Molecular Cloning of a Canine Metallothionein cDNA

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ABSTRACT. A canine metallothionein cDNA obtained from the liver of a cadmium-treated beagle was cloned and sequenced. Asn at position 4 conserved among all mammalian metallothionein-1 and metallothionein-2 is replaced by Asp in the canine metallothionein cDNA clone. Because the acidic amino acid doesn't exist at either position 10 or 11 in the deduced amino acid sequence, it is supposed that this cDNA is derived from canine metallothionein-1 mRNA. Northern blot analysis using the cDNA as a probe revealed the induction of the canine metallothionein mRNA expression in the liver and kidney of a cadmium-treated beagle. Thus, the canine metallothionein cDNA obtained in the present study should provide an useful tool for the molecular investigation of metallothionein in dog. — KEY WORDS: canine, cDNA, metallothionein.

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Metallothioneins (MTs) are low-molecular-weight, cysteine-rich and bivalent metal-binding proteins. It is generally thought that MTs play a role in the detoxification of heavy metals [12]. Besides metals, MT expression is transcriptionally regulated by a variety of factors including glucocorticoid hormones [14, 15], ultraviolet irradiation [13], stresses [25], bacterial lipopolysaccharide [7] and cytokines such as interferon [10] and interleukin-1 [16]. Moreover, the expression of MT genes during early development in several species [2, 18, 32] strongly suggests that MTs are involved in the homeostasis of trace elements including zinc and copper in a developmental processes. The amino acid sequence of canine MTs obtained from the liver of Bedlington terriers affected with inherited copper toxicosis has already been determined by Learch *et al*. [22]. However, little information is available about the gene structure and regulation of the gene expression in dog.

We cloned and sequenced a canine MT cDNA from the liver of a cadmium (Cd)-treated beagle.

A 1.5-year-old female beagle for the purpose of $CdCl_2$ treatment and a 1-year-old male beagle as control animal were supplied from the Safety Research Laboratory, Tanabe Seiyaku Co., Ltd. (Osaka, Japan). $CdCl_2$ was intravenously administered to the female dog at doses of 1.0 mg Cd/kg body weight for the first day and 2.5 mg Cd/kg body weight for the second to fourth day. As the dog wasted away through Cd toxicosis, it was euthanatized by exsanguination under sodium pentobarbital anesthesia after 4 hr from the last administration of $CdCl_2$. The control dog without treatment was euthanatized at the same laboratory. For molecular biological examinations, a piece of the liver, kidney and brain were immediately taken after death, frozen with liquid nitrogen and stored in a freezer at −80°C until the examination. Total cellular RNA from the liver was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [6]. Poly $(A)^+$RNA was purified by oligo (dT)-Latex [21]. cDNA was synthesized using a cDNA Synthesis Kit (TAKARA SHUZO, Shiga, Japan). The first strand cDNA was synthesized on 2 μg of poly $(A)^+$RNA denatured for 5 min at 65°C using 40 U of reverse trancriptase and an oligo $(dT)_{18}$ primer. Following ethanol precipitation, the second strand cDNA was synthesized using *E. coli* DNA polymeraseI and *E. coli* RNase H/*E. coli* DNA ligase mixture. Phosphorylated BstXI adaptor (Invitrogene, San Diego, CA) was linked to this second strand cDNA. The cDNA was inserted into the *Bst*XI site of pcDNAI (Invitrogene, San Diego, CA.). The recombinant plasmids were introduced into *E. coli* MC1061/P3 (Invitrogene, San Diego, CA.) by electroporation. The transformants were isolated on LB-agar containing 12.5 μg ampicillin/mL and 7.5 μg tetracycline/mL. From a total of 12,000 ampicillin and tetracycline-resistant transformants, MT cDNA clones were selected for further screening.

