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cDNA cloning and nucleotide sequence comparison of Chinese hamster metallothionein I and II mRNAs

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ABSTRACT

Polyadenylated RNA was extracted from a cadmium resistant Chinese hamster (CHO) cell line, enriched for metal-induced, abundant RNA sequences and cloned as double-stranded cDNA in the plasmid pBR322. Two cDNA clones, pCHMT1 and pCHMT2, encoding two Chinese hamster isometallothioneins were identified, and the nucleotide sequence of each insert was determined. The two Chinese hamster metallothioneins show nucleotide sequence homologies of 80% in the protein coding region and approximately 35% in both the 5' and 3' untranslated regions. Interestingly, an 8 nucleotide sequence (TGTAAATA) has been conserved in sequence and position in the 3' untranslated regions of each metallothionein mRNA sequenced thus far. Estimated nucleotide substitution rates derived from interspecies comparisons were used to calculate a metallothionein gene duplication time of 45 to 120 million years ago.

INTRODUCTION

Metallothioneins are highly evolutionarily conserved, metal-binding proteins which have been implicated in the basic cellular processes of zinc homeostasis and heavy metal detoxification (1). The two polymorphic forms of metallothionein, metallothionein I (MT-I) and metallothionein II (MT-II), are metal-inducible in the same cells (1,2) and have been conserved evolutionarily in mammals (1), but differ in amino acid sequences and metal-binding properties (1,3,4). Although the specific function of each isometallothionein remains to be determined, the evolutionary conservation of both isometallothioneins and their characteristic physiocochemical differences suggest that each isometallothionein may play a distinct role in metal metabolism.

To study the evolutionary conservation of the two isometallothioneins and their mRNAs, as well as to derive isometallothionein gene-specific DNA probes for molecular analyses of the mechanisms underlying cellular metal resistance, we have isolated and determined the nucleotide sequence of two cDNA clones, pCHMT1 and pCHMT2, encoding two Chinese hamster isometallo-

thioneins. In this paper, the mRNA sequences of MT-I and MT-II are compared and discussed with respect to the evolutionary conservation of both metallothionein proteins and mRNAs, and the search for metallothionein gene-specific nucleotide sequences which may be involved in metallothionein mRNA expression.

MATERIALS AND METHODS

Construction of Recombinant cDNA Plasmids

Polyadenylated cytoplasmic RNA was extracted from cadmium-induced Cd^r20F4 Chinese hamster cells, and a 300-500 nucleotide (NT) long RNA fraction enriched in metal-induced, abundant sequences was purified by methyl-mercury agarose gel electrophoresis as previously described (5). Double-stranded cDNA synthesized from the size-selected RNA (6,7) was inserted into the Bam HI site of pBR322 by AT tailing with terminal transferase (8). The recombinant plasmids were introduced into E. coli HB101 cells by the method of Mandel and Higa (9). A total of 206 recombinant DNA-containing bacterial clones were identified by their respective antibiotic and cadmium resistances (10).

Selection and Characterization of MT-I and MT-II cDNA Clones

^{32}P-labelled DNA complementary to metal-induced, abundant RNA sequences (cDNAa) was synthesized from Cd^r20F4 Chinese hamster cell polyadenylated RNA (5,6) and used as a DNA probe for in situ colony hybridization by the method of Thayer (11). The plasmid DNA from one colony (pCd^r2) of the 47 colonies which hybridized with cDNAa was isolated (12) and identified by hybrid-enhanced cell-free translation (13,14) to be complementary to metallothionein mRNA (data not shown). Using the technique of Maxam and Gilbert (15), the DNA sequence of the pCd^r2 insert was determined to encode the 3' end of metallothionein-II like mRNA, and two Hpa II-Alu I restriction fragments (Fig. 1B) representing distinct sequences of a metallothionein protein encoding region (62 NT fragment) and the 3' untranslated region (50 NT fragment) were defined. The 62 NT and 50 NT restriction fragments were radiolabelled and individually hybridized to replicate filters (11) containing the 47 cDNA clones which had previously hybridized cDNAa. Based on differential colony hybridization to these two DNA probes, three classes of colonies were delineated. Class 1 hybridized both DNA probes and represented a set of MT-II-like cDNA inserts, class 2 hybridized only the 3' untranslated region DNA probe and represented incompletely copied MT-II-like cDNA inserts, and class 3

hybridized only the protein-encoding region DNA probe and represented MT-I-like cDNA inserts. The largest plasmids from class 1 (pCHMT2) and class 3 (pCHMT1), were characterized by their restriction maps. Then the nucleotide sequence of each indicated restriction fragment (Fig. 1A and 1B) was determined by the technique of Maxam and Gilbert (15), with the addition of the thymidine residue reaction as described by Rubin and Schmid (16).

