Induction of a New Metallothionein Isoform (MT-IV) Occurs during Differentiation of Stratified Squamous Epithelia[†,‡]

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ABSTRACT: A new member of the metallothionein (MT) gene family was discovered that lies about 20 kb 5′ of the MT-III gene in both mouse and human. The MT-IV proteins are highly conserved in both species and have a glutamate insertion at position 5 relative to the classical MT-I and MT-II proteins. Murine MT-IV mRNA appears to be expressed exclusively in stratified squamous epithelia associated with oral epithelia, esophagus, upper stomach, tail, footpads, and neonatal skin. The MT derived from tongue epithelium contains both zinc and copper. Many of these epithelia develop parakaratosis during zinc deficiency in the rat. *In situ* hybridization reveals intense labeling of MT-IV mRNA in the differentiating spinous layer of cornified epithelia, whereas MT-I is expressed predominantly in the basal, proliferative layer; thus, there is a switch in MT isoform synthesis during differentiation of these epithelia. We suggest that MT-IV plays a special role in regulating zinc metabolism during the differentiation of stratified epithelia.

Mammalian metallothioneins (MTs) were traditionally subdivided into two classes: MT-I and MT-II. They both contain 61 amino acids but can be resolved by ion-exchange chromatography. These MTs contain 20 cysteines in identical positions that coordinate various heavy metals, including the essential metals zinc and copper (Kägi & Kojima, 1987). The functions of MTs are not established, but the isoforms are thought to have similar properties. They have been shown to protect against heavy metal toxicity in cultured cells (Durnam & Palmiter, 1987), and they may play a role in the homeostasis of essential metals *in vivo* (Bremner & Beattie, 1990). In the mouse, the MT-I and MT-II genes are about 6 kb apart on chromosome 8, and the two genes are expressed coordinately in most organs of the body (Searle et al., 1984; Yagle & Palmiter, 1985). In human, there is one MT-II gene and a cluster of 13 closely-linked MT-I genes on chromosome 16 (West et al., 1990). The human MT genes appear to be differentially expressed in various organs and established cell lines (Hamer, 1986). More recently, a third, brain-specific MT was discovered by virtue of its ability to inhibit survival of cultured neurons (Uchida et al., 1991). That MT gene, designated MT-III, also maps close to the other MTs on mouse chromosome 8 and human chromosome 16, but physical linkage to the other genes was not established (Palmiter et al., 1992). In the process of characterizing the cosmid carrying the human MT-III gene, we discovered another sequence that cross-hybridized with MT cDNAs. That sequence turns out to represent a new member of the MT gene family, which we designate MT-IV. We discovered a similar gene upstream of the murine MT-III gene and in the process linked all four of the murine MT genes. It was previously thought that all the MT genes in the mouse and human had been identified; thus, we did not know whether this new gene was functional and, if so, where it would be expressed.

Our analysis of MT-IV expression in the mouse revealed that it is restricted to cornified, stratified, squamous epithelium, a tissue that provides a protective surface on skin, tongue, the upper part of the alimentary tract, and the vagina of rodents (Gude et al., 1982). Differentiation of stratified epithelium involves movement of keratinocytes out of the basal, proliferative layer into the overlying region where synthesis of unique cytoskeletal proteins begins (Stenn, 1988; Fuchs, 1990; Holbrook, 1991). As differentiation proceeds, the cells are pushed toward the outermost stratum corneum where they become flattened, cytoskeleton-filled cellular ghosts. We show that differentiation of this tissue involves a switch in expression of MT-I in the basal layer to MT-IV expression in the next layer, the stratum spinosum. This switch occurs in the same cells as the well-characterized switch in keratin isoforms (Sun et al., 1984; Ouhayoun et al., 1985; O'Guin et al., 1990). The switch in MT isoform synthesis suggests that MT-IV may play a unique role in the differentiation of this specialized epithelia.

MATERIALS AND METHODS

Preparation and Screening of Mouse Cosmids. A partial *Sau*3A digest of DNA from cadmium-resistant hepalA cells (Durnam & Palmiter, 1984) was centrifuged on a sucrose gradient; fractions containing DNA of about 40 kb were isolated, ligated with a *Bam*HI-cut cosmid vector, pWE 15 (Stratagene), and introduced into bacteria as recommended by Little (1987). About 38 000 clones (on four 15-cm plates) were screened with unique probes from the ends of DNA inserts from λ clones containing mMT-I, mMT-II, and mMT-III genes. Unique probes from the ends of these cosmids were isolated to continue the chromosomal walk. Because the MT locus is amplified about 30-fold in these Hepa 1A cells, there were several positive clones per filter.

DNA Sequencing. Various restriction fragments from the cosmids were subcloned into Bluescript or M13 for sequencing by the dideoxy chain termination method. Both strands were

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[‡] The human and mouse MT-IV gene sequences have been submitted to GenBank under Accession Numbers U07807 and U07808, respectively.

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sequenced, and ambiguities were resolved with 7-deaza-dITP instead of dGTP, or in somes cases by tailing the reaction products with terminal transferase.

Primer Extension and S_1 Nuclease Mapping. MT-IV mRNA was enriched from tongue total nucleic acids by hybridization to single-stranded M13 DNA complementary to exon 3 (Palmiter et al., 1992). For primer extension, an end-labeled primer (387, see below) was extended with reverse transcriptase, and the products were electrophoresed on an acrylamide gel next to a sequencing ladder produced with the same primer. To map the 3′ end, a 300 bp *Ava*I fragment spanning the two potential polyadenylation sites was labeled by filling in the ends with DNA polymerase with [α-^{32}P]-dCTP and three unlabeled dNTPs. It was denatured and hybridized with enriched RNA under conditions that favored RNA–DNA hybrids (0.6 M NaCl, 75% formamide, 50 °C). The reaction was diluted into S_1 nuclease buffer and treated with varying amounts of S_1 nuclease, and the products were run on a gel next to a sequencing ladder generated with a primer that abuts the *Ava*I site in exon 3.

