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Human Metallothionein Genes: Structure of the Functional Locus at 16q13

A. K. WEST,*,[1] R. STALLINGS,† C. E. HILDEBRAND,† R. CHIU,‡ M. KARIN,‡ AND R. I. RICHARDS§

*Department of Biochemistry, University of Tasmania, Australia; †Genetics Group, Life Sciences Division, Los Alamos National Laboratory, New Mexico; ‡School of Medicine, University of California, San Diego, California; and §Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, South Australia, Australia

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The functional human metallothionein (*MT*) genes are located on chromosome 16q13. We have physically mapped the functional human *MT* locus by isolation and restriction digest mapping of cloned DNA. The mapped region contains all sequences on chromosome 16 that hybridize to metallothionein gene probes and comprises 14 tightly linked *MT* genes, 6 of which have not been previously described. This analysis defines the genetic limits of metallothionein functional diversity in the human genome.

INTRODUCTION

Metallothioneins (MTs) are small, cysteine-rich proteins that bind heavy metals (reviewed in Kagi and Kojima, 1987). They are found in all animals and have been implicated in the detoxification and homeostasis of heavy metals (Hamer, 1986), although other roles have been postulated (Karin, 1985). Several closely related isoforms of *MT* exist and the number present in humans is unknown, although at least 6 isoforms have been described in human liver (Hunziker and Kagi, 1985). Southern blot analysis shows that these proteins are encoded by a multigene family of at least 12–15 members (Karin and Richards, 1982b) which are distributed to 5 autosomes (Schmidt *et al.*, 1984). A locus on chromosome 16 that contains all functional human *MT* genes (Karin *et al.*, 1984) has been partially mapped (Richards *et al.*, 1984; Varshney *et al.*, 1986) and it is likely that the chromosomal loci external to this site are inactive processed pseudogenes such as *MT2B* (Karin and Richards, 1982b), which is found on chromosome 4 (Schmidt *et al.*, 1984). The functional *MT* locus was originally assigned to 16q22 by *in situ* hybridization (Le Beau *et al.*, 1985) but more recent reports suggest a location at 16q13 (Simmers *et al.*, 1987; Sutherland *et al.*, 1990).

The complete nucleotide sequence of several human *MT* genes has been determined and their expression pattern characterized in detail (Karin and Richards, 1982b; Richards *et al.*, 1984; Schmidt *et al.*, 1985; Schmidt and Hamer, 1986; Heguy *et al.*, 1986; Sadhu and Gedamu, 1988; Foster *et al.*, 1988), but the total number of genes, both functional and nonfunctional, and their organization in the locus on chromosome 16 have not been investigated.

In this report, we present the structure of the functional human locus on chromosome 16. The locus contains 14 linked *MT* genes within an 82.1-kb region, which includes all expressed human *MT* genes identified to date.

MATERIALS AND METHODS

Isolation of Genomic Clones

Lambda and cosmid clones used in this study were obtained by screening four genomic libraries and a chromosome 16-specific cosmid library (Deaven *et al.*, 1986; Langmire *et al.*, in preparation) with several *MT* gene-specific probes. Charon 4a clones (λs 1, 4, 35, 39, 42, 47, 48, 66, 69, 74, and 95) were isolated from the library of Lawn *et al.* (1978) using a general *MT* gene probe derived from a *MT2A* cDNA clone (Karin and Richards, 1982a). EMBL-3 clones, propagated in the *recA recB recC* host DB1255 (kindly supplied by Dr. D. Botstein), were obtained from a primary plating of a library constructed by B. A. Evans: clones λDB1 and 7 were selected using an 800-bp *Bam*HI/*Hin*dIII restriction fragment containing the 5′ flanking region of *MT2A* as a probe (Karin and Richards, 1982b); clone λDB10 was isolated using a probe derived from a 220-bp *Sac*I/*Apa*I fragment from intron 1 of *MT1E* (Schmidt *et al.*, 1985). Cosmid clones were isolated by screening two libraries provided by Choo

[1] To whom correspondence should be addressed at Department of Biochemistry, G.P.O. Box 252C, Hobart, Australia 7001.

