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CLONING, NUCLEOTIDE SEQUENCE AND CHARACTERIZATION OF A NEW ZEALAND RABBIT METALLOTHIONEIN-I GENE

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SUMMARY: We have isolated a rabbit metallothionein-I gene from a lambda gt10 library. The coding sequence of this gene is interrupted by two introns occurring at amino acid positions 9 1/3 and 30 1/3. Comparison of the promoter sequence of this gene with the promoters of other metallothionein genes identified a number of oligonucleotide sequences which are recognized by trans-acting proteins involved in the regulation of these genes by heavy metals, glucocorticoids and alpha interferon.

Metallothioneins (MTs) are small, cysteine-rich proteins that bind heavy metals (1-3). Their roles are thought to be in either homeostasis or detoxification (4-7). These polypeptides are ubiquitous. They are found in almost every living organisms (8-22) and are inducible by metals, glucocorticoids, alpha-interferon and iodoacetate, etc...(22-25). As a first step in the study of the promoter sequences and the trans-acting factors involved in the regulation of the synthesis of metallothioneins in the rabbit, we have cloned the rabbit metallothionein-I gene and identified a multitude of regulatory elements in its promoter.

METHODS

Cloning of the MT1 rabbit metallothionein gene. DNA from a New Zealand female rabbit was purified (26) by phenol-chloroform extraction and alcohol precipitation. The 4-6 Kb EcoRI-digested DNA fragments were separated by sucrose gradient (27), inserted at the EcoRI site of lambda gt10 (28) and packaged (29). The phage recombinants were selected on E. coli C600 Hf1. This library was screened (30) with a nick-translated mouse MTI cDNA (31). Hybridization was carried out in 50% formamide at 37°C for 16-60 hours. The filters were washed 30 min at 25°C in 0.5xSSC, 0.1%SDS. One lambda MT44 recombinant was isolated. Its insert was subcloned in M13, mp18 and mp19 (32-33) and sequenced by the chain-termination method (34).

Genomic blot. Hybridization of the EcoRI-digested rabbit DNA was at 42°C and washing was at 62°C in 0.1xSSC, 0.1%SDS.

RESULTS AND DISCUSSION

Figure 1 shows the restriction map of the 4.4 Kb clone. Southern blot analysis of the 4.4 Kb using MT1 mouse cDNA, reveals that the hybridizing

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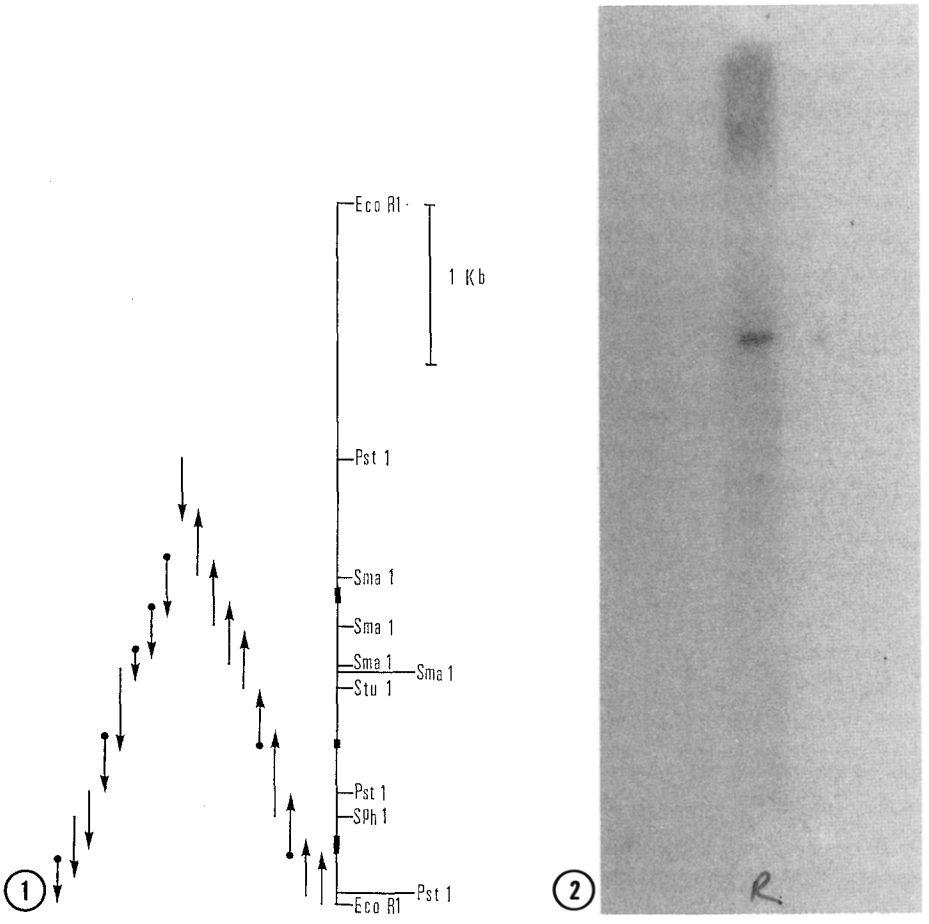