Solution Hybridization. C57Bl mice were killed under ether/CO_2 anesthesia and tissues removed, placed on dry ice, and then stored at −70 °C. Total nucleic acids were isolated as described by Durnam and Palmiter (1983). Briefly, frozen tissue was homogenized in 1× SET buffer (1% sodium dodecyl sulfate, 10 mM Tris-HCl, and 5 mM EDTA, pH 7.5) containing 200 μg/mL proteinase K. After incubation at 45 °C for 1 h, samples were extracted with phenol and chloroform, followed by chloroform alone. The nucleic acids were precipitated with ethanol, the pellet was washed with ethanol and then dissolved in 0.2× SET. The epithelium was removed from the tongue by placing the organ in phosphate-buffered saline (PBS) at 65 °C for 15–20 s and then quickly peeling it from the underlying tissue. The amount of MT-I and MT-IV mRNA was determined by solution hybridization using end-labeled oligonucleotide probes essentially as described (Durnam & Palmiter, 1983) except the formamide concentration was reduced to 10% and hybridization and S_1 nuclease treatments were both at 45 °C. Complementary single-stranded M13 standards were used to estimate the absolute amount of mRNA, and the values are presented as molecules of mRNA per cell, assuming 6.4 pg of DNA/cell (Durnam & Palmiter, 1983). For MT-I, oligonucleotide 57 (5′-GAAAACGGGGGTTTAGTAAACAGGG-3′), which is complementary to the 3′ untranslated region, was used; for MT-IV, oligonucleotide 387 (5′-GGTGTGTGAGCAGCTAA-GAAAGGG-3′), which is complementary to the 5′ untranslated region, was used.

In Situ Hybridization. In situ hybridization and autoradiography were performed essentially as described by Marks et al. (1992). In brief, tissues were placed in PBS containing 4% paraformaldehyde at 4 °C for 4–5 h, rinsed, and soaked overnight at 4 °C in PBS. The tissues were dehydrated, transferred into xylenes, and then embedded in paraplast x-tra (Oxford Labware, St. Louis, MO) according to standard techniques (Sheehan & Hrapchak, 1980). Five-micrometer sections were cut from the paraffin blocks and placed on RNase-free microscope slides which had been dipped in 2% 3-aminopropyltriethoxysilane (Sigma) in acetone, followed by three quick changes of distilled H_2O (Rentrop et al., 1986). The sections were air-dried overnight at 37 °C, deparaffinized and hydrated under RNase-free conditions, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 9.5), followed by incubation in 0.2 M Tris/0.1 M glycine (pH 7.5) for 10 min. The slides were then rinsed in PBS, excess liquid was removed, and then 16 μL of hybridization mix [50% formamide, 1× Denhardt's solution, 10% poly(ethylene glycol) 6000, 200 mM dithiothreitol, 500 μg/mL yeast tRNA, 0.6 M NaCl, 10 mM NaOAc, and 1% SDS, pH 7.5] containing about 1 μg/mL probe RNA was placed on the sections, and coverslips were applied and sealed with a 50:50 mixture of petroleum ether and rubber cement (Sanford). The slides were incubated overnight at 45 °C, the coverslips removed, and the sections rinsed in 4× SSC (20× SSC is 3 M NaCl/0.3 M trisodium citrate) containing 10 mM dithiothreitol (DTT). Nonhybridized probe was digested by incubating the slides in a buffer containing 0.5 M NaCl, 10 mM Tris-HCl (pH 8), 10 mM DTT, and RNase A at 20 μg/mL for 30 min at 37 °C. This was followed by a series of washes: (1) 0.5 M NaCl, 10 mM Tris-HCl (pH 8), and 10 mM DTT at 37 °C for 30 min; (2) 2× SSC with 50% formamide and 10 mM DTT at 45 °C for 30 min; (3) 1× SSC containing 50% formamide and 10 mM DTT at 45 °C for 30 min; and (4) two 15-min washes with 0.1× SSC at 37 °C. The sections were air-dried prior to being coated with emulsion (NTB-2; Eastman Kodak, Rochester, NY) for autoradiography. Coated slides were incubated in the dark at 4 °C for 2–6 weeks prior to development. Some sections were stained with 0.1% nuclear fast red in 5% aluminum sulfate (Sheenan & Hrapchak, 1980) while others were viewed and photographed without stain or coverslip. A Nikon Microphot FX microscope was used for photography.

Riboprobes were prepared by drying down 12 μL of [α-^{35}S]-UTP (150 μCi) and resuspending it in 3.5 μL of reaction mixture [7.5 μL of H_2O, 1.25 μL of 0.2 M DTT, 3.75 μL of 3.75 mM ATP, CTP, and GTP, 1.5 μL (30 units) of RNAguard (Pharmacia), and 5 μL of 5× transcription buffer (Promega)]; then, 1 μL of template (500 ng of linearized plasmid DNA) was added along with 10 units (0.5 μL) of the appropriate RNA polymerase (T3 or T7). The reaction mixture was incubated for 1 h at 37 °C. Then it was diluted with 35 μL of H_2O, and DNA was removed by addition of 5 μL of 10× DNase I buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, and 500 μg/mL BSA), 2.5 μL of 0.2 M DTT, 5 μL (5 units) of RNase-free DNase I (Boehringer Mannheim), and 2 μL (40 units) of RNAguard and incubated for 30 min at 37 °C. The mixture was then extracted with phenol/chloroform or chloroform alone; the RNA was precipitated with ethanol and dissolved in the hybridization solution.

The MT-I template was prepared by cloning a 335 bp *Rsa*I fragment that includes the entire coding region of MT-I cDNA (Glanville et al., 1982) into the *Bam*HI site of Bluscript (KS). The MT-IV template was prepared by making cDNA from a preparation of mouse tongue RNA using reverse transcriptase and oligo(dT) as a primer. The coding region of MT-IV was then amplified by PCR using oligos 381 (5′-CAGCCTCCCTTTCTTAGCTG-3′) and 382 (5′-CCCT-TGACTCAGGTACTGTG-3′). The reaction products were phosphorylated with T4 polynucleotide kinase, and the ends were made flush with T4 DNA polymerase; then they were cloned into the *Eco*RV site of Bluescript (KS). White colonies were picked, and one with an insert of the expected size was sequenced.