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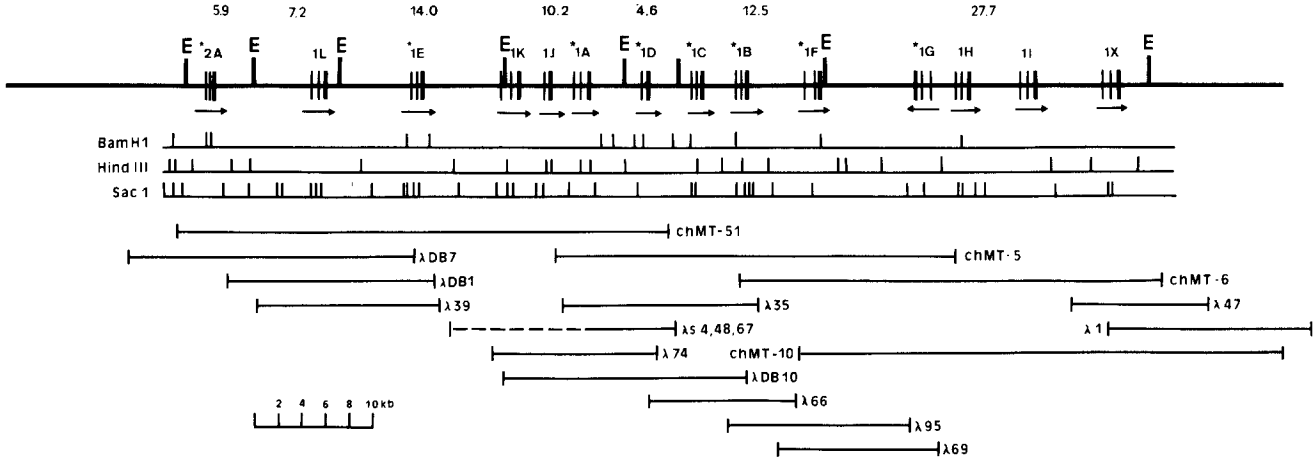

FIG. 1. Physical map of the human metallothionein locus on chromosome 16. The restriction map of the locus was derived from analysis of lambda and cosmid genomic clones as shown: *Bam*HI, *Hin*dIII, and *Sac*I cleavage sites are indicated below and *Eco*RI sites are designated within the map (E). The size (kb) of each *Eco*RI fragment is shown above. The position and transcriptional orientation of the *MT* genes (arrows) were established by use of exon-specific probes (exons are represented by vertical bars and are arbitrarily positioned within the smallest hybridizing restriction fragment): 2 genes (*MT1J* and *MT1D*) did not hybridize to the exon 1-specific probe. *MT* genes characterized previously are indicated by an asterisk. λs 4, 48, and 67 are not depicted individually—the structure of their putative parental clone is shown and the region in which deletions were observed is dotted (see Fig. 3 and text).

et al. (1986), one in the vector pCV001 which yielded chMT-10 (isolated by J. Savige) and one in the vector pAVCV007 from which chMT-5 and chMT-6 were isolated. Cosmid chMT-51 was isolated from a chromosome 16-specific cosmid library, based on the vector sCos-1 (Evans *et al.*, 1989). In all, approximately 18 genomic equivalents were screened.

λDNA was prepared by a "mini-prep" procedure described earlier (Richards *et al.*, 1984), and cosmid DNA was isolated by the alkaline lysis method (Maniatis *et al.*, 1982). Restriction fragments bearing *MT* genes were subcloned into pUC13 or pUC19 and restriction analysis was carried out by standard techniques.

