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Molecular analysis of cytosolic and mitochondrial malate dehydrogenases isolated from domestic cats (*Felis catus*)

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ABSTRACT. Malate dehydrogenase (MDH) plays crucial roles in energy and cellular metabolism. In this study, we describe the identification and characterization of cytosolic MDH (MDH1) and mitochondrial MDH (MDH2) in liver of domestic cat (*Felis catus*). To clone the feline full-length *MDH* genes, we performed rapid amplification of cDNA ends. The *MDH1* gene encoded a protein of 334 amino acids and the *MDH2* gene encoded a protein of 338 amino acids, containing a 24-amino acid mitochondrial target sequence. The feline MDH1 and MDH2 proteins shared, respectively, 98.8-93.7 and 96.7-94.4% homology with dog, giant panda, horse, cow, pig, human, mouse, and rat. The feline MDHs had a highly conserved active motif, which contained important residues for catalysis and coenzyme binding. The putatively acetylated lysine residues that regulate MDH activity were also conserved at K118, K121, and K298 in MDH1, and K185, K301, K307, and K314 in MDH2. Both *MDH1* and *MDH2*

mRNAs were ubiquitously expressed, but these expression levels varied in a tissue-specific manner. Both *MDH* genes were expressed at considerably high levels in heart and skeletal muscle, but at low levels in lung and spleen.

Key words: Cytosolic malate dehydrogenase; Lysine acetylation; Mitochondrial malate dehydrogenase; Domestic cat; *Felis catus*

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD)-dependent malate dehydrogenase (MDH; EC1.1.1.37) catalyzes the reversible conversion of malate to oxaloacetate linked to oxidation and reduction of the coenzyme NAD. The detailed aspects of MDH have been discussed in several reviews (Goward and Nicholls, 1994; Musrati et al., 1998; Minárik et al., 2002). Most eukaryotes express two types of MDH, cytosolic MDH (MDH1) and mitochondrial MDH (MDH2). The MDH enzymes are essential for both gluconeogenesis and the tricarboxylic acid (TCA) cycle and therefore play crucial roles in energy metabolism. MDH1 is the key enzyme in the malate/aspartate shuttle, which is the dominant pathway in the liver and heart (Scholz et al., 1998), while MDH2 is known to be a key enzyme in the TCA cycle. Cytosolic MDH, mentioned as MDH2 in yeast, has been shown to work as a gluconeogenic enzyme, because it is required for the growth of yeasts on a minimal medium in the presence of gluconeogenic precursors (Gibson and McAlister-Henn, 2003).

Feline liver requires the high enzymatic activities of MDHs to produce glucose via gluconeogenesis. As domestic cats (*Felis catus*) rely mainly on hepatic glucose production to maintain blood glucose levels, gluconeogenesis from amino acids is highly constitutive even after eating (MacDonald et al., 1984).

Cellular metabolic rate and glucose utilization status affect the expression and activity of MDHs. For example, MDH1 expression level in leukocytes is lower in cats than in dogs (Washizu et al., 2002). In addition, MDH activity and expression is decreased in diabetes (Magori et al., 2005), suggesting that this glucose metabolic disorder affects its expression and/or activity in cats. However, the structure, regulatory mechanisms, and distribution of feline MDHs are still unclear.

Recent proteomic studies have shown that lysine acetylation regulates many metabolic enzymes involved in glycolysis, gluconeogenesis and fatty acid synthesis, as well as the TCA and urea cycles (Kim et al., 2006; Choudhary et al., 2009; Wang et al., 2010; Zhao et al., 2010). MDH activity is regulated by protein acetylation, which is a crucial post-transcriptional regulator of energy and cell metabolism. Zhao et al. (2010) demonstrated that MDH2 activation is not observed when putative lysine residues are replaced by arginine. We speculated, therefore, that the activity of feline MDH is regulated similarly by lysine acetylation and deacetylation.

The aims of this study were: 1) to identify the primary structure of feline MDHs; 2) to explore the functional active motif and putative lysine acetylation residues; and 3) to determine the expression patterns of *MDH* mRNAs in feline tissues. Our findings provide a foundation for further functional analyses of feline MDHs.

