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Human metallothionein genes: molecular cloning and sequence analysis of the mRNA

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ABSTRACT

From a cDNA clone bank prepared from cadmium-treated HeLa cells, we isolated clones representing mRNAs whose concentration is increased after cadmium induction. Several metallothionein cDNA clones were isolated by cross-hybridization to mouse metallothionein-I cDNA. The nucleotide sequence of one of these clones, containing a nearly full-length cDNA copy of human metallothionein-II mRNA, was determined. The homology between the human and mouse metallothionein sequences is strictly limited to the coding region of the mRNA. Codon usage in metallothionein mRNA is not random. Seventy-nine percent of the codons have G or C residues at the third position, resulting in a GC-rich sequence.

INTRODUCTION

Metallothioneins (MT) are a family of low-molecular-weight, heavy metal binding proteins, characterized by a high cysteine content and the lack of aromatic amino acids. Metallothioneins bind 6 to 7 moles of heavy metals, such as Zn, Cd, Cu and Hg, per mole of protein. They are ubiquitously distributed in the animal and plant kingdoms and are also found in prokaryotes (1). Human MTs have been isolated from the liver, where they are present as two major forms known as MT-I and MT-II (2). The regulation of human MT synthesis has been studied in several human cell lines, including the well characterized HeLa cells. In HeLa cells, MT synthesis can be induced by either Zn^{++} or Cd^{++} (3) and by glucocorticoid hormones (4). In the past we have shown that either heavy metals or glucocorticoids are "primary" inducers (5) of the genes and lead to a rapid increase in the amount of translatable MT mRNA (6). In order to investigate the molecular mechanisms regulating MT genes' expression, it is necessary to have specific hybridization probes for the genes and the mRNAs. In this commun-

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ication we describe the molecular cloning and primary structure of the mRNA for human MT-II.

MATERIALS AND METHODS

Total RNA was extracted by the method of Chirgwin et al. (7) from HeLa cells maximally induced to synthesize MT by incubation in 10^{-5}M $CdCl_2$ and 10^{-4}M cycloheximide for 8 hr before harvest (5,6). Poly(A)-containing RNA was selected by hybridization to oligo(dT)-cellulose (8) and used as template for the synthesis of double-stranded cDNA by sequential reverse transcriptase reactions. After removal of hair-pin loops by S_I nuclease digestion, homopolymeric tracts (tails) with an average of 12 dCMP residues were added to the 3' termini of the cDNA by incubation with terminal transferase (Ratcliffe) and dCTP (9). The double-stranded, dCMP-tailed cDNA sequences were annealed to plasmid pBR322 DNA, previously linearized with restriction endonuclease Pst I (BRL) and 3'-tailed with an average of 10 dGMP residues. The resultant chimeric plasmid DNA was used to transform E. coli RRI. The yield of recombinant colonies was $4 \times 10^3/\mu g$ cDNA, greater than 90% being of Amp^s, Tet^r phenotype. Bacterial colonies containing recombinant plasmids were grown and fixed on 0.45-μ nitrocellulose filters, essentially as described by Grunstein and Hogness (10). Duplicate filters were hybridized with ^{32}P-labelled cDNA synthesized from poly(A)-containing RNA from either induced or uninduced HeLa cells, as described above. Twenty-four colonies were judged by radioautography to give a stronger hybridization signal with induced cDNA. Plasmid DNA was prepared from each of these colonies and digested with Bam HI and Ava II. Four colonies were found to contain a Bam HI site within the cloned insert sequence and also to hybridize relatively strongly with a restriction fragment of a cloned mouse MT-I cDNA (11) containing only coding sequences. The identity of these sequences as coding for human MT was verified by nucleic acid sequence analysis.