Metal Analysis. Tongue epithelium was prepared as described above, homogenized in 25 mM NaCl/20 mM Tris-HCl, pH 8.6, and centrifuged for 1 h at 27000*g*. The supernatant, representing total soluble protein, was heated to 100 °C for 1 min and the insoluble protein removed by centrifugation for 5 min at 15000*g*. The heat-stable fraction was applied to a Sephadex G-75 superfine column equilibrated with 25 mM NaCl/20 mM Tris-HCl, pH 8.6. Metals in the column fractions were measured using a Jarrel Ash 955

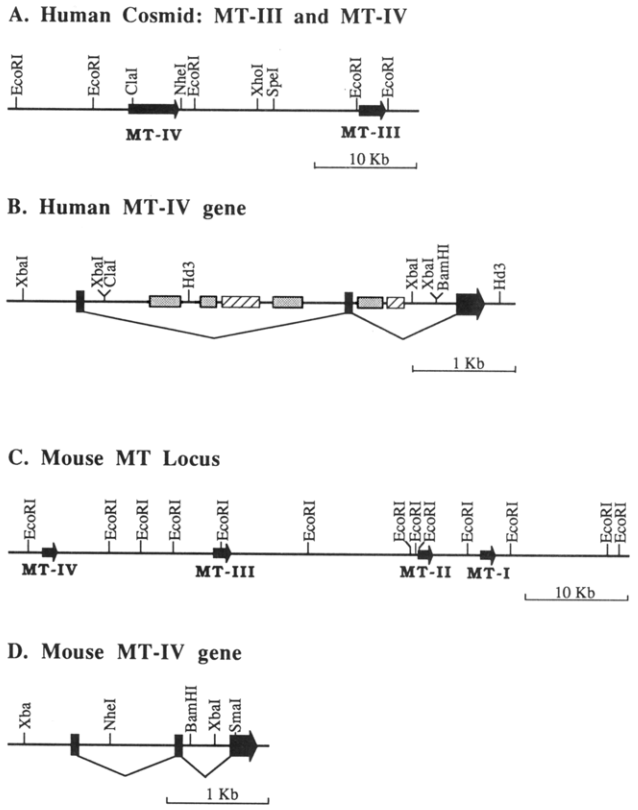

FIGURE 1: Schematic of the human and mouse MT-IV genes. (A) Human cosmid containing hMT-IV and hMT-III genes. (B) The region including hMT-IV that was sequenced; solid boxes are exons; stippled and hatched boxes are repeats of the Alu and $(AARG)_n$ types, respectively. (C) The mouse MT locus as pieced together from overlapping cosmids and λ phage. (D) The region including the mouse MT-IV gene that was sequenced; solid boxes are exons.

inductively coupled plasma emission spectrophotometer (Fassel, 1978).

RESULTS

Cloning the Human and Mouse MT-IV Genes. Previously, we screened a human cosmid library and isolated one clone that contained the human MT-III gene (Palmiter et al., 1992). A Southern blot of this cosmid that had been cut with various restriction enzymes was hybridized at low stringency with a mixture of probes from mouse MT-I, MT-II, and MT-III cDNAs. These probes revealed bands containing the human MT-III gene as expected, but they also hybridized to other bands. The location of these other bands was mapped to a position about 20 kb upstream of the hMT-III gene (Figure 1A). This region was subcloned into Bluescript or M13 for sequencing. The sequence revealed that it had all the features of other mammalian MT genes; thus, we call this the human MT-IV gene. The major difference is that this gene spans about 5 kb, and the introns have many repeat sequences, whereas other mammalian MT genes usually span 1–2 kb (Figure 1B). The transcriptional orientation is the same as the hMT-III gene. The sequence of the proximal promoter region and the exons of the hMT-IV gene is shown in Figure 2.

To ascertain whether a similar gene exists in the mouse, a cosmid library was made from cadmium-resistant hepa 1A cells that have amplified their MT-I and II genes about 20–40-fold (Durnam & Palmiter, 1984). Low-stringency screening of mouse genomic libraries with MT cDNAs is not feasible because there is a minisatellite sequence in the mouse that cross-hybridizes with these probes (Elliott, 1986). Therefore, we decided to do a chromosomal "walk" from the known MT genes with the hope that they would all be clustered and perhaps coamplified in the hepa 1A cells. Hence, we screened the cosmid library with probes from the extremities of available λ clones containing the mouse MT-I, MT-II, and MT-III genes. This strategy resulted in a cosmid contig that spans about 80 kb (Figure 1C). This contig not only linked the mouse MT-III gene to the MT-II and MT-I cluster but also allowed us to find another MT gene about 20 kb upstream of the MT-III gene (Figure 1C). This region was subcloned (Figure 1D), and sequenced (Figure 2), which revealed that this new mouse MT gene is more similar to the human MT-IV gene than any of the other mouse MT genes. Because of the conservation of sequence as well as genomic organization, we believe that this gene is the murine counterpart of hMT-IV. We have walked about 20 kb 5′ of MT-IV gene and about 20 kb 3′ of MT-I without discovering any more sequences that hybridize with MT cDNAs at low stringency.

Characterization of the Mouse MT-IV Gene. The sequence of the genomic DNA (Figure 2) revealed three exons with splice sites at the same positions as in other MT genes. This was subsequently confirmed by comparison of an MT-IV cDNA with the genomic sequence. The last exon of mouse MT-IV gene contains two potential AATAAA sequences that might direct the choice of polyadenylation site whereas there is only one in the human gene.

To determine whether either or both mouse polyadenylation sites are used, a genomic DNA probe labeled by filling in an *Ava*I site that lies within exon 3 was annealed with enriched mouse MT-IV mRNA and then digested with S_1 nuclease. The protected products were electrophoresed on an acrylamide gel next to a sequencing ladder generated from the same region. Two protected products of about equal abundance were observed: one terminated 19 bp downstream of the more distal AATAAA sequence, as expected if it were used (see Figure 2); the other terminated 12 bp upstream of the proximal AATAAA sequence, which was unexpected.

The transcription start site was mapped by primer extension of enriched MT-IV mRNA using reverse transcriptase and an oligonucleotide complementary to exon 1. The extension products were electrophoresed next to a dideoxy sequencing ladder derived from the genomic clone using the same primer. Figure 3 shows that there was a tight cluster of extension products with the main one being 28 nucleotides 3′ of the first T of a consensus TATAAA box. The lengths of the 5′ and 3′ untranslated regions of mMT-IV are relatively short and similar to other mammalian MTs. The locations of the transcription start site and poly(A) addition sites for human MT-IV were not determined. However, the conservation of sequence, including TATAAA and AATAAA sites in human MT-IV, was used to predict these sites.

Comparison of MT Sequences. MT-IV has an extra amino acid at position 5 relative to MT-I and MT-II, and this insertion is in the same position as one of the two insertions in MT-III (Palmiter et al., 1993). The mouse and human MT-IV proteins predicted from the genomic sequences are very similar, differing in only 4 of 62 positions (Figure 2). In fact, they are more conserved than any other pair of mouse and human MT proteins; there are 9 differences between mouse and human MT-III, 10 differences for MT-II, and 10–13 differences for the various MT-I comparisons.