A 2-week-old male mongrel dog housed in conventional environment was used for synthesis of canine MT cDNA fragments. The dog was euthanatized by excessive administration of sodium pentobarbital. A piece of the liver was immediately taken after death, frozen with liquid nitrogen and stored in a freezer at −80°C until use. Total cellular RNA from the liver was isolated by the guanidine/cesium chloride centrifuge method [5]. Poly $(A)^+$ RNA was purified by oligo (dT)-cellulose chromatography [4]. First strand cDNA was synthesized using MuLV reverse transcriptase and oligonucleotide NotI $(dT)_{18}$ in a T-primed First-Strand Kit (Pharmacia Biotech., Uppsala, Sweden). For amplification of canine MT cDNA fragments, mixed oligonuceotide primers representing all possible coding combinations were designed from the reported amino acid sequence of the canine MT [22]. The sequences of the primers were 5'-TG(C/T)AA(A/G)TG-(C/T)AA(A/G)GA(A/G)TG(C/T)AA (the sense primers constructed from the amino acid residues 19–25) and 5'-CC(C/T)TT(A/G)CA(A/G/T)AT(A/G)CA-(A/C/G/T)CC(C/T)TG (the antisense primers constructed from the amino acid residues 46–52). The primers were obtained from Sawadei Technology Co., Ltd. (Tokyo, Japan). PCR amplification of canine MT cDNA fragments was performed using the above mixed primers. The cDNA fragments were amplified for 35 cycles in a thermal cycler (ATTO, Tokyo, Japan). Each cycle of amplification consisted of 1 min of denaturation at 95°C, 2

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min of annealing at 32°C and 1 min of elongation at 72°C. The amplified band equivalent to 100 bp after 1.5% agarose gel electrophoresis was recovered, the cDNAs were extracted from the gel slice and these were again amplified by PCR as the template DNA. The PCR products were recovered by the above method, generated blunt ends by Klenow fragment and phosphorylated 5'-termini by T4 polynucleotide kinase. The modified PCR fragments were subcloned at the *Eco*RV site of pBluescript II KS+ (Stratagene, La Jolla, CA). Nucleotide sequences of the recombinant plasmid DNA were analyzed by the dideoxy chain-termination method [29]. The MT cDNA fragment subcloned into pBluescript II KS+ was used as a probe and labeled with [α-^{32}P] dCTP (111 TBq/mmole, ICN, Irvine, CA) by the random-primed method [9]. This probe was used to screen the MT cDNA clones from the cDNA library by colony hybridization [28]. The cDNA library grown overnight on LB-agar containing ampicillin and tetracycline was transferred to a nylon membrane (Hybond N, Amersham, UK). Colonies were lysed and plasmid DNAs were fixed onto the filter by ultraviolet. After prehybridization in 50% formamide, 5 × Denhardt's solution, 5 × SSC, 50 mM Na_2HPO_4 (pH 7.0), 1% SDS and 100 μg/m*l* of heat-denatured salmon sperm DNA for 2 hr at 37°C, the filter was hybridized under the same conditions with heat-denatured and ^{32}P-labeled MT cDNA fragment for 20 hr. It was washed twice in 2 × SSC containing 0.1% SDS and once in 1 × SSC containing 0.1% SDS for 15 min at 37°C. The filter was autoradiographed for 2 days at −80°C.