RESULTS

Representation of Metallothionein cDNA Clones

Cadmium-induced, abundant, polyadenylated RNA sequences comprise approximately 3-5% of the total polyadenylated RNA extracted from maximally cadmium-induced Cd^r20F4 Chinese hamster cells, and are enriched approximately 10-fold more in a size-fractionated, 300-500 nucleotide long RNA class (5). Accordingly, when cDNA synthesized from this RNA class was cloned as recombinant DNA plasmids, 23% (47/206 clones) of the transformed bacterial colonies were shown by *in situ* hybridization to contain DNA sequences complementary to induced, abundant polyadenylated RNA (data not shown). This 23% frequency of occurrence agrees reasonably well with the maximally expected 30 to 50% frequency.

Of the 47 colonies which hybridized cDNAa, 34 (72%) of the colonies also hybridized the metallothionein specific DNA probes from pCd^r2. Class 1 contained 9 clones, class 2 contained 5 clones, and class 3 contained 20 clones (data not shown).

Characterization of MT-I and MT-II cDNA Clones

The two selected cDNA clones, pCHMT1 and pCHMT2, were characterized by differences in their restriction maps, nucleotide sequences, and decoded amino acid sequences (Fig. 1A and 1B). Since the CHO metallothionein I and II amino acid sequences have not been determined previously, designation of the two cDNA clones as representatives of MT-I and MT-II mRNAs depended upon a comparison of the decoded amino acid sequence with the known mouse MT-I and MT-II amino acid sequences. Murine metallothioneins were chosen as the standard for comparison for two reasons; both MT-I and MT-II amino acid sequences from mouse are defined (1) and Chinese hamster is evolutionarily closer to mouse than to other species with known MT-I and MT-II amino acid sequences (1). If the two cDNA sequences each encode a different isometallothionein, then each should show greater amino acid sequence homology to its mouse counterpart than to the other Chinese