There is 88% identity of nucleotide sequence in the coding regions of mouse and human MT-IV, but progressively less in the short 5′ untranslated regions and 3′ untranslated regions and virtually no similarity of intronic sequences. In the promoter region, there is a remarkable block (box 2, Figure 2) of identity (45 of 46 nucleotides) that starts 22 bp 5′ of the

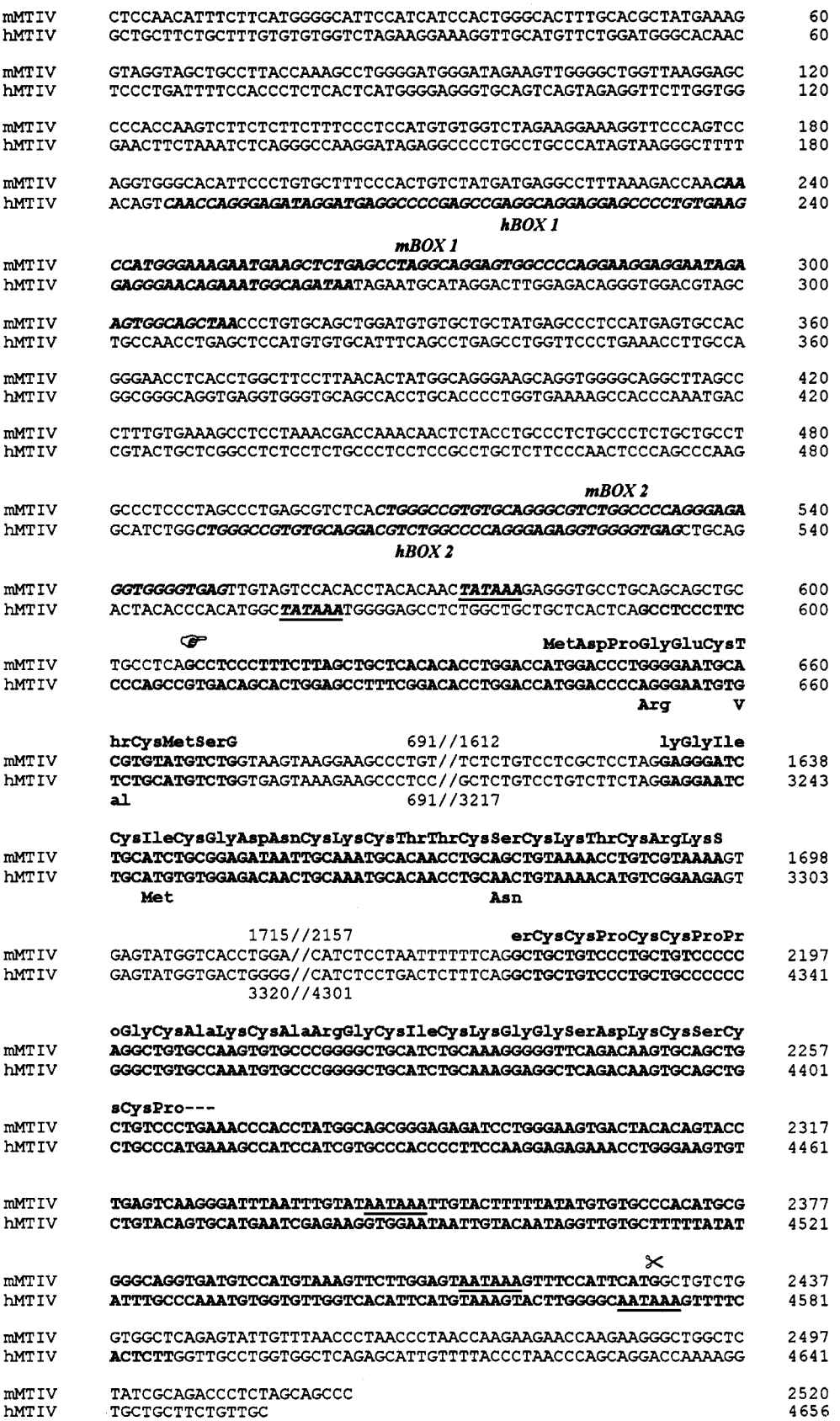

FIGURE 2: Comparison of mouse and human MT-IV DNA and protein sequences. The mouse sequence lies above the human sequence. The exonic sequences are boldface, the TATAAA boxes and AATAAA polyadenylation elements are italic boldface and underlined, and two conserved sequences in the 5′ flanking region are indicated in underlined boldface type and labeled box 1 and box 2. The complete protein sequence for mMT-IV is shown; only the differences in hMT-IV are indicated. The transcription start site for mMT-IV is indicated by the extended finger, and one of the polyadenylation sites in indicated by scissors; these were determined experimentally. The comparable hMT-IV sites were assigned by alignment with the mouse sequence. Not all the intronic sequence is included here, but it has been submitted to the database.

conserved TATAAA element, and there is another larger block (box 1, Figure 2) of similarity (62 of 78 nucleotides) located about 200 bp further upstream. There are several potential metal response elements (Searle, 1990) in the promoter-

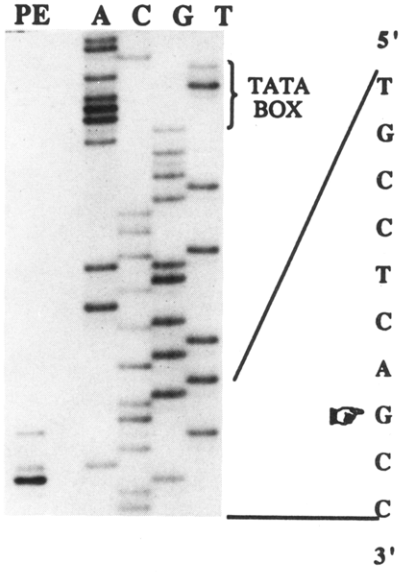

FIGURE 3: Determination of the transcription start site by primer extension. An end-labeled oligonucleotide was hybridized with enriched mMT-IV mRNA and extended with reverse transcriptase. The products were electrophoresed next to a sequencing ladder obtained with the same primer. The location of the TATAAA box is indicated.

proximal region of the hMT-IV gene, but they are not conserved in the mMT-IV gene.

To assess the ability of the hMT-IV promoter to be regulated by metals, 800 bp of 5′ flanking region was fused to βGeo, a lacZ-neomycin resistance gene fusion (Freidrich & Soriano, 1991), and transfected into baby hamster kidney (BHK) cells. A population of stable transformants was selected with G418, and then they were tested with metals, glucocorticoids, TPA, and cAMP. Treating the cells with 100 μM zinc for 16 h resulted in a 2-fold increase in β-galactosidase activity construct compared to 5-fold with a comparable MT-I/βGeo construct. The other treatments had little effect on expression from either construct.

