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Genetics

Human metallothionein genes are clustered on chromosome 16

(heavy metals/gene mapping/somatic cell hybrids/trace-metal metabolism/gene duplication)

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ABSTRACT **The metallothioneins are a family of heavy-metal binding proteins of low molecular weight. They function in the regulation of trace metal metabolism and in the protection against toxic heavy metal ions. In man, the metallothioneins are encoded by at least 10–12 genes separated into two groups, MT-I and MT-II. To understand the genomic organization of these genes and their involvement in hereditary disorders of trace metal metabolism, we have determined their chromosomal location. Using human–mouse cell hybrids and hybridization probes derived from cloned and functional human *MT1* and *MT2* genes, we show that the functional human genes are clustered on human chromosome 16. Analysis of RNA from somatic cell hybrids indicated that hybrids that contained human chromosome 16 expressed both human *MT1* and *MT2* mRNA, and this expression is regulated by both heavy metal ions and glucocorticoid hormones.**

The metallothioneins (MTs) are a group of low molecular weight proteins that bind heavy metal ions belonging to groups Ib and IIb, such as Cd^{2+}, Zn^{2+}, and Hg^{2+} and Cu^{+}, Ag^{+}, and Au^{+}, respectively. They are characterized by their high cysteine content and capacity for metal binding; they exist in several molecular forms and are classified by their electrophoretic behavior as either MT-I or MT-II (1). Yet, each class might represent more than a single protein species. Their major role is in the regulation of trace metal (Zn^{2+}, Cu^{+}) metabolism, acting in the storage of these ions in the liver. However, since the transcription of *MT* genes increases dramatically after exposure to heavy metals (2), they also can serve as a protective system against heavy metal toxicity. The control of *MT* gene expression by glucocorticoid hormones (3, 4) is probably involved in the regulation of plasma Zn^{2+} concentration (5). Defects in *MT* gene expression have been suggested to be the cause for several disorders of trace metal metabolism in man—i.e., Menkes kinky hair syndrome (6, 7), Wilson disease (8), and acrodermatitis enteropathica (9). Yet, no direct involvement of the *MT* genes in any one of these diseases has been demonstrated. *MT* genes also have been suggested to be involved in the genetic predisposition of mice to the toxic effects of Cd, such as testicular necrosis (10), and Cd-induced hypertension (11) in rats.

In man, MTs are encoded by a multigene family containing about 12 members (12). To learn more about the chromosomal organization of *MT* genes, we have isolated bacteriophage clones containing human *MT* genes (*hMT*) and established physical linkage between some of them encoding various hMT-I proteins. We have identified a cluster of five linked genes for hMT-I, which were named arbitrarily: *hMT-I_A*, *hMT-I_B*, . . . etc. (13). It was not clear whether these genes are physically linked to the functional *hMT-II_A* gene we described earlier (12). [Following human gene nomenclature guidelines (14), the *MT* genes are designated *MT1A*, *MT1B*, . . . etc. and *MT2A*, . . . etc., respectively.] Using these probes, we present data showing that the functional human *MT* genes are clustered on chromosome 16.

MATERIALS AND METHODS

Parental and Hybrid Cells. A total of 36 cell hybrids were used in these studies. They were isolated and genetically characterized from 16 independent hybrid sets involving 14 unrelated human parental cells and 4 different mouse enzyme-deficient cell lines (see ref. 15).

Human Chromosome Composition of Cell Hybrids. Human–mouse somatic cell hybrids were characterized for their human chromosome content as described (15–17). On the same cell passage, cell hybrids were examined for 31 human chromosome-specific enzyme markers as described (17) to confirm the chromosome analysis and to recognize regions of human DNA not identifiable by microscopy.

Southern Blotting Analysis. DNA was isolated from human, mouse, and hybrid cells on the same cell passage as chromosome and enzymes were tested (18). Ten micrograms of each DNA sample was cleaved with *Eco*RI or *Hin*dIII (4 units/μg of DNA) for 3 hr at 37°C in the buffer recommended by the manufacturer. DNA fragments were transferred onto nitrocellulose as described by Southern (19). The *MT* probes were labeled by nick translation (20); hybridization and washing of blots were as described (12).