RESULTS AND DISCUSSION

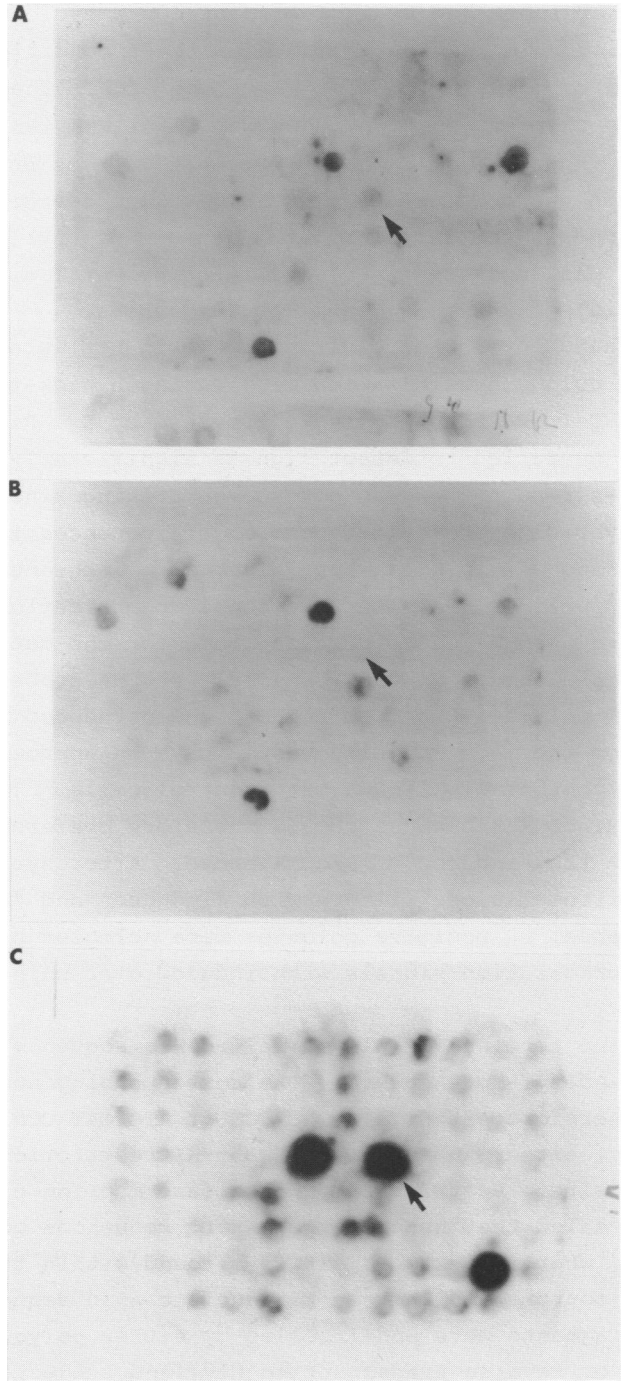
Construction and isolation of the cDNA clone

Maximal levels of MT-mRNA are achieved 8 hr after addition of 10^{-5}M Cd^{++} to HeLa cells (6). The level of translatable MT-mRNA

can be further increased by inclusion of 10^{-4}M cycloheximide during the induction period (5). Under these conditions, MT-mRNA is estimated to constitute about 2% of the mRNA. The starting material for the cloning experiment was total RNA isolated from HeLa cells, induced with Cd^{++} and cycloheximide as described above. Messenger RNA was selected by oligo(dT)-cellulose chromatography (8) and served as template for the synthesis of double-stranded cDNA, which was subsequently cloned into the Pst I site of the cloning vehicle pBR322, as described in Methods.

Seven hundred of the resultant recombinants were initially screened by colony hybridization (10), using a nick-translated (12) mouse MT-I cDNA probe (kindly provided by Dr. Richard Palmiter). We failed to detect signals significantly higher than background--a result that was not too surprising since the mouse MT-I cDNA hybridizes to a large number of sequences in the mouse genome (11) and weakly cross-hybridizes to a large number of human sequences as well (R. Palmiter, personal communication). Therefore, we have used a less direct approach for the detection of human MT-mRNA clones. Single-stranded ^{32}P-cDNA probes were transcribed from poly(A)$^+$-RNA from induced and uninduced HeLa cells. The mRNA from the induced cells should have an approximately 50-fold higher content of MT-mRNA than control cells (6). A clone containing MT-cDNA sequences should therefore preferentially hybridize with the "induced" ^{32}P-cDNA probe. After hybridization of duplicate nitrocellulose filters with "induced" and "uninduced" ^{32}P-cDNA probes, 24 positive colonies were selected by virtue of stronger hybridization signals with induced over uninduced probe (Fig. 1).

