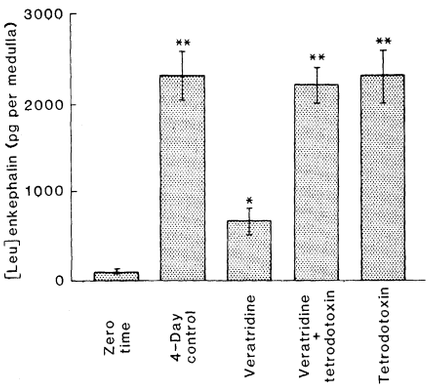
Fig. 4. Effects of veratridine. Groups of seven medullas were cultured in the presence of veratridine ($10^{-5}M$), tetrodotoxin ($10^{-7}M$), or both, added on day 3. Medullas were examined for [Leu]enkaphalin content on day 4. Veratridine-induced depolarization prevented the accumulation of [Leu]enkephalin. Tetrodotoxin blocked the effects of veratridine, while having no effect alone. Values are expressed as in Fig. 1. *Differs from zero time at $P < 0.05$. **Differs from zero time and veratridine at $P < 0.001$ (one-way analysis of variance and Newman-Keuls and Kruskal-Wallis tests).



[Leu]enkephalin after decreased transsynaptic activity in vivo or in vitro reflects increased synthesis, decreased release, decreased catabolism, or a combination of these processes. In contrast, however, depolarization did not significantly alter TH or PNMT activities (Fig. 3).

Our studies indicate marked differences in the regulation of [Leu]enkephalin and catecholaminergic characters in the adrenal medulla. Transsynaptic stimulation, which biochemically induces adrenomedullary TH and PNMT (*17*), dramatically decreases [Leu]enkephalin content. Reduction in peptide content is mediated by transmembrane Na^+ influx (Fig. 4), which mediates TH induction in sympathetic neurons in vitro (*18*). Consequently, the same or similar molecular events, depolarization and Na^+ influx, elicit opposite changes in peptidergic and catecholaminergic characters, which are found in the same medullary chromaffin cells. Although abundant evidence indicates that enkephalins and CA's, critical physiologic neurohormones, are co-localized and co-released by medullary cells, intracellular processing is distinct. Our observations suggest that diverse physiologic effectors, elaborated by the same cells, may be independently expressed and regulated. This study complements recent work from our laboratory, indicating that the peptides substance P and somatostatin are expressed and regulated differently from catecholamines in sympathetic neurons (*19*). Therefore, neuropeptides and "classical" neurohormones, such as catecholamines, may be differentially regulated in a wide variety of cell types.

In a somewhat different context, the independent regulation of sympathoadrenal neuropeptides and enzymes for catecholamines implies that physiological responses to stress (increased transsynaptic activity) may be analyzed in discrete molecular terms. It may be possible to identify metabolic regulatory and effector molecular mechanisms differentially elicited by variation in the local environment. Consequently, new therapeutic approaches to shock, hypoxemia, and stress-induced analgesia, for example, may evolve through the use of agents which differentially alter peptidergic or catecholaminergic metabolism.

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Chromosomal Location of Human Metallothionein Genes: Implications for Menkes' Disease

Abstract. *Human metallothioneins are encoded by a complex multigene family. The chromosomal location of these genes has been determined by gel transfer hybridization analysis of the DNA from human-rodent cell hybrids. Chromosome 16 contains a cluster of metallothionein sequences, including two functional metallothionein I genes and a functional metallothionein II gene. The remaining sequences, including a processed pseudogene, are dispersed to at least four other autosomes. The absence of metallothionein sequences from the X chromosome indicates that Menkes' disease, an X-linked disorder of copper metabolism, affects metallothionein expression by a* trans*-acting mechanism.*

Metallothioneins (MT's) are cysteine-rich, low molecular weight proteins that bind heavy metal ions including zinc, copper, cadmium, and mercury (*1*). They are believed to play a role in both heavy metal homeostasis and detoxification. All vertebrates examined synthesize two major isoforms, MT-I and MT-II, that have closely related but distinct amino acid sequences. In man, results obtained by protein sequencing suggest the presence of at least three different forms of MT-I. In addition, gel transfer hybridization analysis has demonstrated multiple MT-related sequences in the human genome (*2*). To further our understanding of the organization and evolution of this multigene family, we have performed chromosome mapping experiments on a series of human-rodent cell hybrids. We were particularly interested in determining whether any MT sequences are located on the X chromosome because of the evidence that Menkes' disease (*3*), an X-linked disorder of copper metabolism, alters either