Eight clones which hybridized to the canine MT cDNA fragment were isolated from 12,000 transformants. Double stranded plasmid DNAs were prepared from the obtained clones. These clones showed the same molecular size and pattern of restriction enzyme digestion (data not shown). These nucleotide sequences were analyzed by the dideoxy chain-termination method [29] using *Bca*BEST™ Dideoxy Sequencing Kit (TAKARA SHUZO, Shiga, Japan). We sequenced two of the eight clones and confirmed that the sequence data of one clone was identical to that of the other clone (data not shown). Two clones contained the entire coding region for canine MT. Figure 1 shows the sequencing strategy and restriction map of the canine MT cDNA. Figure 2 shows the nucleotide and the deduced amino acid sequence. This clone contained an initiation codon (ATG) followed by an open reading frame of 61 amino acids. The encoded protein contained abound cysteine residues, many of which showed the characteristic sequence (Cys-X-Cys) and had neither aromatic amino acids nor histidine; this is in agreement with the reported features of the MTs [19]. The conserved sequence common in all mammalian MT-1 and MT-2 (Met-Asp-Pro-Asn residues from position 1 to 4 [12]) has not been determined in the amino acid sequence analysis of canine MT previously reported [22]. In the present study, we determined that the canine MT cDNA encoded Met-Asp-Pro-Asp residues at position 1 to 4. Asp at position 4 has been found in fowls and frogs [24]. Significance of the alteration in the evolution is not certain at this moment.

The deduced amino acid sequence showed 93.4%, 93.4%, 85.2% and 85.2% homology to human MT-1e [30], human MT-2A [17], mouse MT-1 [8] and mouse MT-2 [31], respectively (Fig. 3). The classification of the major mammalian subgroups MT-1 and MT-2 is based on the respective absence and presence of an acidic amino acid residue in position 10 or 11 of the standard sequence [19]. In this clone, Gly exists at position 10 and 11. Therefore, it is likely that this cDNA from the Cd-treated dog is derived from canine MT-1 mRNA. The amino acid sequence analysis based on different elusion profiles from anion exchangers should be required for the confirmation of the MT isoforms [19].

The nucleotide sequence flanking the initiation codon (GAGATGG, position −3 to 4) agrees with the consensus

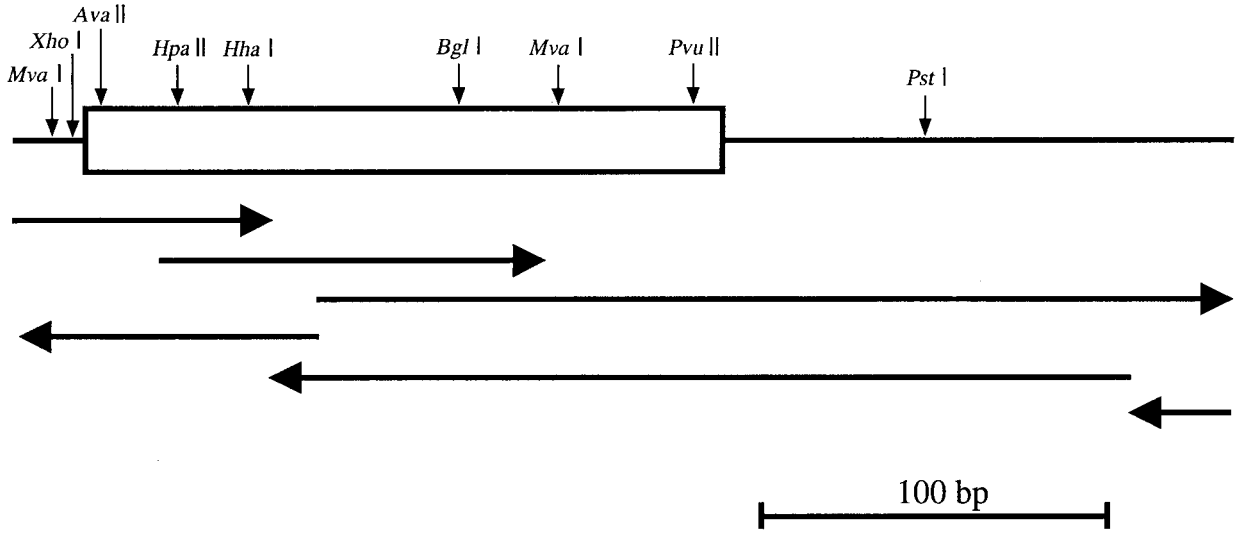


Fig. 1. Sequencing strategy and restriction map of canine MT cDNA clones. The open box shows the open reading frame. The line represent 5'- and 3'-noncoding regions. Each arrow indicates the direction and extent of sequence determination.