Southern Blots

Genomic Southern blotting was performed by a modification of the alkaline-blot procedure of Reed and Mann (1985). Human placental DNA (10–20 μg) was digested to completion with *Eco*RI and electrophoresed through a 0.75% agarose gel in 2× TBE (100 m*M* Tris–HCl, pH 8.3, 84 m*M* boric acid, 2 m*M* EDTA). After ethidium bromide staining and photography, the DNA was transferred to Zeta-probe nylon membranes (Bio-Rad) using 0.4 *M* NaOH as the blotting medium. After two rinses in 2× SSC (20× is 3 *M* NaCl, 0.3 *M* Na citrate), the membrane was prehybridized at 60°C in 1.5× SSPE (0.27 *M* NaCl, 15 m*M* sodium phosphate, pH 7.0, 15 m*M* EDTA), 1% SDS, and 0.5% milk powder (Diploma nonfat). After 6 h, a radiolabeled probe (approximately 10^9 cpm/μg) was added and hybridization continued for 24 h at 68°C. The membrane was washed three times in 2× SSC at room temperature and then in 0.1× SSC, 1% SDS at increasing temperatures up to 65°C. High stringency washes were continued until excess counts on the membrane were reduced to background. Autoradiography was performed at room temperature for 1–3 days using Kodak X-AR film.

Exon-Specific Probes for Human MT Genes

The nucleotide sequences of all available human *MT* genes were compared to derive consensus sequences unique for each of the three exons. Complementary oligodeoxyribonucleotides were synthesized for each of these sequences (exon 1, 5′ CTGGAGCAGTTGGGGTCCAT 3′; exon 2, 5′ CTTCTTGCAGGAGGTGCATTTGCA 3′; exon 3, 5′ GAGCCCACGGGGCAGCAGGAGCAGCAGC 3′). After end-labeling and hybridization under conditions described by Ullrich *et al.* (1984), these probes enabled mapping of specific exons to restriction fragments.

RESULTS AND DISCUSSION

Lambda and cosmid clones containing *MT* genes were obtained by screening four human genomic libraries and a chromosome 16-specific cosmid library with a cDNA clone of *MT2A* (Karin and Richards, 1982a), a probe that is likely to hybridize to all members of the *MT* gene family. Alternatively, EMBL-3 clones were identified with two region-specific probes as described under Materials and Methods. Sixty-two positive clones survived several rounds

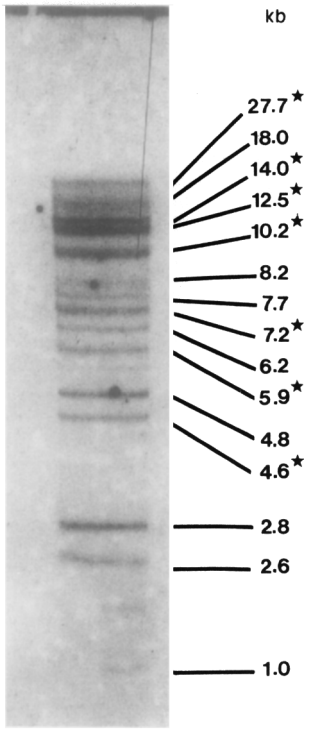

FIG. 2. Southern blot analysis of the human *MT* locus. *Eco*RI-digested genomic DNA was fractionated by electrophoresis through a 0.75% agarose gel and then blotted onto a nylon membrane. The membrane was hybridized to the *MT2A* cDNA probe: 15 major bands can be visualized. In particular, the upper section of the autoradiograph can be resolved into 5 hybridizing fragments of 27.7, 18.0, 14.0, 12.5, and 10.2 kb. An asterisk (*) denotes *Eco*RI fragments that are derived from the chromosome 16 locus (see text and Fig. 1).