RNA Blot Hybridization Analysis. Total cytoplasmic RNA was extracted from cells that were cultured for the last 10–12 hr in the presence of either 5 μM $CdCl_2$ or 1 μM dexamethasone, or no inducer at all (21). Samples (40 μg) were analyzed by electrophoresis on 1.5% agarose gels, and RNA blot hybridization was carried out as described (21, 22).

RESULTS

Experimental Approach. The principle approach to mapping cloned genes has been to test a probe for human sequences in human–mouse cell hybrids retaining different human chromosomes by restriction enzyme analysis and blot-hybridization techniques (17). This approach was used for mapping the human *MT* gene family; additionally, we have undertaken the new approach of analyzing cell hybrid RNA for expression of cloned human *MT* gene by RNA blot hybridization (22). This allowed assignment of human *MT1* and *MT2* genes to a specific chromosome with a high degree of confidence and the demonstration of gene regulation in cell hybrids by both heavy metal ions and glucocorticoid hormones. This combined approach is very useful for the analysis of complex gene families, especially those that contain pseudogenes, since it allows discrimination between func-

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Abbreviations: MT, metallothionein; hMT, human MT; kb, kilobase(s).

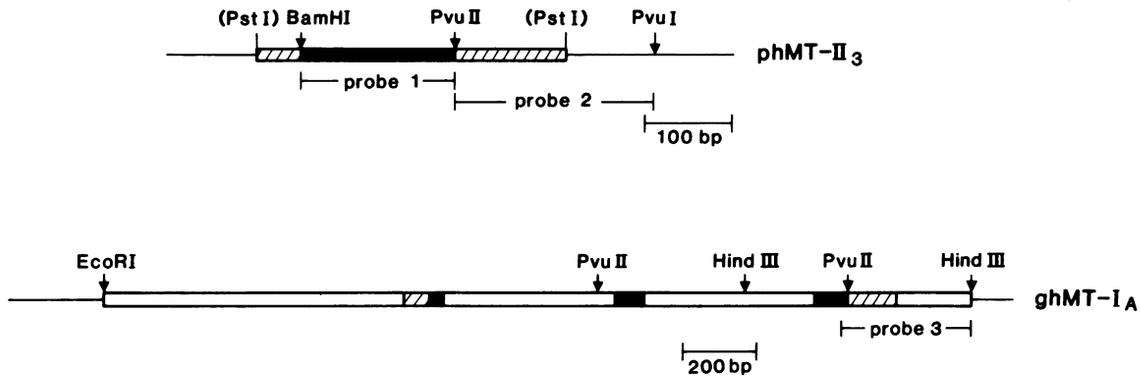

FIG. 1. Restriction maps of MT DNA probes phMT-II$_3$, a hMT-II mRNA cDNA clone (23), and ghMT-I$_A$, which contains the *MT1A* gene (13). ■, Protein-encoding regions; ▨, 5′ and 3′ untranslated regions; and □, both 5′ flanking regions and intron sequences. The fragments used to prepare the different probes are indicated. bp, Base pairs.

tional and nonfunctional genes. RNA blot hybridization also reduced the level of cross-hybridization between mouse and human sequences and background hybridization difficulties with some of the probes.

Cell hybrids were analyzed for their human chromosome complement by chromosome-specific enzyme markers and Giemsa/trypsin chromosome staining. We tried to perform all of the different analyses on hybrid cells derived from the same passage to minimize variation caused by loss of human chromosomes, which can occur during prolonged cultivation.