MATERIAL AND METHODS

RNA extraction and partial-cDNA cloning

Feline liver RNA was extracted with TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) and then purified using Oligotex-dT30 <Super> (Takara Bio Inc., Otsu, Shiga, Japan). First-strand cDNA was synthesized (SuperScript II reverse transcriptase; Invitrogen, Carlsbad, CA, USA) with oligo d(T) as the primer. To obtain partial cDNAs of *MDH*, polymerase chain reaction (PCR) primers were designed considering the canine *MDH1* (XP_531844) and *MDH2* (XP_849944) mRNA sequences (Table 1). Reverse transcription PCR (RT-PCR) was performed with feline liver cDNA as the template using these primers. The PCR products were ligated into the pT7Blue T-vector (Novagen, Inc., Madison, WI, USA) and then transformed into NovaBlue competent *Escherichia coli* cells (Novagen). The inserts were sequenced using BigDye Terminator v3.1 and an ABI PRISM 310 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Full-length cDNA cloning of feline MDH1 and MDH2

After determining partial MDH fragments, the rapid amplification of cDNA ends (RACE) method was used to clone full-length feline *MDH* cDNA, using the GeneRacer kit (Invitrogen) according to the manufacturer protocol. One of the 3'-RACE primers for *MDH1* (MDH1-for2; Table 1) was designed on the basis of a partial sequence. Reactions for 5'- and 3'-RACE were performed with GeneRacer primers and gene-specific primers (Table 1). The amplified PCR products were purified and subcloned. Both strands of the longest 5'-RACE and 3'-RACE cDNA inserts were sequenced using the primer walking method.

Table 1. Sequences of primers used in cDNA cloning and real-time PCR.

Primer	Sequence (5'-3')	Gene	Aplication
MDH1-for	AGC CAA TAC CAA CTG CCT GAC TGC	*MDH1*	RT-PCR
MDH1-rev	GGA GAT AAT GCC CAT GGA CAC AAA	*MDH1*	RT-PCR, 5'-RACE
MDH1-rev2	AAT GTC TGC TGC AAA AGC CAT CTC	*MDH1*	3'-RACE
MDH2-for	GCA TGA CAC GGG ATG ATC TGT TC	*MDH2*	RT-PCR, 3'-RACE
MDH2-rev	CAC TGA GAG ATC AGG GGG ATG ATG G	*MDH2*	RT-PCR, 5'-RACE
GeneRacer oligo d(T) primer	GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG (T)18		RT
GeneRacer 5'-primer	CGA CTG GAG CAC GAG GAC ACT GA		5'-RACE
GeneRacer 3'-primer	GCT GTC AAC GAT ACG CTA CGT AAC G		3'-RACE
qMDH1-for	AAG ATG TTG CCT TCA AAG ACC T	*MDH1*	qPCR
qMDH1-rev	TTC CCA CCA CAA TAA CCT TAA CTG	*MDH1*	qPCR
qMDH2-for	CAA GAA GCA TGG AGT ATA CAA CCC	*MDH2*	qPCR
qMDH2-rev	ACC TTG GGA GTA CAC TGA GAG	*MDH2*	qPCR
qCYPA-for	CAA AGT TCC CAA GAC AGC AGA GA	*CYPA*	qPCR
qCYPA-rev	AGT GCC ATT ATG GCG TGT GAA	*CYPA*	qPCR

Sequence analyses

A search for the mitochondrial targeting sequence in MDH2 was conducted using the MitoProt program (http://ihg.gsf.de/ihg/mitoprot.html; Claros and Vincens, 1996). Active mo-

tif searches were performed against the PROSITE motif database via the MOTIF search program (Falquet et al., 2002) available on the website of the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/tools/motif/MOTIF.html). Alignment of multiple sequences was performed using the ClustalW program.

The protein sequence reference numbers used in the alignment analysis of MDH1 and MDH2 are respectively as follows: dog (XP_531844, XP_849944); giant panda (XP_002921852, XP_002923512); horse (XP_001494315, NP_001182455); cow (NP_001029800, AA109598); pig (NP_999039, NP_00121082); human (NP_005908, NP_005909); rat (NP_150238, NP_112413); and mouse (NP_032644, NP_032643).

Determination of tissue distribution by quantitative real-time PCR (qPCR)

Tissue distribution of *MDH1* and *MDH2* mRNA was determined by qPCR. Total RNAs from various feline tissues, including heart, lung, liver, pancreas, stomach, ileum, colon, kidney, spleen, skeletal muscle, and adipose tissue were purchased from Zyagen (San Diego, CA, USA). The relative expression of *MDH1*, *MDH2*, and cyclophilin A (*CYPA*) mRNAs were determined by qPCR. The primers for qPCR are shown in Table 1. The primers used for *CYPA* were reported in the study by Zini et al. (2010). First-strand cDNA was synthesized (PrimeScript RT Reagent kit, Takara Bio) and the subsequent qPCR was carried out on a GeneAmp 7500 Sequence Detection System (Applied Biosystems) with SYBR Premix Ex Taq II (Takara Bio) following the manufacturer protocol. The comparative Ct method ($\Delta\Delta^{Ct}$ method) was used to analyze the relative fold-changes of MDHs (Bookout and Mangelsdorf, 2003). Expression levels of *MDH1* and *MDH2* in each tissue were normalized to *CYPA*. The ratios of *MDH1*/*CYPA* and *MDH2*/*CYPA* from each tissue were standardized to the liver ratio of 1.0-fold. Each sample was analyzed in duplicate and the experiment was repeated three times.