Fig. 1 Restriction enzyme map of the cloned insert of lambda MT44. The arrows indicate the direction and extent of sequencing runs. (⟶): Sequencing with universal primer; (•⟶): sequencing with synthetic primer. Transcription proceeds from left to right. The three exons are shown with thickened lines.

Fig. 2 Southern blot analysis of New Zealand rabbit genomic DNA probed with a 1.4 Kb 3' end fragment of the MTI gene. A 10 ug amount of high-molecular weight DNA isolated from rabbit lever was digested with EcoRI and subjected to electrophoresis through a 1% agarose gel. The DNA was transferred to nitrocellulose, hybridized to the nick-translated DNA, probed and autoradiographed (Southern, 1975). Hybridization was done at 42°C and washing of the nitrocellulose filter was carried out at 62°C.

region is in a small fragment spanning the SphI-PstI-EcoRI sites. Sequence analysis indicates that this fragment codes for the third MT exon with the 3' untranslated end towards the right of the map. Figure 2 shows the result of a genomic blot of New Zealand rabbit liver DNA digested with EcoRI and probed with the 1.4 Kb SmaI-StuI-PstI-SphI-PstI-EcoRI fragment (third exon). A 4.4 Kb band is clearly visible (fig. 2). This indicates the cloned fragment has not been rearranged during subcloning and may represent a unique DNA sequence in the rabbit genome. About 2.8 Kb was sequenced with the strategy illustrated (fig. 1).

Exons and introns. From the sequence (fig. 3), this gene codes for a 61 amino acid protein. The intron-exon junctions of the gene were deduced by comparing the DNA and the amino acid sequence of the rabbit metallothionein

