and MT-II in brain (Table 1).

A fascinating aspect of hMT-III is its function in regulating neuronal survival in culture and its depletion in people with AD. Uchida *et al.* (1) showed that brain extracts from people with AD stimulated neuronal survival in culture much better than extracts from normal aged individuals, and this led to their discovery that normal extracts have high levels of an inhibitor, which turned out to be MT-III. Furthermore, addition of purified MT-III to AD extracts inhibited neuronal survival, and this inhibition could be blocked by an antibody directed against MT-III (1). MT-I and MT-II were ineffective in this assay. A puzzling aspect of these observations is that MT-III apparently functions in the culture medium but MTs are generally thought to be cytoplasmic proteins because they have no signal peptides or other hydrophobic regions that might be involved in secretion, although low levels of MTs have been detected in blood (17). The sequence of MT-III genes does not provide any insight into how MT-III might be released from the cell. Perhaps MTs are released only from dying cells; alternatively, they may exit the cell in association with some carrier protein.

The amplification of MT-I and MT-II genes in cadmium-resistant cells provides strong genetic evidence that these proteins can function in metal detoxification (2, 15). Their ability to sequester essential metals such as zinc and copper supports their role in metal homeostasis. However, the fact that MTs are not expressed in certain cell lines argues that they are not essential for synthesis of zinc or copper metalloenzymes. The results of Uchida *et al.* (1) suggest that we should also consider roles of MTs in cell–cell interaction. The MT-III genes described here will facilitate a variety of genetic experiments in cells and transgenic animals that may provide further insight into MT-III function. These experiments may also provide new perspectives on MT-I and MT-II function. The fact that MT-III gene was not detected by cross-hybridization with MT-I or MT-II gene probes raises the possibility that yet other specialized MTs exist in the mammalian genome.

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