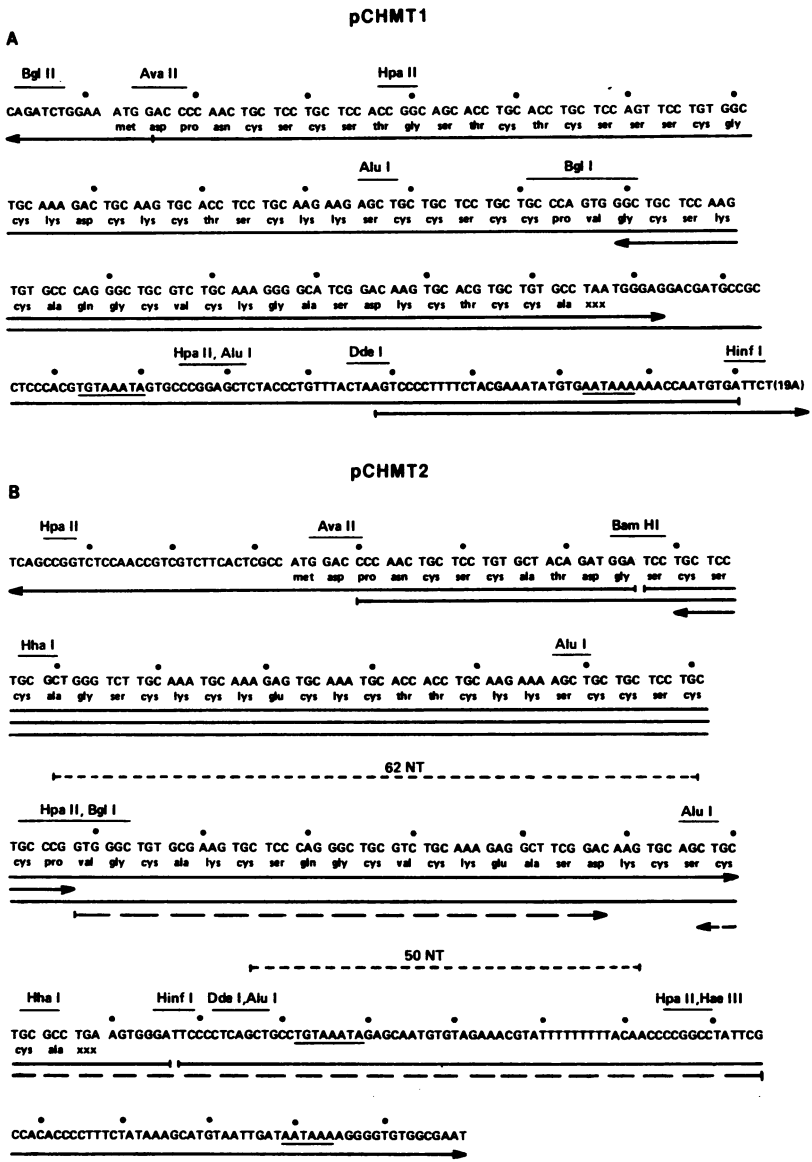

Figure 1. DNA sequences and decoded amino acid sequences of pCHMT1 (A), pCHMT2 (B), and pCd 2 (B). Solid arrows (⟵) indicate the direction and extent of DNA sequencing for pCHMT1 and pCHMT2. Dashed arrows (⟵ - - -) indicate the direction and extent of DNA sequencing for pCd 2. Dotted lines (----) indicate the protein coding region and 3' untranslated region DNA probes derived from pCd 2. Restriction endonuclease enzyme cleavage sites confirmed by experimental analysis are indicated. Both TGTAAATA sites and AATAAA signals are underlined.

hamster metallothionein or the other mouse metallothionein. As summarized in Table 1, the decoded amino acid sequence from pCHMT1 showed greater homology to mouse MT-I (56/61; 92%) than to mouse MT-II (45/61; 74%) or to pCHMT2 (47/61; 77%). The decoded amino acid sequence from pCHMT2 showed greater homology to mouse MT-II (55/61; 90%) than to mouse MT-I (48/61; 79%) or to pCHMT1 (47/61; 77%). By these criteria, pCHMT1 represents a Chinese hamster metallothionein I mRNA and pCHMT2 represents a Chinese hamster metallothionein II mRNA. Obviously, the absolute characterization of Chinese hamster MT-I and MT-II cDNA clones awaits comparison with the metallothionein amino acid sequences derived from Chinese hamster.

Both pCHMT1 and pCHMT2 represent nearly full-length copies of their respective mRNAs. Based on a previous approximation of 400 nucleotides for the length of abundant, induced RNA from Chinese hamster (5) and based on the 391 and 372 nucleotide lengths of mouse MT-I mRNA and human MT-II mRNA (17,18,19,20), respectively, pCHMT1 and pCHMT2 represent up to 89% of the full mRNA sequence. The plasmids pCHMT1 and pCHMT2 contain 323 and 349 nucleotides of mRNA sequence, respectively. Though neither cDNA clone may contain a complete 5' untranslated region, both cDNAs appear to have complete 3' untranslated regions. Both cDNAs contain a poly (A) addition signal (AATAAA) at 15 nucleotides from the 3' terminus, and the pCHMT1 sequence also contains a poly (A) tail. Definition of the complete mRNA sequences will be derived from genomic clones encompassing these two genes.

Intraspecies and Interspecies Nucleotide Sequence Comparisons

The intraspecies and interspecies nucleotide sequence homologies for each region of the MT mRNA were examined. As seen in Table 1, the MT-I nucleotide sequences of mouse (17,18,19) and Chinese hamster show the following interspecies homologies: 91% in the protein coding region, 82% in the 5' untranslated region, and 55% in the 3' untranslated region. The MT-II nucleotide sequences of human (20) and Chinese hamster show the following interspecies homologies: 87% in the protein coding region, 67% in the 5' untranslated region, and 65% in the 3' untranslated region. However, the intraspecies nucleotide sequence comparison of Chinese hamster MT-I and MT-II show the following lower homologies: 81% in the protein coding region, 36% in the 5' untranslated region, and 37% in the 3' untranslated regions. Thus each Chinese hamster MT shows greater interspecies nucleotide sequence homology with a mouse or human counterpart than intraspecies nucleotide sequence homology with the other Chinese.

TABLE I
AMINO ACID SEQUENCE HOMOLOGY

	Homology	%
CHO MT I vs Mouse MT I	56/61	92
CHO MT II vs Mouse MT II	55/61	90
CHO MT I vs CHO MT II	47/61	77
Mouse MT I vs Mouse MT II	46/61	75
CHO MT I vs Mouse MT II	45/61	74
CHO MT II vs Mouse MT I	48/61	79

NUCLEOTIDE SEQUENCE HOMOLOGY

	5' Untranslated Region		Protein Coding Region		3' Untranslated Region	
	Homology	%	Homology	%	Homology	%
CHO MT-I vs						
Mouse MT-I	9/11	82	167/183	91	60/110	55
CHO MT-II	4/11	36	149/183	81	41/110	37
Human MT-II	3/11	27	156/183	85	53/110	49
CHO MT-II vs						
Human MT-II	22/33	67	160/183	87	87/133	65
CHO MT-I	4/11	36	149/183	81	41/110	37
Mouse MT-I	14/33	32	144/183	78	66/133	50

hamster MT. As expected from the amino acid sequence homology, the greatest nucleotide homology is seen between the MT-I protein coding regions of mouse and Chinese hamster and MT-II protein coding regions of human and Chinese hamster. Consistent with data from other multigenic families (e.g., ref. 21,22,23), the 3' and 5' untranslated regions show an overall lower level of interspecies homology. Surprisingly, the MT-I 5' untranslated regions of mouse and Chinese hamster show more extensive interspecies homology than expected, although the comparison was only made with the 11 nucleotides presently available from the Chinese hamster MT-I sequence.