Table 1. Assignment of the five MT loci to human chromosomes. The MT loci A to E are as defined in Fig. 2. This table summarizes the data obtained for all 77 of the hybrid lines and subclones that were analyzed. For a given locus, percent discordancy is defined as 100 times the total discordancy with a particular chromosome, divided by 77. A discordancy with a particular chromosome occurs when a hybrid contains the human chromosome, yet lacks the MT locus, or when a hybrid lacks the human chromosome, yet contains that MT locus. The chromosome giving the lowest discordancy contains that MT locus. For loci A, D, and E the lowest discordancy is not 0 and this is probably due to differences in the sensitivity of the gel transfer hybridization experiments and the isozyme analysis. The somatic cell hybrids have already been characterized (*8*).

MT locus	Percent discordancy of human chromosome																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
A	36	52	55	43	31	43	52	32	30	51	32	35	39	26	49	3	39	52	35	40	51	45	48
B	42	60	27	25	50	20	50	38	48	50	31	46	67	38	35	46	21	63	23	0	19	35	42
C	0	25	32	34	22	20	48	39	26	29	26	39	48	45	45	36	39	64	18	23	54	23	54
D	32	54	24	3	31	33	76	39	32	51	34	44	76	46	71	36	63	61	34	32	37	34	53
E	66	51	51	65	64	73	40	57	57	62	65	54	22	45	48	50	48	1	75	73	49	70	44

MT protein structure (*4*) or MT biosynthesis (*5*).

Figure 1A shows a gel transfer hybridization experiment in which 10 μg of human chromosomal DNA was digested with Eco RI, separated on a 1 percent agarose gel, blotted onto nitrocellulose, and hybridized with various cloned MT probes (*6*). Hybridization to a mixture of monkey MT-I and MT-II complementary DNA's (cDNA's) revealed a complex pattern of 14 distinct bands. Because the coding regions of the MT genes have been highly conserved throughout primate evolution (*1*, *7*), and because an identical pattern of bands is observed with a human MT-II probe (*2*), we believe that the monkey coding sequences recognize the complete human MT gene family. Figure 1A also shows the patterns observed when parallel nitrocellulose strips carrying Eco RI–digested human DNA were annealed to noncoding region probes isolated from functional human MT genomic clones (*8*). This permitted us to identify the specific human genes contained in several of these bands.

To determine the chromosomal location of the MT genes (Table 1), we performed similar gel transfer experiments on the DNA isolated from 77 different rodent-human somatic cell hybrids and hybrid subclones (*9*). These hybrids, which were generated by polyethylene glycol fusion of either Chinese hamster or mouse cells with human cells, randomly lose human chromosomes during continuous culture. After the hybrids were cloned, the human chromosomes retained in each cell line were determined by gel electrophoretic analysis of isozymes known to be encoded on each human chromosome. By correlating the gel transfer hybridization patterns with the human chromosome content of each line it was possible to assign the MT genes to specific chromosomes.

A typical DNA blot is shown in Fig. 1B, and the results for 14 somatic cell hybrids are summarized in Fig. 2. We find that the MT genes are located on at least five different autosomes. Chromosome 16 contains five hybridizing bands of 25, 14.5, 10.0, 6.8, and 5.9 kilobase pairs (kbp). As indicated above, we have used unique flanking region probes from human MT genomic clones to identify the genes contained in specific bands. The results presented in Fig. 1A demonstrate that the 5.9-kbp band contains a human MT-II gene, whereas the 14.5- and 10.0-kbp bands contain at least two functional MT-I genes. Consequently, at least three functional MT genes are clustered together on chromosome 16. These bands appear to be closely linked, since they segregated concordantly in all 77 cell hybrids examined, including some hybrids containing breaks in chromosome 16 at two different sites.