The amino terminal of all mammalian MTs sequenced has the structure N-Acetyl-Met-Asp-Pro. The corresponding nucleotide sequence is therefore either ATG GAC CCN or ATG GAT CCN, containing, respectively, either an Ava II or a Bam HI restriction endonuclease recognition site. Restriction endonuclease mapping of the selected plasmids revealed that none of the insert sequences contained an Ava II site, whereas a Bam HI site was found within the inserted sequence of four recombinants. For nucleic acid sequencing, restriction fragments were end-labelled by either polynucleotide kinase (13) or reverse transcriptase (14) and subjected to chemi-


A
B
C

cal degradation by the method of Maxam and Gilbert (13). These four plasmids also gave relatively strong hybridization signals with mouse MT-cDNA probe (11) on a Southern blot (15) (data not shown). Of these plasmids, the one with the longest insert was chosen for further analysis.

The nucleotide sequence of MT-II mRNA

Plasmid DNA was prepared from the clone described above; it was labelled at its Bam HI site with α-^{32}P-dGTP and reverse transcriptase (14), and the nucleotide sequence around this site was determined by the method of Maxam and Gilbert (13). The sequence in one direction encoded almost the complete peptide sequence (amino acids 3 - 61) of human MT-II. This insert sequence was subsequently completely derived (Fig. 2) and the plasmid designated phMT_{II}-3. The coding region in phMT_{II}-3 is flanked by 57- and 128-nucleotide-long 5' and 3' untranslated regions, respectively.

The coding region predicts a peptide sequence clearly identical to human MT-II, as previously reported (16), with the exception of residues 58 and 59, which are actually conserved with respect to the rodent MT sequences (1,18) and have recently been reassigned in the human (Kägi, J.H.R., personal communication). Both the location of the Pvu II site and repeated nucleotide sequence analysis across this region verified the sequence shown (Fig. 2). The 5' untranslated region, although apparently almost full-length (73 nucleotides in mouse MT-I; ref. 19), may contain ambiguities introduced during the cloning procedure (20,21) and will therefore require verification by sequence analysis of the chromosomal gene. The 3' untranslated region may also be incomplete since it lacks a poly(A) tract. Nevertheless, it does contain two AAUAA sequences commonly found near the 3' end of eukaryotic mRNA sequences (17).

Amino acid position 11 in the human MT-II sequence is an aspartic acid residue (16) and its codon (GAY) forms part of a

Figure 1. Identification of cloned cDNA sequences coding for human MT. Shown is one of seven triplicate sets of filters used to screen the HeLa cell cDNA library with A. "induced" ^{32}P-cDNA, B. "uninduced" ^{32}P-cDNA, and C. ^{32}P-nick-translated Bam HI-Pvu II fragment from phMT_{II}-3.