Three different probes were used for the mapping studies; they are described in Fig. 1. The first probe was derived from the coding region of the cDNA clone phMT-II$_3$ (23). This probe hybridized to most of the human *MT* genes (both *MT1* and *MT2*) and to a lower extent to the mouse genes (see Fig. 2). At the RNA level, under stringent washing conditions, this probe hybridized to human MT mRNA but not to mouse MT mRNA (see Fig. 4). The second probe was derived from the 3′ untranslated region of phMT-II$_3$ and is specific for the *MT2A* gene and its mRNA (13, 21). This probe was not very useful for the analysis of DNA blots because of high background hybridization. However, it gave satisfactory results when hybridized to RNA blots and was used to detect specific expression of hMT-II mRNA in the hybrids (see Fig. 4). The third probe is derived from the 3′ untranslated region of the *MT1A* gene (13). It was used to identify the presence of the gene (Fig. 3) and its mRNA in cell hybrids. It does not hybridize to hMT-II sequences and, therefore, is hMT-I specific (13).

DNA Blot-Hybridization Analysis. Genomic DNA was prepared from many human–mouse somatic cell hybrids, digested with *Hin*dIII (Fig. 2) or *Eco*RI (Fig. 3), and analyzed for the presence of hMT-specific bands by blot hybridization with probes 1 or 3, respectively. Probe 1 reacted with at least 12 different bands from human DNA (Fig. 2, lane 1) (12). Of these, the 3-kilobase (kb) band contains the *MT2A* functional gene (indicated by an arrow in Fig. 2), and most of the other bands presumably contain *MT1* genes (13). In mouse DNA (Fig. 2, lane 2), this probe hybridized to six different bands, which also appear in all of the somatic cell hybrids examined. However, on top of this background of mouse-specific bands, several bands corresponding in size to those present in human DNA were present in some hybrids but not in others. Of particular diagnostic value was the 3-kb band containing the *MT2A* gene (12), and this band was scored in the hybrids (two negative and two positive hybrids are shown in lanes 3–6 of Fig. 2). This band segregated in hybrids only with human chromosome 16 and its markers (see Table 1). Thus, the *MT2A* gene can be assigned to chromosome 16.

Several other bands, presumably containing *MT1* genes cosegregated with the 3-kb band. To examine this, we examined *Eco*RI-digested genomic DNA prepared from the hybrids with probe 3, which is derived from the *MT1A* gene. In human DNA this probe hybridized strongly to two bands and faintly to another one, of which the 13.5-kb band contains the *MT1A* gene (13). This probe did not hybridize to mouse DNA. When the presence of the 13.5-kb band (indicated by the arrow in Fig. 3) in the various hybrids was examined against their content of human chromosomes, segregation with chromosome 16 was observed (Table 1). Because the

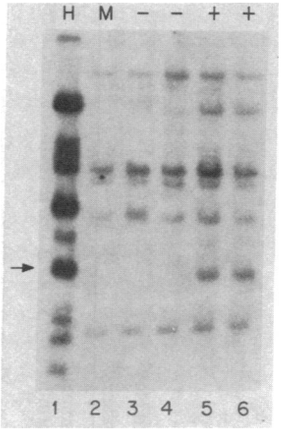

FIG. 2. Metallothionein II (*MT2*) in cell hybrids. The human probe 1 for *MT2* was hybridized to *Hin*dIII digests of cell hybrid DNA. The 3-kb fragment (arrow) hybridizing with the probe contains the functional gene; this fragment was not observed in mouse DNA. Lanes: 1, human (H) control; 2, mouse (M) control; 3 and 4, *MT2*-negative hybrids; 5 and 6, *MT2*-positive hybrids.

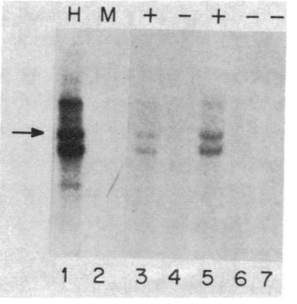

FIG. 3. Metallothionein I (*MT1*) in human, mouse, and hybrid cells. Human probe 3 for *MT1* was hybridized to *Eco*RI digests of cell hybrid DNA. Human DNA contains a 13.5-kb fragment containing the functional gene. No signal was observed for mouse DNA. Lanes: 1, human control; 2, mouse control; 3 and 5, *MT1* positive hybrids; 4, 6, and 7, *MT1*-negative hybrids.