Although the 3' untranslated regions of the two metallothioneins show little overall sequence homology, short regions of sequence homology can be aligned. For example, the sequence TGTAAATA is conserved in location within the 3' untranslated region of both Chinese hamster metallothionein mRNA sequences and within the mouse MT-I (17,18,19) and human MT-II (20)

mRNA sequences. This octanucleotide begins at nucleotide 24 to 26 3' relative to the termination codon of the metallothionein mRNAs and only human MT-II shows a single nucleotide substitution to TGTAAAGA. By computer analysis of the nucleotide sequence data available on 235 eukaryotic DNA sequences (approximately 300,000 bp) (24), we were unable to detect this octanucleotide (TGTAAATA) in approximately the same location in any other mRNA and the frequency of occurrence for this octanucleotide at any location fits the expected random frequency. The evolutionary conservation of this octanucleotide amongst the metallothionein mRNAs suggests a regulatory or structural function for this sequence.

With the availability of both metallothionein mRNA sequences from Chinese hamster, an estimated time of metallothionein gene duplication can be calculated using the method of Kimura (25). A nucleotide substitution rate for metallothionein I can be derived from a Chinese hamster and mouse interspecies comparison and for metallothionein II can be derived from a Chinese hamster and human interspecies comparison. Assuming that mouse and Chinese hamster diverged approximately 20 million years ago (26), their metallothionein I's show an evolutionary rate per site of 2.4×10^{-9} per year. Assuming that human and Chinese hamster diverged approximately 78 million years ago (27), their metallothionein II's show an evolutionary rate per site of 0.90×10^{-9} per year. These two different evolutionary rates give rise to a gene duplication time of 45 million to 120 million years ago. A similar duplication time can be derived from metallothionein I and II amino acid sequence comparisons by the method of Kimura and Ohta (28).

DISCUSSION

Metallothionein is a highly conserved protein which is found in organisms ranging from blue-green algae (29) to plants (30) and humans (1). The present characterization of two Chinese hamster metallothioneins provides amino acid and nucleotide sequence data confirming that the vertebrate interspecies homology for each isometallothionein is greater than the intraspecies homology for the two isometallothioneins. The calculation of the gene duplication time further indicates that the mammalian MT-I and MT-II may be characterized by different rates of evolution. All of the differences between vertebrate MT-I and MT-II could reflect separate functions rather than random divergence between functionally equivalent isomers.

Interestingly, the calculated time of mammalian metallothionein gene duplication does not coincide with the evolutionary appearance of MT-I and MT-II. The invertebrate crab, Scylla serrata, has two well characterized metallothioneins (31,32), yet diverged from vertebrates approximately 450 million years ago (33). This implies that metallothionein gene duplication may not have been a unique event in an ancestor primordial to the vertebrate and invertebrate divergence.

Since the two metallothioneins are coordinately induced by an array of agents in many species and tissues (1), they also provide an excellent system for studying coordinate gene expression. Several short DNA sequences with known regulatory functions, such as the "TATAA" box (34), "CCAAT" box (35), and polyadenylation signal (AATAAA) (36), have been conserved in position in a variety of gene sequences. However, to be implicated in differential gene expression, regulatory nucleotide sequences would be expected to be not only conserved, but also unique amongst a set of coordinately regulated genes. Consistent with these requirements, the octanucleotide TGTAAATA, which is highly conserved in sequence and position in all the metallothionein mRNA sequences examined thus far, may have a regulatory function unique to metallothionein gene expression. Although a transcription initiation role for this octanucleotide is unlikely since the mouse metallothionein I gene has a transcription signal in the 5' flanking region (37,38), the octanucleotide could be involved in mRNA processing or on a preferential translation of the metal-induced metallothionein mRNA sequences. Though the mechanism is unknown, the heat shock-induced mRNAs of Drosophila provide an example of preferentially translated mRNAs (39). Future studies will examine the potential functions presently only speculated for this octanucleotide.

In addition to fostering evolutionary and regulatory function speculations, the nucleotide sequence data allows the isolation of DNA probes unique to each of the Chinese hamster metallothionein mRNA sequences. The DNA probes will further our examination of the coordinate amplification, transcription, methylation, genomic organization, and chromosomal localization of the genes for metallothionein I and II.

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