Mouse MT-IV Gene Expression. A solution hybridization assay, using a labeled oligonucleotide that is complementary and unique to mMT-IV, was used to measure the expression of this MT-IV mRNA. Total nucleic acid samples from 15 different organs isolated from adult mice were examined. The only positive sample was the tongue. The epithelium of the tongue can be readily stripped from the underlying muscle layers. When epithelium and muscle were tested separately, MT-IV mRNA was found exclusively in the epithelial fraction, and it was 8-fold more abundant than MT-I mRNA (Table 1). When it became apparent that MT-IV mRNA was expressed specifically in the tongue epithelium, other tissues with cornified, stratified epithelium were analyzed. In the adult, MT-IV mRNA was found in other oral tissues including the buccal mucosa and soft and hard palate, as well as in esophagus and the upper portion of the stomach. The highest levels were found in tongue and upper stomach (Table 1). In addition, lower levels of MT-IV were found in tail and foot pads. During development, MT-IV mRNA became detectable in tongue and stomach only after day-7 post-partum (Table 1). When samples derived from mice treated with 25 mM zinc in their drinking water for 5 days were assayed, MT-IV mRNA levels were elevated in the upper stomach but not in any of the other samples (Table 1). Curiously, adult skin from the abdominal region was negative; however, a low level of MT-IV mRNA was detected in day-7 and day-14 skin (Table 1).

Table 1: MT-I and MT-IV mRNA Levels in Selected Organs[a]

	(I) Adult MT-I (molecules/cell)		MT-IV (molecules/cell)	
	uninduced	induced[b]	uninduced	induced
tongue	49	44	95	61
tongue epithelium	23	*c*	210	*c*
soft palate	35	*c*	45	*c*
hard palate	38	*c*	52	*c*
buccal mucosa	33	*c*	20	*c*
upper stomach	380	1700	110	450
feet	59	58	37	*c*
skin	24	*c*	<5	<5
tail	29	19	5	<5
liver	64	2500	<5	<5

	(II) Developing Pup MT mRNA (molecules/cell)					
	MT-I days postpartum			MT-IV days postpartum		
	day 0	day 7	day 14	day 0	day 7	day 14
tongue	36	45	41	<5	<5	140
stomach	76	90	110	<5	<5	33
skin	<5	44	24	<5	62	51
feet	<5	44	21	<5	70	18
liver	210	640	420	<5	<5	<5

[a] Numbers represent the average of either three (uninduced) or two (induced) samples. [b] Animals were given $ZnSO_4$ (25 mM) in their drinking water for 5 days prior to sacrifice. [c] Not done.

Cellular Location of MT-IV mRNA. In situ hybridization was used to determine which cells in the epithelium express MT-IV. The coding regions of mMT-I and mMT-IV were cloned into Bluescript for preparation of ^{35}S-labeled sense and antisense riboprobes. These longer probes were used instead of the oligonucleotides to gain sensitivity. Control experiments indicated that the MT-I and MT-IV probes do not cross-hybridize under the very stringent conditions used for the *in situ* experiments. This was apparent from the *in situ* results in that the two probes did not hybridize to the same cells; for example, the MT-IV probe did not hybridze with pancreas or liver sections that have abundant MT-I mRNA.

Histological observation shows that the majority of the dorsal surface of the tongue is covered with specialized epithelial structures called filiform papillae, whereas the ventral surface of the tongue has a much simpler organization (Figure 4A,B). The antisense MT-IV probe hybridized to mRNA in epithelial cells of both dorsal and ventral surfaces in a band of cells, referred to as the stratum spinosum, which lies immediately above the basal, proliferative layer (Figure 4C,F,I). Cells in this suprabasal region are beginning their differentiation program. The intensity of the hybridization signal in the dorsal epithelium varies with location, with the anterior surfaces of the filiform papillae being consistently more intense than the posterior surfaces (Figure 4C,F). In contrast, the signal in the ventral epithelium is relatively uniform (Figure 4I). There was no specific labeling of the underlying muscle cells as indicated by comparison of sense and antisense probes (Figure 4H).

Solution hybridization revealed that MT-I was also expressed in tongue, so we examined its expression pattern by *in situ* hybridization as well. We observed a narrow band of hybridization with the antisense probe that was confined to the basal cell layer of the epithelium (Figure 4D,G) rather than the overlying stratum spinosum as observed with the MT-IV probe. As with MT-IV, there was variation in the intensity of the hybridization signal in dorsal epithelium with