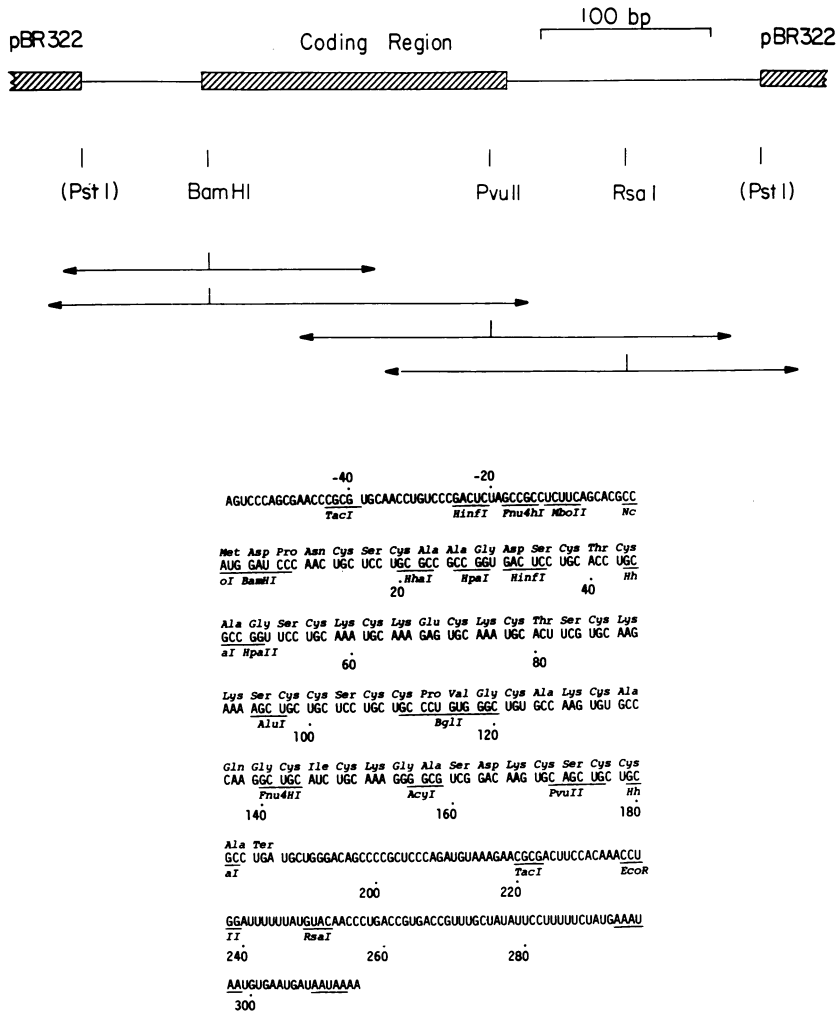


Figure 2. Nucleotide sequence of human MT-II mRNA, as deduced from the cloned cDNA insert in plasmid phMT$_{II}$-3. (Top) The sequencing strategy and physical map of the phMT$_{II}$-3 insert sequence cloned into the Pst I site of pBR322, as described in Methods. Both Pst I sites were lost during the cloning procedure, probably due to exonuclease contamination of terminal transferase. (Bottom) Nucleotide sequence of human MT-II mRNA showing coding potential for the MT-II protein sequence (ref. 16; Kägi, J.H.R., personal communication). Nucleotides are numbered from the AUG initiation codon. Restriction endonuclease recognition sites discussed in the text are underlined, as are the AAUAA sequences commonly found in the 3' untranslated regions of eukaryote mRNA sequences (17). The cloned sequence is flanked by 21 G residues and 18 C residues at the 5' and 3' termini, respectively.

Hinf I restriction endonuclease recognition site (Fig. 2). In the human MT-I sequence this amino acid residue is a glycine (GGN) (1), and therefore this Hinf I site should be absent. In each of the cloned MT cDNA sequences analyzed, this Hinf I site is present; we therefore conclude that they all encode the MT-II peptide and not MT-I.

Table I shows the codon usage in human MT-II mRNA. Codon usage is not random: 78.5% of the codons have G or C residues at the third position; 90% of the cysteine codons, for example, end with C. Similar observations have been made for the mouse MT-I mRNA (11). The two MTs are 81% and 85% homologous in their nucleotide coding regions and amino acid sequences, respectively. The homology is strictly limited to the coding region of the mRNAs; the 3' and 5' untranslated regions do not exhibit any homology whatsoever.