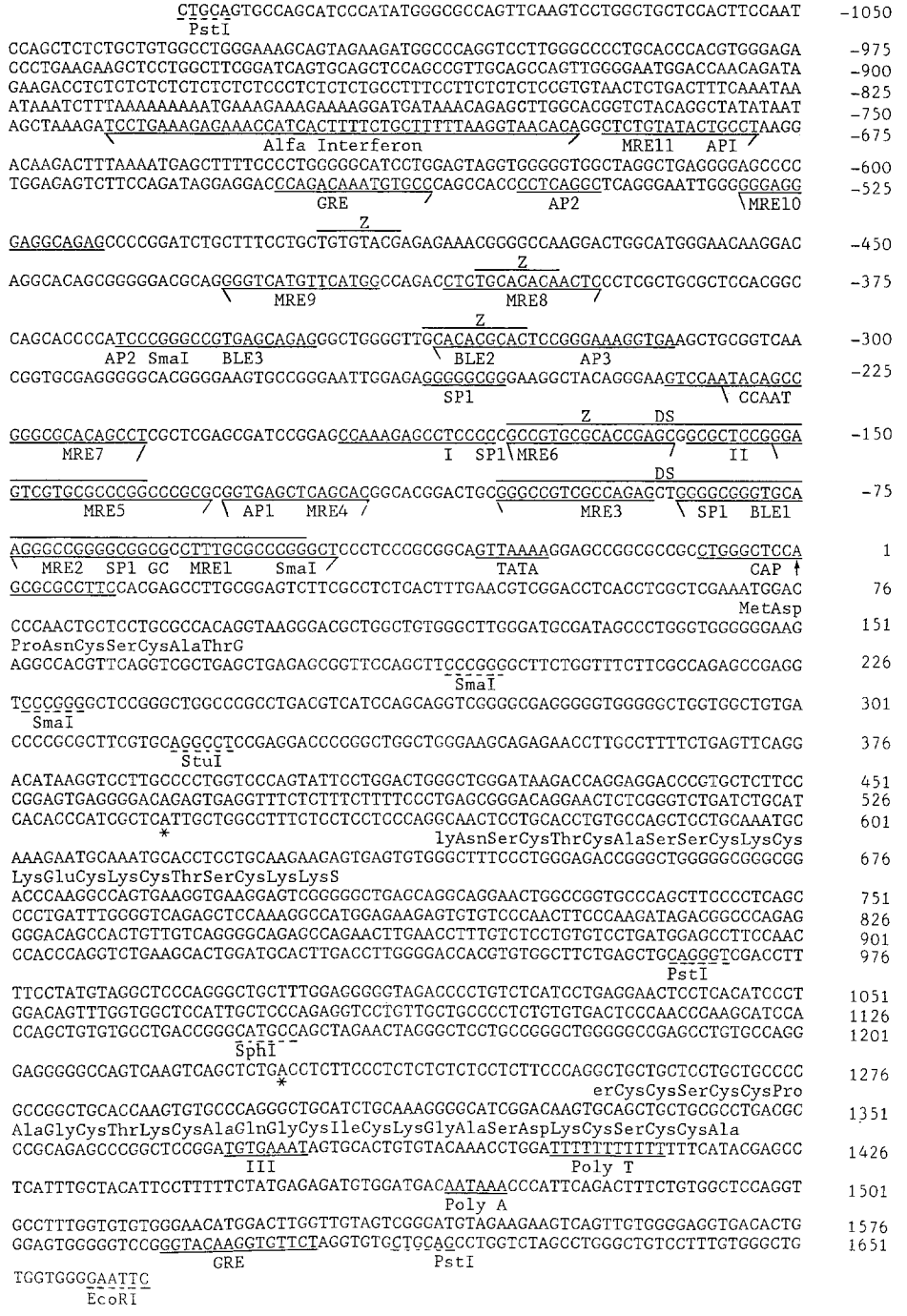


Fig. 3 DNA sequence of the MTI gene. Numbering is from the proposed mRNA capping site which is arrowed. The poly (A) addition site AATAAA is underlined. Several proposed regulatory elements with the 5' promoter region and the conserved elements in the 3' untranslated region which are discussed in the text are underlined and labeled.

protein (35). Like in other mammalian MTs (23, 36-41), two introns interrupt three exons at the same amino acid positions. Exon 1 encodes the 5' untranslated region and amino acids $1\text{-}9^{1/3}$; exon 2 encodes amino acids $9^{2/3}\text{-}30^{1/3}$; exon 3 encodes amino acids $31^{2/3}$-61 and the 3' untranslated region. That this gene belongs to the MTI family is indicated by the absence of an aspartate at position 10 or 11 of the peptide chain while the presence of aspartate is characteristic of the MTII gene family (42).

Introns 1 and 2 (468 bp and 624 bp long respectively) obey the GT-AG rule (43-44). The donor sequences of the exon-intron boundaries are 5'-CAG/GTAAGG-3' and 5'-AGA/GTGAGT-3' for introns 1 and 2 respectively. They conform with the consensus 5'-$^{A}_{C}$AG/GT$^{A}_{G}$AGT-3' (44). The receptor intron-exon boundaries are 5'-$(Y)_{14}$CCAG/GC-3'(Y=pyrimidine) and 5'-$(Y)_{25}$CCAG/GC-3' for introns 1 and 2 respectively. They are identical to the consensus 5'-$(Y)_{n}$NYAG/G-3' ($n \geqslant 11$; N being any base) (45-46). In fig. 3, the asterisks indicate the putative lariat branch points of the two introns. The surrounding CGCTCAT of the branch point of intron 1 matches the consensus YNYTRAY (R= purine) (47) in 6 out of the 7 nucleotides. For intron 2, CTCTGAC matches the consensus sequence.

The 3' untranslated region. First, two peculiar sequences TGTAAATA and polyT are present here. TGTAAATA (21 bp after the termination codon and labelled element III) is also in the mouse MTII gene (37) and the Chinese hamster MTI and MTII cDNAs (48). A poly T sequence is also in the mouse MTII (37) and the monkey MT cDNAs (49). The functions of these 2 sequences are not yet known. Second, the typical polyadenylation signal (labelled poly A in fig. 3) is 119 bp from the stop codon. The 12 bp TT$^{G}_{A}$NNNTTTTTT located at 5 to 20 bp downstream from the poly(A)-addition site in some transcription units (50) is absent.