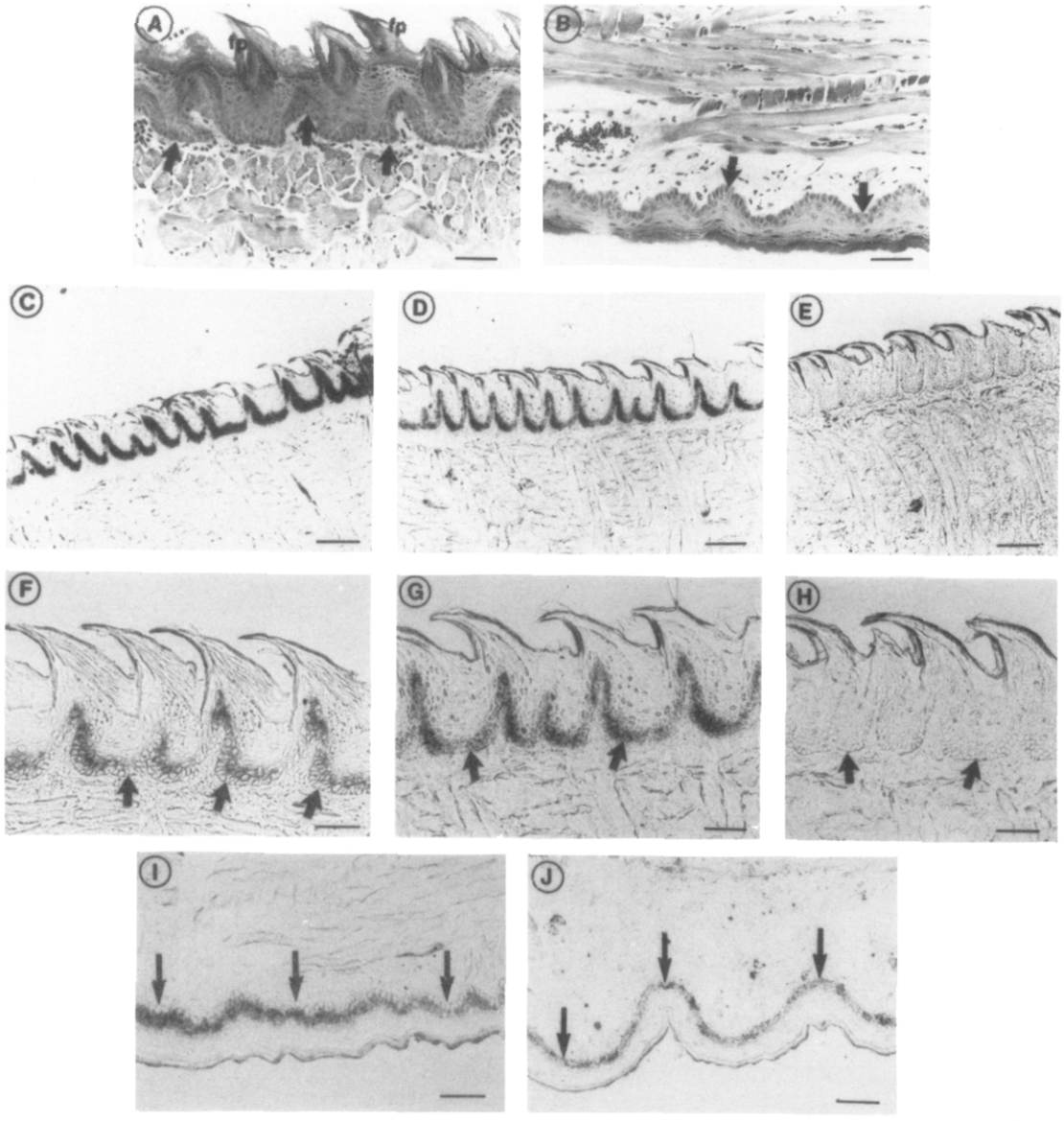

FIGURE 4: Localization of MT-I and MT-IV mRNA in tongue by *in situ* hybridization. (A) A hemotoxylin and eosin-stained paraffin section shows epithelial filiform papillae (fp) on the dorsal surface of the tongue. (B) The epithelium on the ventral surface of the tongue is less specialized. The basal, proliferative layer of the epithelium is indicated by arrows in (A) and (B). (C) MT-IV mRNA expression pattern in dorsal tongue epithelium as revealed by *in situ* hybridization with antisense probe. (D) MT-I expression pattern in similar section to (C). (E) No specific signal was observed with MT-IV sense probe. (F) Higher magnification of signal obtained with MT-IV antisense probe and (G) MT-I antisense probe. (H) Control hybridized with MT-IV sense probe. The arrows in panels F, G, and H point to the junction between the basal cell layer and underlying muscle cells. (I) MT-IV expression pattern observed with the antisense probe on the ventral surface of the tongue. (J) MT-I expression pattern on the ventral surface. The arrow tips in panels I and J rest on the basal lamina. Bars = 50 μm.

anterior surfaces of the filiform papillae being more heavily labeled. The hybridization signal in the ventral epithelium was also over the basal layer and was relatively homogeneous along its length (Figure 4J). The MT-I antisense probe also hybridized to the underlying muscle, but the signal was patchy (not shown); no specific signals were observed with the MT-I sense probe over either epithelia or muscle (not shown).

Other organs with stratified, squamous epithelia were also examined for the presence of MT-IV mRNA by *in situ* hybridization. In the rodent, the esophagus and upper portion of the stomach are lined by this type of epithelium (Gude et al., 1982). In both organs, specific hybridization to the MT-IV antisense probe was observed over the stratum spinosum (Figure 5B,D). Sections of muscle, pancreas, and gut were included in this study, and they were uniformly negative. The abdominal skin, foot pads, and tail skin were also examined. There was an intense signal localized over the suprabasal cells of the footpads (Figure 5F) and tail skin (Figure 5H), but a MT-IV signal was not observed in sections of abdominal skin (Figure 5J). No specific signal was observed in any of these sections with the MT-IV sense probe (not shown).

MT-IV Protein. MTs can be readily purified by virtue of their heat stability and small size. Epithelia were isolated from 60 mouse tongues, homogenized, and heated to 100 °C for 1 min, and the insoluble protein was removed by centrifugation. The supernatant was applied to a Sephadex G-75 column, and the column fractions were monitored for various metals. There was a distinct peak of zinc and copper in the fractions where MT elutes from this column (data not shown). The zinc to copper ratio was approximately 2.6 in all the fractions. No cadmium was detected. Using the amount of these metals recovered in the MT fractions, we calculate that there is about 240 ng of MT per mouse tongue, assuming 12 copper ions or 7 zinc ions are bound per molecule of MT (Winge, 1991). We estimate that about 80% of the MT is MT-IV. MT-IV represents about 0.20% of the total soluble protein or 4.5 million molecules per epithelial cell.