Fig. 2. Nucleotide sequence of the canine MT cDNA with the deduced amino acid residues. Nucleotide sequence is numbered beginning with the 5' end residue of ATG initiation codon as +1 and the nucleotide sequence of the upstream region is numbered by negative. Cysteines and a termination codon is encircled by narrow line or broad line, respectively.

	1									10										20										30
Canine MT	M	D	P	D	C	S	C	S	T	G	G	S	C	T	C	A	G	S	C	K	C	K	E	C	K	C	T	S	C	K
Mouse MT-1	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	T	S	-	-	A	-	-	N	-	-	-	-	-	-
Mouse MT-2	-	-	-	N	-	-	-	A	S	D	-	-	-	S	-	-	-	A	-	-	-	-	Q	-	-	-	-	-	-	-
Human MT-1e	-	-	-	N	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human MT-2A	-	-	-	N	-	-	-	A	A	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

										40										50										60
Canine MT	K	S	C	C	S	C	C	P	V	G	C	A	K	C	A	Q	G	C	I	C	K	G	A	S	D	K	C	S	C	C	A
Mouse MT-1	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	V	-	-	-	-	A	-	-	-	T	-	-	-
Mouse MT-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	Q	-	-	-	-	-	-	-
Human MT-1e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	E	-	-	-	-	-	-	-
Human MT-2A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fig. 3. Comparison of canine MT amino acid sequence to mouse and human MTs. Identities are indicated by hyphens. The deduced amino acid sequence in this study was identical to that of the canine MT previously described by Learch *et al.* [22]. Sequence references : Mouse MT-1 (Durnam *et al.* [8]), Mouse MT-2 (Searle *et al.* [31]), Human MT-1e (Schmidt *et al.* [30]), Human MT-2A (Karin and Richards [17]).

sequence (A/GXXATGG) for the start of translation in eukaryotes proposed by Kozak [20]. The polyadenylation signal is ATAAAA instead of AATAAA, which is found in most other mRNAs [28]. This sequence, ATAAAA, was also confirmed in the other canine MT cDNA clone (data not shown). However, whether ATAAAA is recognized by the polyadenylation system or not is unknown. Highly conserved nucleotide sequence (TTTCTA) in the 3'-noncoding region [1, 8, 11, 17, 26] was observed in the canine MT cDNA (position 279 to 284); this sequence is