We used two markers on chromosome 16 to further localize the functional MT genes. The gene for phosphoglycolate phosphatase (PGP) has been assigned to the short arm of human chromosome 16 (16p12-16p13), and the gene for diaphorase-4 (DIA-4) has been assigned to the proximal portion of the long arm of 16 (16q12-16q21) (*10*). In one series of 11 subclones, eight retained PGP and the functional MT locus, but none expressed DIA-4. In another series, three hybrids with the phenotype PGP^-, $DIA4^+$ retained the functional locus. We conclude that the functional locus is between the PGP and DIA-4 loci and is situated either

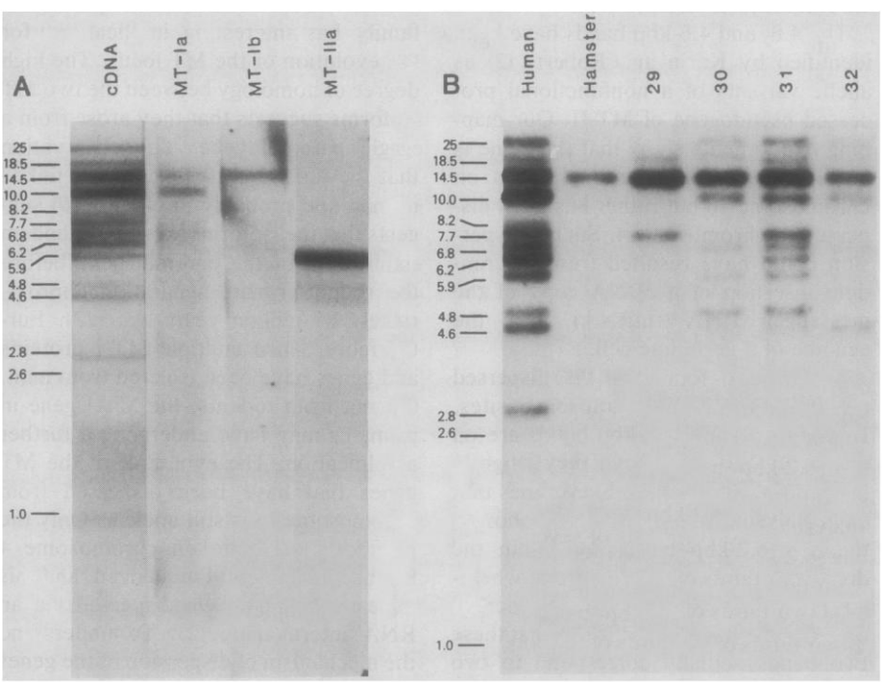

Fig. 1. Autoradiograms of gel transfer experiments. In each lane, 10 μg of chromosomal DNA was digested with Eco RI, subjected to gel electrophoresis, transferred to nitrocellulose, and subsequently annealed to various probes (6). (A) Parallel nitrocellulose strips carrying human placental DNA annealed to the probe indicated above the respective strip. The cDNA probe was the mixed monkey MT cDNA's; MTIa, MTIb, and MTIIa refer to unique probes isolated from the respective human genes. (B) Various cell line DNA's were hybridized with the mixed cDNA probe. The lanes labeled human and hamster contain DNA from the parental lines, whereas lanes 29 to 32 contain DNA from human-hamster cell hybrids. The numbers along the sides of each panel indicate the length of each band in kilobase pairs.

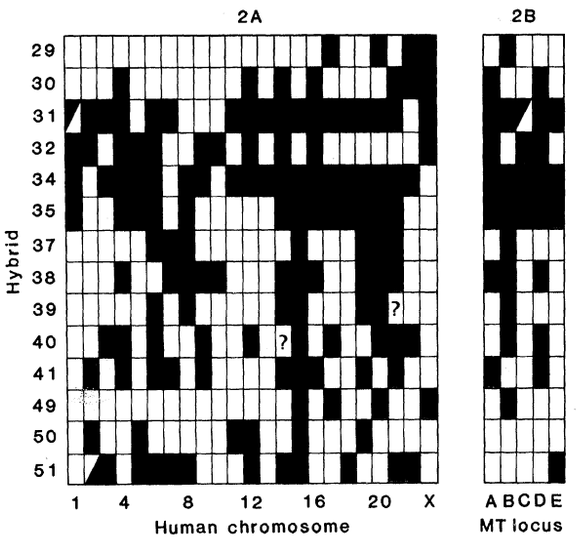

Fig. 2. (A) The distribution of human chromosomes in 14 different Chinese hamster–human hybrids. Ordinate, individual hybrid lines; abscissa, specific human chromosomes. Solid boxes indicate the presence of that human chromosome in a hybrid line. Question marks indicate that the assignment of that chromosome is uncertain. When only the lower right corner of the box is darkened only the long arm of the chromosome was retained in that hybrid. When the upper left corner is darkened only the short arm of that chromosome was retained. (B) The distribution of the five different MT loci (denoted A to E) in these hybrid cell lines. The half-darkened box in hybrid 31, locus C, indicates that this hybrid contains only the 6.2-kbp band. Locus A represents the 25-, 14.5-, 10.0-, 6.8-, and 5.9-kbp bands; locus B the 7.7-kbp band; locus C the 6.2- and 2.8-kbp bands; locus D the 4.8- and 4.6-kbp bands; and locus E the 1.0-kbp band.