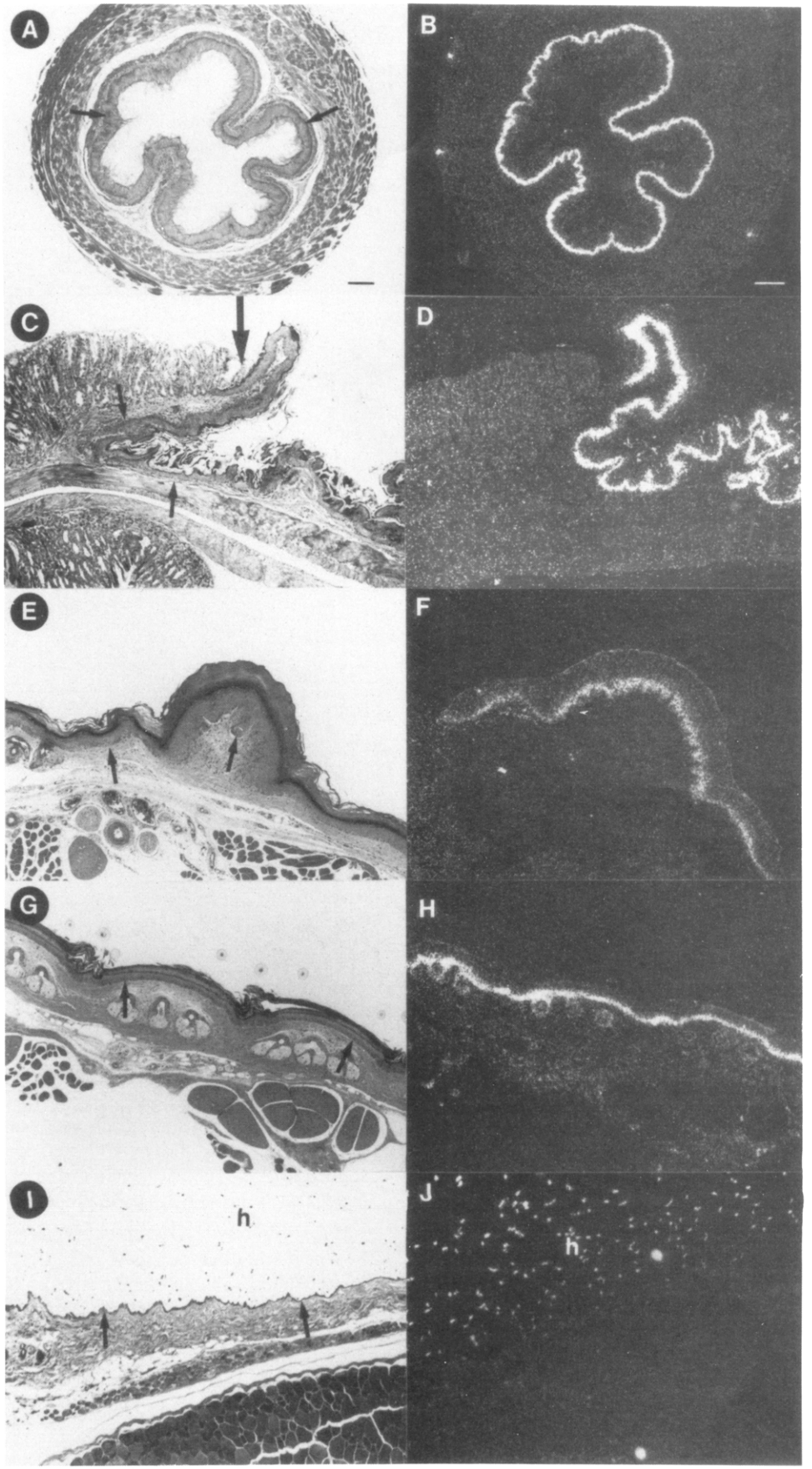

FIGURE 5: Demonstration of MT-IV mRNA in stratified squamous epithelium of several organs by *in situ* hybridization. Hemotoxylin and eosin-stained paraffin sections from (A) esophagous, (C) stomach, (E) footpad, (G) tail skin, and (I) abdominal skin are compared with dark-field images of adjacent sections from (B) esophagous, (D) stomach, (F) footpad, (H) tail skin, and (J) abdominal skin. MT-IV appears as a bright signal in the epithelium of each organ except abdominal skin. The small arrows in bright-field images indicate the junction of the basal cell layer and the basal lamina. The large arrow in panel C indicates the junction of the stratified squamous epithelium and the columnar epithelium of the mucosa; note in panel D that hybridization is restricted to the former. In panel J, the hair (h) is bright, when viewed in dark-field; however, this is not due to silver grains. Bar = 50 μm.

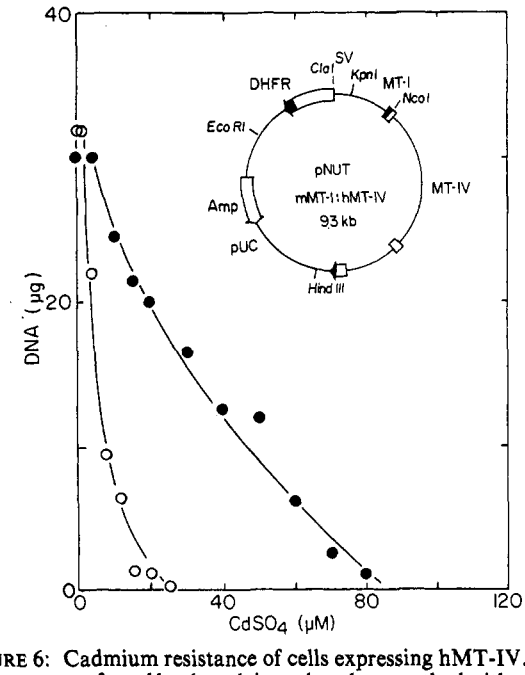

FIGURE 6: Cadmium resistance of cells expressing hMT-IV. BHK cells were transfected by the calcium phosphate method with a pNUT construct (Palmiter et al., 1987) in which the mMT-I promoter was fused to the hMT-IV gene at the *Nco*I site in exon 1. A population of cells that was stably resistant to 200 μM methotrexate was selected. The cells were plated in 24-well dishes and grown in DMEM containing 10% fetal calf serum plus varying amounts of $CdSO_4$ for 3 days. Then the cells were washed, dissolved in 0.2× SET, and sonicated briefly, and the DNA content was determined by the fluorescence of H33258. *Open symbols*, control BHK cells; *solid symbols*, transfected BHK cells.

To ascertain whether MT-IV could bind cadmium and protect against toxicity, the mMT-I promoter was fused to the hMT-IV gene in an expression vector that contains the mutant DHFR gene that allows selection for high levels of expression. This construct (Figure 6) was transfected into BHK cells, and a stable population of cells resistant to 200 μM methotrexate was selected. These transfected cells and control BHK cells were plated in varying concentrations of $CdSO_4$, and the total DNA per well was measured 3 days later. Figure 6 shows that the transfected cells were about 8-fold more resistant to the toxic effects of cadmium compared to parental BHK cells, indicating that hMT-IV can effectively sequester cadmium. Similar results are obtained with contructs expressing mMT-I or mMT-III (data not shown).

DISCUSSION

The MT-IV genes were discovered by virtue of their close linkage to the MT-III genes in both human and mouse. In both cases, the MT-IV genes are about 20 kb 5′ of the MT-III genes and have the same transcriptional orientation. In the mouse, we have been able to establish the physical linkage of all the known MT genes, but this has not yet been accomplished for human. *In situ* hybridization indicates that the human MT-III gene is closely linked (probably within 85 kb) to the other 14 MT genes on chromosome 16 (West et al., 1990), but we do not know whether the MT-IV and MT-III genes lie upstream of the MT-IIA gene or downstream of the last MT-I gene in the cluster.

There are 24–27 amino acid differences when comparing MT-IV to any of the other murine MTs (Palmiter et al., 1993). Considering that about 30 residues are identical in all MTs, that means that virtually every nonessential amino acid in MT-IV differs from those in the other MTs, but like other mammalian MTs there are no aromatic amino acids. Considering the differences between the four mouse MTs, it is remarkable that there are only four amino acid differences between mouse and human MT-IV.

The sequence conservation between mouse and human MT-IV is also observed at the nucleic acid level in the coding region and promoter, but not in 5′ or 3′ untranslated regions or introns. There are two large patches of sequence similarity upstream of the promoter that may be recognized by transcription factors responsible for the epithelia-specific expression that we have observed. This type of sequence conservation is not observed in comparisons of the other mouse and human MT 5′ flanking regions.