The promoter region. The 5' flanking region shows the typical CAP site, TATA and CCAAT boxes. The CTGGGCTCCAGCGCGCCTTC CAP site (indicated by an arrow in fig. 3) was located by comparison with other MT genes (23, 36-38, 40). It agrees with the consensus CTRNRCYCCAYCACGCCTYC (R=purine) (40). Located at 23 to 28 bp upstream from the CAP site is TTAAAA (labelled TATA in fig. 3) which is similar to the consensus 5'-TATA$^{T}_{A}A^{T}_{A}$-3' (43). Because in most eukaryotes TATA boxes are 25 to 30 bp upstream from the CAP site, we suggest that the sequence TTAAAA shares similar function. Between -229 and -237 is GTCCAATAC (labelled CCAAT) in fig. 3 which has 6 out of 9 bases in common with the CCAAT consensus GGTCAATCT (51). The CCAAT sequence, binding site of nuclear factors, modulates the basal and inducible transcription levels during differentiation and induction (52-53).

Comparison of this promoter sequence with the promoters of other metallothionein (23, 38, 40) identified a number of sequences recognized by proteins involved in either basal level transcription or in the induction by metals, glucocorticoids and alfa interferon. In this promoter region, 13 sequences (MRE1-11, BLE1 and 3) show 9 or more bases identical to this 15 bases consensus metal regulatory elements (MRE) and basal level elements (BLE) (table 1).

These same MRE1, MRE2 and BLE1 elements between -44 to-86 are also found in rat (41), mouse (36, 37), human (23, 38), sheep (40) and now the rabbit MT promoter. MRE1 and MRE2 control the regulation of the MT gene by metals (25) while BLE1 controls the basal level expression (37, 40). BLE1 (GGGCGGGTG) differs from the consensus GGGCGYGTG (37, 40) at only 1

Table 1

Element	Sequence	Orientation	# of identical bases
MRE1	-58 CTTTGCGCCCGGGCT -44	normal	11
MRE2	-60 CGCCGC CCCGGCCC -73	reverse	10
BLE1	-72 CCTTGCACCCGCCCC -86	reverse	11
MRE3	-89 CTCTGGCGACGGCCC -103	reverse	10
MRE4	-130 CGGTGAGCTCAGCAC -116	normal	9
MRE4	-124 CTCACCGCGCGGGCC -138	reverse	10
MRE5	-148 TCGTGCGCCCGGCCC -134	normal	11
MRE5	-137 CCGGGCGCACGACTC -151	reverse	9
MRE6	-176 CCGTGCGCACCGAGC -162	normal	9
MRE6	-165 CGGTGCGCACGGCGG -179	reverse	10
MRE7	-226 CCGGGCGCACAGCCT -212	normal	9
MRE7	-215 CTGTGCGCCCGGCTG -229	reverse	11
BLE3	-346 CTCTGCTCACGGCCC -363	reverse	12
MRE8	-408 CTCTGCACACAACTC -394	normal	10
MRE9	-415 CCATGAACATGACCC -429	reverse	9
MRE10	-515 CTCTGCCTCCCTCCC -530	reverse	9
MRE11	-693 CTCTGTATACTGCCT -679	normal	9
Consensus	CTNTGCRCNCGGCCC (R, N are purines and any base respectively)		

position. Similar BLE sequences are at position -326 to -334 (BLE2) and -353 to -362 (BLE3). BLE2 (GTGCGTGTG) is in the reverse orientation and differs from the consensus at only 1 position. BLE3 (GGGCCGTG) has one less G than the consensus.