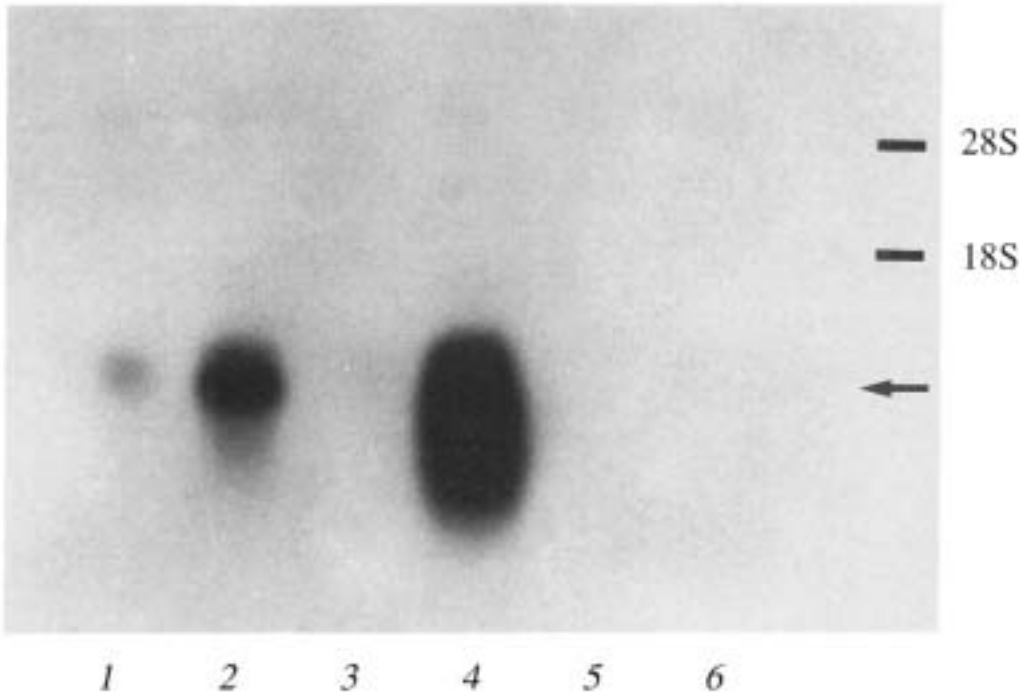


Fig. 4. Northern blot analysis of MT mRNA in the liver, kidney and brain of normal and Cd-treated dogs. Twenty μg of total cellular RNA was electrophoresed on 1.0% agarose/formaldehyde gel (Lehrach *et al*. [23]), blotted onto positive-charge nylon membrane (Hybond N+, Amersham, UK) and UV cross-linked. The filter was hybridized with ^{32}P-labeled canine MT cDNA obtained from the Cd-treated beagle for 20 hr. The filter was autoradiographed for 2 days at −80°C without an intensifying screen. Lane 1: RNA from the liver of the normal dog. Lane 2: RNA of the liver of the Cd-treated dog. Lane 3: RNA from the kidney of the normal dog. Lane 4: RNA from the kidney of the Cd-treated dog. Lane 5: RNA from the brain of the normal dog. Lane 6: RNA from the brain of Cd-treated dog. The position of MT mRNA signals is indicated by an arrow.

not present in the human MT-1e and MT-1f [30]. On the other hand, the conserved octanucleotide (TGTAAATA) noted by Griffith *et al*. [11] is altered to TGTATATA in the canine MT cDNA (position 209 to 216). Some alterations in this region has also been seen in sheep MT (TGTCAACA) [26], human MT-2A (TGTAAAGA) and MT-1f (TGTAACA) [30]. The inconsistency of the octanucleotide sequences may imply that this region is irrelevant to the regulation of mRNA translation. Further studies will be necessary to determine the potential functions of the octanucleotide sequences.

Total cellular RNA from the liver, kidney and brain of normal and Cd-treated beagles was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [6]. Twenty μg of total cellular RNA was electrophoresed on 1.0% agarose/formaldehyde gel [23], blotted onto positively charged nylon membrane (Hybond N+, Amersham, UK) and cross-linked by ultraviolet. Prehybridization and hybridization were conducted using ^{32}P-labeled MT cDNA under the same conditions as the colony hybridization. It was washed twice in 2 × SSC containing 0.1% SDS and once each in 1 × SSC and 0.1 × SSC containing 0.1% SDS for 15 min at 37°C. The filter was autoradiographed for 2 days at −80°C. Northern blot analysis of the total RNAs extracted from the liver, kidney and brain of normal and Cd-treated beagles revealed a single band using the canine MT cDNA obtained in this study as a probe (Fig. 4). Compared to the slight expression of MT mRNA in the liver of the normal dog, expression of both hepatic and renal MT mRNAs was significantly induced in the Cd-treated dog. On the other hand, MT mRNA expression was not detected in the brain either in the normal or Cd-treated beagles. Cd intravenously administered to rats does not accumulate in the brain parenchyma because of the presence of the blood-brain barrier [3]. Therefore, it was supposed that MT mRNA expression was not induced enough to detect in the brain of Cd-treated dog.

The canine MT cDNA obtained in the present study may be an useful tool for the investigation of tissue localization, gene expression and function.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number D84397.

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