Despite the fact that the four murine MT genes are closely linked, the genes are not all expressed identically. The mouse MT-I and -II genes are regulated coordinately in many different cell types, and they are inducible by a variety of metals, hormones, cytokines, and xenobiotics (Kägi & Schaffer, 1988; Waalkes & Goering, 1990). MT-III, in contrast, is expressed in selected populations of neurons and glia, and no specific environmental regulators have been identified (Uchida et al., 1991; and our unpublished observations). Here we show that MT-IV is only expressed in differentiating cells of stratified squamous epithelia. All of these MTs bind cadmium and can confer resistance to cadmium toxicity when they are overexpressed in tissue culture cells by heterologous promoters. Selection for cadmium-resistant hepa-1A cells results in 20–40-fold amplification of the entire MT locus. However, neither the MT-III nor the MT-IV genes are expressed (data not shown), indicating that these genes are not influenced by the nearby control elements that are important for MT-I and MT-II gene expression even after selection for maximum expression of cadmium binding proteins.

In situ hybridization revealed that MT-IV expression is restricted to suprabasal cells in the stratum spinosum of stratified, squamous epithelia. Keratinocytes in the basal layer proliferate, generating daughters that move outward into the stratum spinosum where they begin their differentiation program. When keratinocytes move away from the basal layer, there is a concomitant change in keratin expression (Sun et al., 1984; Ouhayoun et al., 1985; O'Guin et al., 1990). The particular keratin genes expressed vary from one type of squamous epithelia to another (Moll et al., 1982; Dhouailly et al., 1989; Tobiasck et al., 1992; Yuk-Ying et al., 1993). We have observed a similar switch in MT-I to MT-IV expression when certain subtypes of keratinocytes begin to differentiate. In the tongue, where we have examined expression in most detail, MT-I mRNA is abundant in the basal cells with much lower levels in the adjacent spinous layer cells, suggesting that transcription of MT-I may be restricted to basal cells and a small amount of mRNA persists into the differentiating spinous cells. In contrast, MT-IV mRNA is undetectable in the basal cells and accumulates to high levels in the spinous cells. MT-IV mRNA is not uniformly distributed in epithelia with more complex anatomy, such as in the filiform papillae on the dorsal surface of the tongue. These papillae have at least three separate bands of differentiating cells that make esophageal, hair, or skin-type keratins which give the structure its characteristic properties (Dhouailly et al., 1989; Tobiasch et al., 1992). The expression pattern of MT-IV resembles that of keratins K4 and K13. Perhaps the assembly of this particular type of keratin is regulated by MT-IV. We have shown that MT-IV in normal tongue epithelium contains both zinc and copper. Although we only measured MT-I mRNA, we suspect that the MT-II gene is also expressed in the basal

layer because these genes are coordinately regulated everywhere else.

Is the switch from MT-I to MT-IV expression at the start of terminal differentiation of stratified squamous epithelium of functional significance? As keratinocytes move away from the basal layer, they become increasingly displaced from a source of nutrients, and they may rely on stores that they acquire at early stages of differentiation. Thus, MT-IV might be better suited to sequester essential metals compared to MT-I. Alternatively, MTs may associate with other macromolecules in the process of donating their metals, in which case the surface of the MTs may dictate what interactions are possible. For instance, in this scenario, MT-IV may have evolved to interact with molecules involved in building the cytoskeleton of differentiating keratinocytes. Studies *in vitro* have shown that zinc can accelerate keratin assembly and zinc was one of the most efficient metals tested in this assay (Sakamoto et al., 1980). Perhaps MT-IV regulates this process. Alternatively, MT-IV might regulate proteolytic processing of keratins (Bowden et al., 1984; Hsu et al., 1991b) or other proteins responsible for building the cytoskeleton.

Nutritional studies show that dietary zinc deficiency leads to parakaratosis, a perturbation of the normal pattern of differentiation of stratified, squamous epithelium characterized by hyperplasia, alterations in the synthesis and/or processing of keratins, and retention of nuclei in the cornified layer (Alvares & Meyer, 1968; Fell et al., 1973; Niemi et al., 1989; Hsu et al., 1991a,b). There are regional differences in the parakaratotic response of rats exposed to a mild zinc-deficient diet, with the epithelium of posterior tongue, buccal mucosa, esophagus, and footpads being most sensitive, the epidermis showing intermediate sensitivity, and hard palatal mucosa being insensitive (Alvares & Meyer, 1968). The tissues that are most sensitive to zinc deprivation contain the highest amounts of MT-IV mRNA. We did measure some MT-IV mRNA in hard palate, but our dissections of this tissue may not correspond exactly with the regions examined histologically by Alvares and Meyer (1968). Moreover, most of our mRNA measurements were made on dissected material that included varying amounts of underlying tissues in addition to the epithelia; thus, the relative amount of MT-IV mRNA per spinous epithelial cell cannot be estimated from Table 1. Nevertheless, the correspondence we do observe suggests that a more detailed analysis of the relationship between MT-IV expression and hyperkaratosis during zinc deprivation is warranted. It seems likely that apo-MT synthesized in the presence of insufficient zinc would either bind other metals or be readily degraded. In either event, that might be responsible for the disruption of keratinocyte differentiation.

MT-IV may also have toxicological significance. MTs have been implicated as an important protective mechanism against heavy-metal toxicity. We have shown that MT-IV expression protects tissue culture cells against cadmium toxicity. In addition, MT-IV expression is induced in the upper stomach when mice are exposed to high levels of zinc in their drinking water. These observations suggest that MT-IV, like other MTs, can be regulated by heavy metals and can reduce their toxicity. Cornified, stratified, squamous epithelia form an essential barrier between external and internal environments. MT-IV may contribute to this barrier function by sequestering external (luminal) metal ions and limiting their access to the bloodstream.

It is curious that MT-IV is not present in adult mouse skin. Adult epidermis from the abdominal region is very thin compared to other more specialized regions such as footpads and tail. Consequently, the stratum spinosum is much thinner, being only one or two layers thick. This would account for less MT-IV mRNA, but even careful examination of sections of skin used for *in situ* hybridization failed to reveal a signal in the suprabasal cells. This suggests that MT-IV may only be required in thicker, stratified epithelia with more extensive cornification.

Generating mice in which the MT-IV gene is either inactivated or substituted by the coding region of another MT gene by using homologous recombination in embryonic stem cells, would be the most direct way of ascertaining whether MT-IV is essential and uniquely suited to its role in epithelia.

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