of purification and were sorted into two groups on the basis of the number of restriction fragments that hybridized to the general *MT* probe (data not shown). One group, which probably comprised clones of processed pseudogenes, gave rise to only one hybridizing restriction fragment following digestion with 6-bp restriction enzymes suggesting that one, intron-less gene was present. Sequence analysis of one such λ clone, λ42, confirmed that the hybridizing region was a processed pseudogene (data not shown), which showed marked homology to the transcribed portions of the functional gene *MT1F* (Schmidt *et al.*, 1985). Two examples of human *MT* processed pseudogenes have been described previously (Karin and Richards, 1982b; Varshney and Gedamu, 1984). The remaining clones had several hybridizing fragments, indicating the likelihood of introns and, in most cases, that multiple *MT* genes were present. Restriction mapping and the use of end fragment probes demonstrated that clones from this latter group overlapped and represented a region of 82.1 kb which contained 14 tightly linked *MT* genes (Fig. 1). Exon-specific oligodeoxyribonucleotide probes were used to map individual exons to show that all genes, with the exception of *MT1G*, share the same orientation. Following subcloning into plasmid vectors, each gene was identified by detailed restriction mapping or by partial or complete nucleotide sequencing. The structure and expression of some of these genes have been reported previously: the functional genes *MT2A* (Karin and Richards, 1982b), *MT1A* (Richards *et al.*, 1984), *MT1B* (Heguy *et al.*, 1986), *MT1E* and *MT1F* (Schmidt *et al.*, 1985), and *MT1G* (Varshney *et al.*, 1986); and the nonfunctional genes *MT1C* and *MT1D* (Richards *et al.*, 1984). This work has therefore identified 6 novel *MT* genes, *MT1H*, *MT1I*, *MT1J*, *MT1K*, *MTIL*, and *MT1X*. The presence of *MT2A* and *MT1A* in the cluster confirms that the region of DNA illustrated in Fig. 1 is derived from the functional *MT* locus on chromosome 16 (Karin *et al.*, 1984). Interestingly, one Charon 4a clone described here, λ69, appears to be similar to a clone 14VS reported by Varshney *et al.* (1986). Since both clones were isolated from separate platings of the same Charon 4a library (Lawn *et al.*, 1978), it is probable that λ69 and 14VS represent different isolates of the same clone.

The restriction map presented here accounts for seven intact *Eco*RI fragments that contain *MT* genes from the functional locus. We would therefore expect the *MT2A* cDNA probe to hybridize to bands on a genomic Southern blot at 27.7, 14.0, 12.5, 10.2, 7.2, 5.9, and 4.6 kb. This prediction can be compared to the results of Schmidt *et al.* (1984), who performed Southern blots on DNA from rodent/human somatic cell hybrids and were able to assign *Eco*RI fragments containing *MT* genes to specific human chromosomes. These workers concluded that *MT* genes from chromosome 16 were located on five *Eco*RI restriction fragments of 25, 14.5, 10.2, 6.8, and 5.9 kb which, excepting the omission of the 4.6-kb fragment and the 12.5-kb fragment containing *MT1B*, is in good agreement with the present work. The 4.6-kb fragment containing *MT1D* was not detected by Schmidt *et al.* (1984) because it was consistently obscured by a 4.6-kb fragment from chromosome 4 that contains the processed pseudogene *MT2B:* each hybrid used by these workers that retained chromosome 16 also had chromosome 4. To reconcile the mapping data with the structure of the locus *in situ*, particularly with respect to the predicted 14.0-, 12.5-, and 10.2-kb *Eco*RI fragments, a Southern blot experiment was performed. The high-resolution blotting technique of Reed and Mann (1985) was used and, as shown in Fig. 2, demonstrates that the *Eco*RI fragment assigned to 14.5 kb in earlier reports (Karin and Richards, 1982b; Schmidt *et al.*, 1984; Varshney *et al.*, 1986) can be resolved as two discrete bands of 14.0 and 12.5 kb. To