on the proximal portion of the long arm of 16 (16qcen-16q21) or on the short arm. We suspect the long arm location is correct because it also carries adenyl phosphoribosyltransferase (APRT), a marker that resides on mouse chromosome 8, as does mouse MT-I (*10, 11*). In contrast, the hemoglobin A (HBA) locus, which is found on the short arm of chromosome 16 in humans, is located on chromosome 11 in mice (*10*).

The 4.8- and 4.6-kbp bands have been identified by Karin and Roberts (*2*) as allelic variants of a nonfunctional processed pseudogene of MT-II. Our mapping experiments show that this gene is not linked to the functional locus on chromosome 16 but rather has been dispersed to chromosome 4. Such a dispersion could have resulted from the random insertion of a cDNA copy of the messenger RNA (mRNA) into the genome of a germ line cell.

The remaining MT genes are dispersed to at least four other autosomal sites. Both the 6.2- and 2.8-kbp bands are on chromosome 1, but they are not tightly linked. Analysis of several cell lines that contain breaks in chromosome 1 showed that the 6.2-kbp band lies within the distal two-thirds of the short arm, whereas the 2.8-kbp band is probably located on the long arm. This indicates that these two bands actually correspond to two separate genes rather than one gene separated by an Eco RI site. Finally, the 7.7-kbp band segregates with chromosome 20 and the 1.0-kbp band segregates with chromosome 18.

We were unable to make chromosomal assignments for the 18.5-, 8.2-, and 2.6-kbp bands visible in the human genomic DNA lane (cDNA lane in Fig. 1A). These bands show weak hybridization to the cDNA probes and were frequently obscured by more strongly hybridizing adjacent bands. However, it is clear that none of the MT sequences map to the X chromosome because they were all absent in three different hybrids that retained the entire human X chromosome (data not shown).

The structure of this complex gene family has interesting implications for the evolution of the MT locus. The high degree of homology between the two MT isoforms suggests that they arose from a single parental gene. The observation that the functional MT genes are linked in man and probably in mouse (*10*) suggests that the isoforms arose by a tandem duplication of the parental gene before the rodent-primate separation approximately 85 million years ago (*12*). Furthermore, since multiple MT-I proteins and genes have been isolated from man, but not from rodents, the MT-I gene in primates may have undergone a further amplification. The evolution of the MT genes that have been dispersed from chromosome 16 is still unclear. Only the pseudo-MT-II gene on chromosome 4 has been completely sequenced, and this appears to have been dispersed via an RNA intermediate (*2*). To understand the mechanism of dispersion of the genes to chromosomes 1, 18, and 20 will require further sequence analysis.

These results also have important implications for the molecular basis of Menkes' kinky hair syndrome, an X-linked disorder of copper metabolism leading to severe mental and motor retardation and death at age 2 to 3 years. Menkes' patients have greatly reduced levels of circulating copper but accumulate excess copper and copper MT in some extrahepatic tissues and in cultured cells (*3*). It has been suggested that the accumulation of excess copper MT could result from a change in either MT structure (*4*) or MT biosynthesis (*5*), and recently our laboratory has shown that copper is a more potent inducer of MT mRNA production in Menkes' fibroblasts than in normal fibroblasts (*13*). Our finding that the X chromosome contains no MT-related DNA sequences shows that Menkes' disease cannot be due to mutations or amplification of the MT genes themselves or of their *cis*-acting regulatory sequences. Similarly, the X-linked mottled mutations of mouse (*14*) affect MT synthesis even though the mouse MT-I gene has been mapped to an autosome (*11*). We conclude that the Menkes' mutation affects human MT gene expression through a *trans*-acting alteration in MT transcriptional regulation or, indirectly, by altering copper homeostasis.

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