Table 1. Distribution of human metallothionein genes (*MT2* and *MT1*) in somatic cell hybrids after DNA blot hybridization

Cell hybrid*	*MT2*†	*MT1*†	Presence or absence on human chromosome‡																							TL§
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
WIL-1	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	+	−	+	
WIL-5	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	+	−	+	
WIL-6	−	−	−	+	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	
WIL-7	−	−	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	
WIL-8		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	
WIL-8X	−	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-13	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	−	
WIL-14	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	+	+	−	+	−	−	−	−	−	+	
WIL-15	−	−	−	+	+	+	−	−	+	−	−	+	+	+	+	+	−	−	+	+	−	+	+	−	+	
REW-5		−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	+	+	
REW-7	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
REW-10	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	
REW-11	+		−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+	−	+	
REW-15		−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
NSL-15	+	+	+	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+	−	+	−	+	−	−	−	17/9, 12q$^+$
NSL-9	+	+	−	−	−	−	+	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	+	+	−	17/9
JWR-26C	+		−	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	−	+	+	−	+	1p$^-$
XER-7	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	−	−	−	+	11/X
XER-11	+	+	+	−	+	+	−	+	+	+	−	+	−	+	+	−	+	+	+	+	+	+	+	+	−	11/X, X/11
JSR-17S	+		+	−	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	7/9
EXR-9	+		+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	X/11, 11/X
DUM-13	+	+	+	+	+	−	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	−	X/15, 15/X
ALR-3	+		+	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	−	−	+	+	+	+	+	9/X
XTR-22	−	−	−	+	−	+	+	+	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	−	X/3
TSL-2	−	−	−	+	−	−	+	+	−	−	−	+	−	+	−	−	−	−	−	+	−	+	+	−	+	17/3
ATR-13	+		+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	5/X
MT2, % discordancy¶			22	57	52	52	52	48	43	57	30	52	48	35	35	48	30	0	61	57	48	35	57	30	61	
MT1, % discordancy¶			30	65	65	65	65	55	55	65	25	65	60	40	45	60	40	0	65	65	50	40	70	35	80	

*Reference to somatic cell hybrids is in *Materials and Methods*.

†*MT2* was determined by scoring the presence (+) or absence (−) of the 3-kb fragment after reaction with probe 1. *MT1* was determined by scoring the 13-kb fragment after reaction with probe 3.

‡Chromosome-specific enzyme markers confirmed the chromosome analysis. At least 30 metaphase spreads were examined for each hybrid. Presence (+) or absence (−) or a human chromosome is indicated. Negative hybrids retained no chromosome 16; positive hybrids retained from 17% to 60% of chromosome 16. Human female parental cells were used, eliminating the Y chromosome.

§Reference to human translocation (TL) chromosomes is in *Materials and Methods*.

¶Concordant hybrids either retained or lost *MT* and a specific human chromosome. Discordant hybrids have either retained *MT* but not a specific chromosome (+/−) or the reverse (−/+). % Discordancy indicates the degree of discordant segregation for a marker and a chromosome. No discordancy demonstrates chromosome assignment.

MT1A gene is present in a cluster of at least five *MT1* genes (13), these genes are also all present on human chromosome 16.

Several of the *Hin*dIII and *Eco*RI restriction fragments hybridizing to probe 1 in human DNA did not cosegregate with chromosome 16 in the hybrids. At least two of these fragments correspond to the two forms of *MT2B* processed pseudogenes (12). We assume that the other bands contain *MT1* processed pseudogenes. (Due to either overlap with mouse *MT* gene fragments or weak hybridization signals, we could not derive the chromosomal assignment of these processed pseudogenes.)