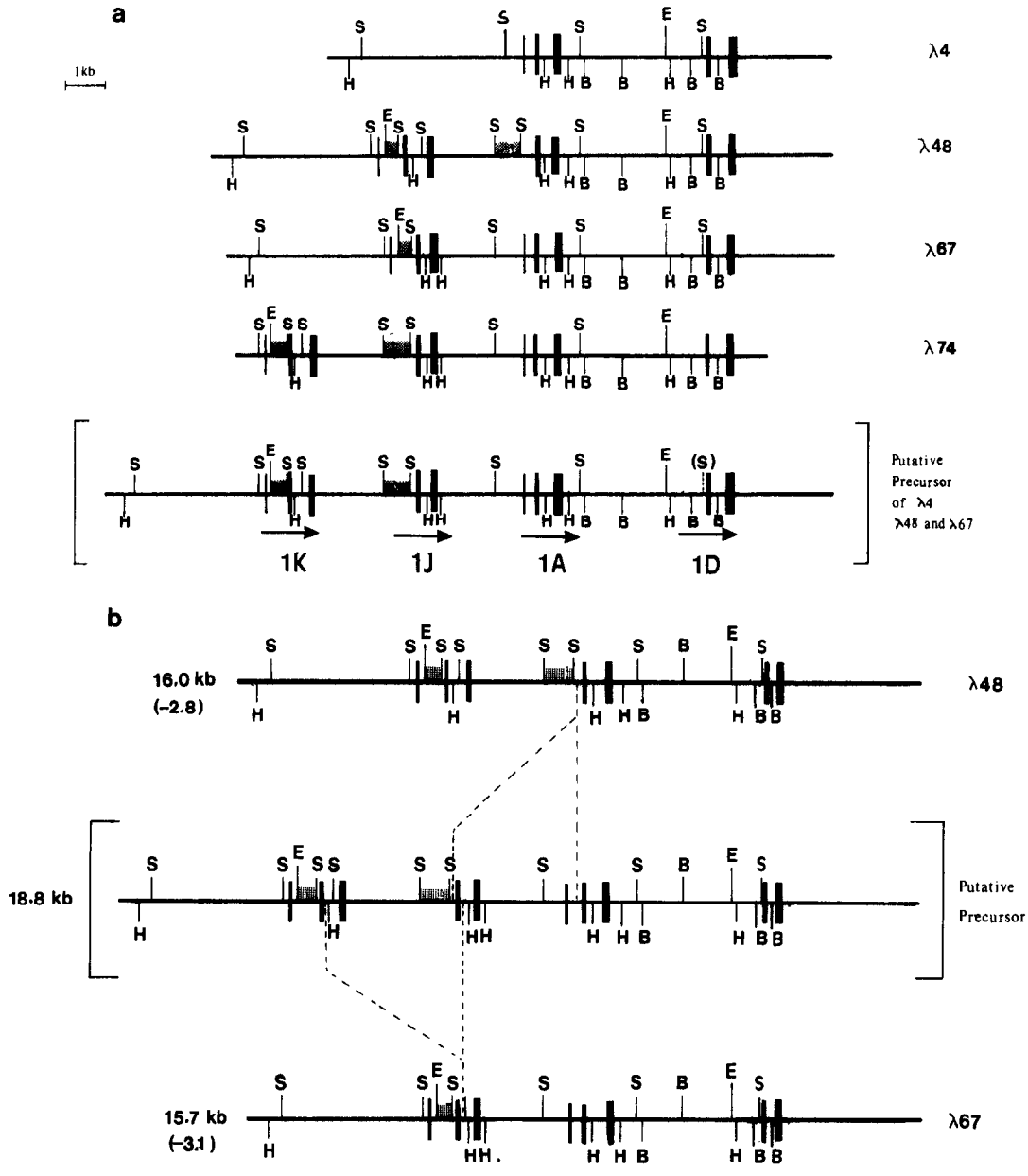

FIG. 3. Restriction analysis of λ4, 48, 67, and 74. (**a**) The structure of Charon 4a clones λ4, 48, 67, and 74. Analysis of the restriction map of these clones was aided by use of exon-specific oligodeoxyribonucleotide probes and by use of a restriction fragment probe, *Sac*I–*Apa*I fragment from intron 1, *MT1E* (22), which hybridized to homologous regions of *MT1K* and *MT1J* (speckled). The polymorphic *Sac*I site, present in λ4, 48, and 67 but not in λ74 (or λDB10) is depicted in parentheses. The structure of the three clones λ4, 48, and 67 suggests that they are derived from a precursor clone which has undergone deletions of 5.8, 2.8, and 3.1 kb, respectively. The structure of the putative precursor clone is predicted from a comparison of λ4, 48, and 67 with λ74. (**b**) λs 48 and 67 may have arisen following deletions (dotted lines) of 2.8 and 3.1 kb, respectively, from the putative precursor clone. A similar argument can be constructed for the origin of λ4.

demonstrate that these new bands and the 10.2-kb band were not artifacts and to confirm their origin from the functional *MT* locus on chromosome 16, the Southern blots were reprobed with radiolabeled restriction fragments identified from the restriction maps of this region. Under stringent hybridization conditions, each of these probes cross-reacted specifically with the predicted *Eco*RI fragment consistent with the structure shown in Fig. 1 (data not shown).