Using phMT_{II}-3 as a specific hybridization probe for the different human MT genes, we can now: a) isolate cDNA clones of the other human MT mRNAs; b) isolate bacteriophage clones of human genomic DNA, containing the MT genes; c) investigate the molecu-

TABLE I

Codon Utilization of Human MT-II mRNA

1	2 U		2 C		2 A		2 G		3
U	Phe		Ser	4	Tyr		Cys	2	U
	Phe		Ser		Tyr		Cys	18	C
	Leu		Ser		Term		Term	1	A
	Leu		Ser	2	Term		Trp		G
C	Leu		Pro	1	His		Arg		U
	Leu		Pro	1	His		Arg		C
	Leu		Pro		GluN	1	Arg		A
	Leu		Pro		GluN		Arg		G
A	Ile		Thr	1	AspN		Ser		U
	Ile	1	Thr	1	AspN	1	Ser	2	C
	Ile		Thr		Lys	5	Arg		A
	Met	1	Thr		Lys	3	Arg		G
G	Val		Ala		Asp	1	Gly	2	U
	Val		Ala	6	Asp	2	Gly	2	C
	Val		Ala		Glu		Gly		A
	Val	1	Ala	1	Glu	1	Gly	1	G

lar mechanisms involved in regulation of MT mRNA synthesis by heavy metals and glucocorticoid hormones; and d) determine whether the inherited defects in human trace-metal metabolism, such as Wilson's disease, acrodermatitis enteropathica and Menkes' kinky hair syndrome, are due to mutations in the MT gene(s), as has been proposed (22-24).

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REFERENCES

1. Kägi, J.H.R. and Nordberg, M., Eds. (1979) Metallothionein, Birkhauser, Basel.
2. Pulido, P., Kägi, J.H.R. and Vallee, B.L. (1966) Biochemistry 5, 1768-1777
3. Rudd, C.J. and Herschman, H.R. (1979) Toxicol. Appl. Pharmacol. 47, 273-278
4. Karin, M. and Herschman, H.R. (1979) Science 204, 176-177
5. Karin, M., Andersen, R.D., Slater, E., Smith, K. and Herschman, H.R. (1980) Nature 286, 295-297
6. Karin, M., Andersen, R.D. and Herschman, H.R. (1981) Eur. J. Biochem. 118, 527-531
7. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
8. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412
9. Chang, A.C.Y., Nunberg, J.H., Kaufman, R.J., Erlich, H.A., Schimke, R.T. and Cohen, S.N. (1978) Nature 275, 617-624
10. Grunstein, M. and Hogness, D.S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965
11. Durnam, D.M., Perrin, R., Gannon, F. and Palmiter, R.D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6511-6515
12. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251
13. Maxam, A.M. and Gilbert, W. (1980) in Methods in Enzymology,

Grossman, L. and Moldave, K., Eds., Vol. 65, Nucleic Acids, Part I, pp. 499-560, Academic Press, New York.
14. Roberts, J.L., Seeburg, P.H., Shine, J., Herbert, E., Baxter, J.D. and Goodman, H.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2153-2157
15. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517
16. Kissling, M.M. and Kägi, J.H.R. (1977) FEBS Lett. 82, 247-250
17. Proudfoot, N.J. and Brownlee, G.G. (1976) Nature 263, 211-214
18. Huang, I.-Y., Yoshida, A., Tsunoo, H. and Nakajima, H. (1977) J. Biol. Chem. 252, 8217-8221
19. Glanville, N., Durnam, D.M. and Palmiter, R.D. (1981) Nature 292, 267-269
20. Richards, R.I., Shine, J., Ullrich, A., Wells, J.R.E. and Goodman, H.M. (1979) Nucleic Acids Res. 7, 1137-1146
21. Weaver, C.A., Gordon, D.F. and Kemper, B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4073-4077
22. Evans, G.W., Dubois, R.S. and Hambidge, K.M. (1973) Science 181, 1175-1176
23. Cousins, R.J. (1976) Lancet 2, 686-687
24. Chan, W.-Y., Garnica, A.D. and Rennert, O.M. (1978) Clin. Chim. Acta 88, 221-228