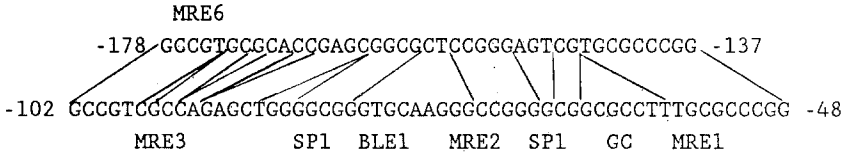
Elements similar to the glucocorticoid and the interferon regulatory consensus sequences can be identified: CCAGACAAATGTGCCC (GRE1) between -560 and -574 has 10 out of 15 bases identical to the human glucocorticoid TCGTACAAATGTTCT (55) and 7 out of 12 bases identical to the glucocorticoid consensus GGTACANNNTGTTCT (56). GGTACAAGGTGTTCT between 1591 and 1601 is identical to the glucocorticoid consensus sequence. This element located after the polyadenylation signal, either regulates MT1 itself or belongs possibly to another gene promoter downstream of the MTI gene. The sequence (Alpha Interferon) between -696 to -740 has 19 bases out of 28 bases of the alpha interferon regulatory consensus element (56). Inside has been inserted the sequence AAAGAGAA. When in the reverse orientation, this insert and its neighboring bases have a 70% similarity to the interferon regulatory element consensus sequence in the reverse orientation:

TTCN$^{G}_{C}$N ACC TCANGCGTTTCTC$^{C}_{T}$TCTCT	Consensus
TCCTGAAAAGAGAAACCATCA CTTTCTGCTTTTTAAGGTAACACA	Element Alpha Interferon
AGA$^{G}_{A}$AAGAGAAAC GCN TGAGGTN$^{C}_{G}$NGAA	Consensus in reverse orientation

Four eukaryotic nuclear transcription regulatory factors: Sp1, AP-1, AP-2 and AP-3 (57-60) bind to specific enhancer sequences of simian virus 40 and MT genes (61). Factor Sp1 binds to GGGCGG or CCGCCC in the reverse orientation (57). This sequence is present four times in the MTI promoter (MREII, BLE1, MRE6 and at -253 to -260). Interaction of AP-1 with Sp1 may be required for transcription of MT genes because AP-1 binds to the BLE region or close to the Sp1 binding site in the h-MTIIa promoter (57). AP-1 binds to $^{C}_{G}$TGACT$^{C}_{A}$A (58). Similarly there are two sequences GTGAGCTCA and CTGCCTAA

close to MRE4 and MREII respectively. The first one differs from the consensus by one extra G; the second one matches the consensus in 7 out of 8 nucleotides. The AP-1 binding site close to MRE4 is about 50 base-pairs away from BLE1 and Sp1 binding sites. Factor AP-2 binds to the enhancer regions of the h-MTIIa promoter between -110 and -130 and between -165 to -230 (59) (consensus CC$^{C}_{G}$C$^{A}_{G}$GGC). Two similar sequences (TCCCGGGC at -350 close to BLE; CCTCAGGC at -551) can be identified in the MTI gene. Both match the consensus in 7 out of 8 positions. The consensus sequence for AP-3 is unknown. However, AP-3 protects TGTGGAAAGTCCCA of the simian virus 40 enhancer (60) from nuclease digestion. Here TCCGGAAAGGTGAAG (-312 to -325) has a similar sequence and may therefore play the same role.

Besides the elements above, a region of diad symmetry and 4 regions of alternating purine and pyrimidine are in this promoter. CCAAAGAGCCTCCCCC (element I at -190) has 12 bases in common with CCAAAGGGNNGGTCCCGC found in all MTI genes except for h-MTIIa gene (42). AGCGGCGCTCCGGGAGTC (element II) is similar to the promoter sequence RGCNGNNYTCCNGGAAYY identified by Peterson and Mercer (40). As in other mammalian MTs but not for the mouse MTII promoter (23), MRE1 is in a region of imperfect diad symmetry (overlined and labelled DS in fig. 3):



The 4 regions of alternating purine and pyrimidine (labelled Z in fig. 3) and similar regions in the sheep MTIa (40) have the potential to form Z-DNA structure (62). Although no function has yet been identified for these 7 sequences, the fact that they are also in most other mammalian metallothionein promoters suggests that they are recognized by not yet isolated trans-acting factors. Indeed, in bacteria, diad symmetrical sequences are part of the regulation of the operons suggesting a similar function here.

In conclusion, the rabbit MTI gene possesses all the structures required for basal transcription, induced transcription as well as mRNA processing. The promoter region, with its numerous potential regulatory sequences, represents one of the more complete and extensive promoters isolated so far. About 30 possible regulatory sites have been identified. This promoter should be an excellent model to study gene regulation and to test the functions of the metal regulatory elements.

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