The relative intensities of the *Eco*RI bands in Fig. 2 do not correlate strongly with the number of *MT* genes mapped to each restriction fragment, suggesting that other factors such as sequence divergence between individual *MT1* genes and the *MT2A* cDNA

probe and the efficiency of Southern transfer were important. Indeed, the relative intensities of *MT* *Eco*RI fragments are not always constant between different experiments (e.g., see Sutherland *et al.*, 1990) but this is most likely due to variables such as transfer method, hybridization stringency, and the particular *MT* probe used. It is not possible, however, to rule out interindividual variation in *MT* gene copy number, although we feel that this is unlikely because mapping of overlapping genomic clones from five independent libraries gave consistent results with regard to copy number.

A pertinent observation about the nature of the locus around *MT1K* was made during the analysis of a group of clones from this region—λ clones 4, 48, and 67. The inserts of these Charon 4a clones had identical left and right termini and shared a polymorphic *Sac*I site absent in independent, overlapping clones (e.g., λ74 and λDB10), but each differed in their internal structure (Fig. 3). Comparison of their restriction maps suggested that these clones are each derived from a common parental precursor phage that has undergone a variety of internal deletions between homologous regions during amplification of the original library. The structure of the parental phage can be surmised from the composite structures of λ4, 48, and 67 and from the restriction maps of λ74 and λDB10, which include part of the same region. The restriction map of this region was confirmed following the subsequent analysis of the independent cosmid clone chMT-51. The unstable nature of some genomic clones during propagation in standard *Escherichia coli* hosts has been noted previously (Wyman *et al.*, 1985).

It is likely that Fig. 1 represents the entire functional *MT* locus. DNA extending 17 kb upstream of *MT2A* and 16.4 kb downstream of *MT1X* has been cloned but no further *MT* genes were identified (data not shown). Given the tight linkage within the gene cluster, it is probable that *MT2A* and *MT1X* are the terminal members of the locus. In support of this, the cloned region accounts for all *MT*-gene-bearing *Eco*RI fragments from chromosome 16 identified by Schmidt *et al.* (1984). In addition, all functional *MT* genes characterized so far have been assigned in this work. It is highly unlikely that any functional *MT* genes remain undetected by hybridization to the *MT2A* cDNA probe because of the high level of amino acid (and nucleotide) sequence conservation of all known metallothioneins (Kagi and Kojima, 1987) and the observation that divergent nonfunctional *MT* pseudogenes within the functional *MT* locus (e.g., *MT1D;* Richards *et al.*, 1984) were readily detected by hybridization.

Elucidation of the structure of the functional *MT* locus will permit the continuing analysis of the molecular biology and physiology of human metallothioneins. It will be possible to design a complete panel of gene-specific probes to investigate tissue- and developmental-dependent expression of each functional member, analogous to the studies with *MT1F* and *MT1E* reported by Schmidt and Hamer (1986). This information may assist in determining the physiological role of metallothionein. Furthermore, it will now be possible to investigate regulatory mechanisms such as those involving chromatin structure and methylation which may operate on the locus as a whole. The completion of the map of the functional human *MT* locus sets an upper limit of 12 possible functional genes encoding metallothioneins. The physiological requirement for this large number of genes is not clear. Potential differences in function of the encoded proteins may now be addressed by manipulated expression of individual cloned genes (Clouston *et al.*, 1990). This will overcome problems encountered in attempts to purify individual *MT* proteins for functional studies. Of particular interest in this regard will be the most divergent *MT* genes in the locus which appear to be coincidentally located at the extremities of the gene cluster (i.e., *MT2A* and *MT1X;* West and Richards, unpublished data).

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