Gene Mapping by RNA Blot Analysis. We also assigned the human *MT* genes to chromosome 16 by RNA blot analysis. MTs were found to be expressed in human–mouse somatic cell hybrids. RNA was prepared from hybrids and analyzed for the presence of hMT mRNA by blot hybridization. This was examined to determine whether functional *MT* genes are present on more than a single chromosome, as some of the probe 1-reactive restriction fragments did not segregate with human chromosome 16 (see Fig. 2). To determine whether the human genes in hybrids are still subject to regulation as in normal human cells, we treated the hybrids prior to extraction of RNA with two inducers of MT mRNA: Cd^{2+} and dexamethasone (3, 4). A representative example of the results obtained by hybridization to probe 2 is in Fig. 4 (results are summarized in Table 2; the same scoring was obtained by hybridization to probes 1 and 3). hMT-I_A and hMT-II_A mRNAs were always expressed in the same hybrids. Based on their expression, both the functional *MT2A* and the *MT1A* genes were assigned to chromosome 16, and all hybrids that contain this chromosome expressed both *MT* genes (as determined by hybridization to probes 2 and 3). The expression of the *MT* genes in the hybrids is regulated by both glycocorticoids and heavy metal ions as it is in normal human cells (3, 4). Thus, the presence of the structural human *MT* genes in hybrid cells is sufficient to allow for their regulated expression. Similar results were obtained in transfection experiments with the cloned *MT2A* gene (21, 24).

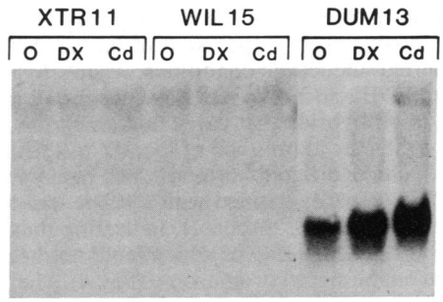

FIG. 4. *MT* gene mapping by RNA blot hybridization. Examples of a positive (DUM13) and two negative (XTR11, WIL15) hybrids are shown. Cytoplasmic RNA was extracted from control cultures (0) or cultures treated for 12 hr with either 1 μM dexamethasone (DX) or 5 μM Cd^{2+} (Cd). Samples (40 μg) were analyzed by electrophoresis on 1.5% agarose gel and blot hybridization (21, 22). This figure shows hybridization to probe 2. The same results were obtained when probes 1 or 3 were used (not shown).

DISCUSSION

Using the combined approach of DNA and RNA blot hybridization, we found that the functional members of the human *MT* gene family are clustered on chromosome 16. Analysis of genomic DNA from cell hybrids with a probe prepared from the coding region of hMT-II cDNA (probe 1) that hybridized to both *MT1* and *MT2* coding sequences revealed several different restriction fragments that segregate with human chromosome 16. One fragment contained the functional *MT2A* gene (12), and the functional *MT1A* gene (13) was revealed by hybridization to a probe (probe 3) prepared from the 3′ untranslated region of that gene.

Table 2. Distribution of human metallothionein genes in somatic cell hybrids after RNA blot hybridization

Cell hybrid*	*MT1* and *MT2*†	Chromosomes determined by enzyme markers‡																							TL§
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
ALR-3¶	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	−	−	+	+	+	+	+	9/X
DUM-13¶	+	+	+	+	−	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	−	X/15 15/X
REX-11 BsAgB¶	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	
TSL-2	−	−	+	+	−	+	+	−	−	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	
WIL-6	−	−	+	−	+	+	−	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	
WIL-8X	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-15	−	−	+	+	+	−	−	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+	−	+	
XTR-11 BsAgA¶	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	$20p^+$
JSR-1	+	−	−	−	+	−	−	−	+	+	−	−	−	+	−	−	+	−	+	−	−	−	−	+	
JSR-29¶	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7/9
REW-11	+	−	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	−	−	−	+	+	−	+	
SIR-1	−	−	−	−	+	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	
XER-7	−	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	
XER-11	+	+	−	+	+	−	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	
JSR-17S	+	+	+	+	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	
% discordancy‖		20	53	53	47	47	40	47	40	27	53	33	40	33	53	33	0	47	47	40	40	40	20	40	

*Reference to cell hybrids is in *Materials and Methods*.

†*MT1* and *MT2* were both identified by scoring the presence (+) or absence (−) of mRNA hybridizing respectively to probe 3 (MT-I_A-specific) and probe 2 (MT-II_A-specific). In all instances *MT1* and *MT2* were coordinately expressed in hybrids.

‡Chromosome-specific enzyme markers were used to identify human chromosomes. Human female parental cells were used, eliminating the Y chromosome. Hybrids identified by ¶ were tested for both chromosomes and enzyme markers. Hybrids tested in this table have later cell passages than corresponding hybrids in Table 1 and occasionally retained a slightly different human chromosome complement. Chromosome 16 results were identical for the same cell hybrids in Tables 1 and 2. All markers were examined on the same cell passage.

§Reference to translocation (TL) chromosomes is in *Materials and Methods*.

‖% discordancy indicates the percentage the *MT* genes do not cosegregate with a specific human chromosome; see the legend to Table 1.

To confirm and map functional *MT* genes, the same hybrids were analyzed for expression of human MT mRNAs under different induction conditions. Using hybridization probes for MT-II and MT-I_A mRNAs, we found them to be expressed in all hybrids that carry human chromosome 16. In these hybrids the expression of the MT mRNAs is inducible by both glucocorticoid hormones and heavy metal ions. Identical results were obtained with a probe that hybridizes to all MT RNA species (probe 1), indicating that all of the expressed *MT* genes, some of which have not been characterized, are on the same chromosome (Fig. 4). Therefore, the restriction fragments that react with this probe (Fig. 2) but do not segregate with chromosome 16 must represent nonfunctional genes. Most likely, these are processed pseudogenes similar to the *MT2B* gene we described earlier (12). Since processed pseudogenes are derived from reverse transcripts of MT mRNA, which apparently integrate at random into genomic DNA, they are likely to be dispersed.

This combined approach allowed us to assign without doubt the functional human *MT* gene cluster to chromosome 16. Such a dual approach should be useful for mapping any gene that is expressed and whose structural sequences segregate in somatic cell hybrids. This approach should diminish difficulties that might be derived from a complex hybridization pattern for genomic DNA, especially in cases where a gene family contains a large number of dispersed pseudogenes, which are not expressed but contribute to the overall hybridization pattern.

The localization of the *MT* gene cluster to chromosome 16 in man rules out the involvement of strucutral or regulatory mutations at this locus in the etiology of Menkes kinky hair syndrome because this defect is X chromosome-linked (7). There is still a possibility, however, that the X chromosome contains a regulatory gene whose *trans*-acting product affects the expression of *MT* genes. The *MT* genes themselves, however, could be involved in Wilsons disease and in acrodermatitis enteropathica since both are autosomal defects (8, 9). Family studies of these diseases and analysis of *MT* gene expression in mutant fibroblasts should elucidate the possible involvement of the *MT* gene family in the defects.

An autosomal recessive gene (*Cdm*) determining resistance to cadmium-induced testicular necrosis has been demonstrated in inbred mouse strains (10). This gene was assigned to mouse chromosome 3 (25). It also was shown that this gene affects the extent of induction of MTs by heavy metals (10) but is not directly linked to the *MT1* locus in mice, since the latter has recently been mapped to chromosome 8 (26). Genes controlling resistance to heavy metal toxicity have not been described in man; however, due to the growing significance of Cd and Hg as industrial and environmental pollutants, it is important to search for such alleles and investigate their relationships to the *MT* gene cluster.

Previously, studies have suggested a region of homology between human chromosome 16 and a region on mouse chromosome 8 (27). The genes glutamicoxaloacetic transaminase-2 (*GOT2*), chymotrypsinogen-B (*CTRB*), and adenine phosphoribosyltransferase (*APRT*) have been assigned to human chromosome 16 and mouse chromosome 8. In human, *GOT2* and *APRT* have been assigned to the q12→q22 region of chromosome 16. In mouse, evidence is not available to show close linkage. Now that human and mouse *MT* genes have been assigned to human chromosome 16 and mouse chromosome 8, this generates speculation that they may reside in this evolutionarily conserved chromosomal region. If this is the case, then it is expected that the *MT* genes will be located on the long arm of human chromosome 16 near or within the q12→q22 region.

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