RESULTS

Primary structures of feline MDH1 and MDH2

The nucleotide and deduced amino acid sequences of the feline *MDH1* cDNAs are shown in Figure 1A. Feline *MDH1* consists of 1292 bp, which includes a 5'-untranslated region (UTR) of 75 bp and a 3'-UTR of 212 bp (Figure 1A). Feline *MDH1* cDNA contains a polyadenylation signal (AATAAA) 21 bp upstream from the poly(A) tail. This cDNA encodes a polypeptide of 334 amino acids (aa). The calculated molecular mass of the polypeptide is 36.4 kDa. The complete feline *MDH1* nucleotide sequence was submitted to the DNA Data Bank of Japan (DDBJ) as accession number AB113364.

The nucleotide and deduced amino acid sequences of feline *MDH2* cDNA are shown in Figure 1B. Feline *MDH2* cDNA consists of 1281 bp, including a 5'-UTR of 81 bp and a 3'-UTR of 180 bp (Figure 1B). Feline *MDH2* cDNA contains a polyadenylation signal 26 bp upstream from the poly(A) tail. Feline *MDH2* encodes a polypeptide of 338 aa with a calculated molecular mass of 35.5 kDa. The feline *MDH2* nucleotide sequence was deposited in the DDBJ as accession number AB751611.

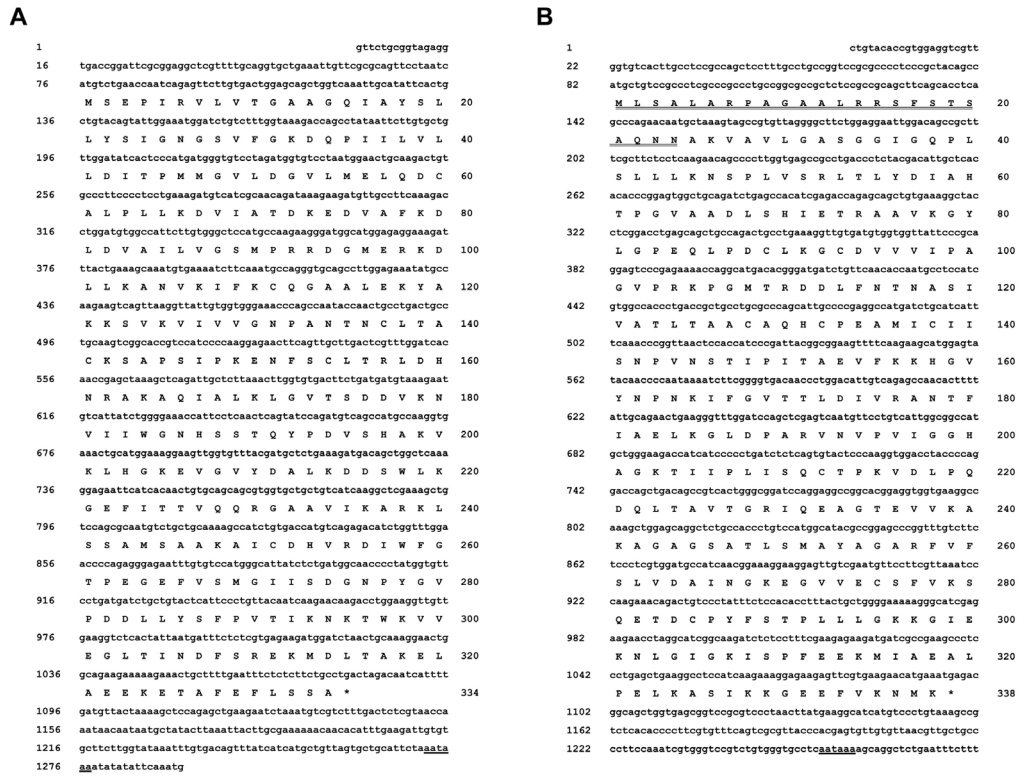

Figure 1. Nucleotide and deduced amino acid sequences of feline MDH1 and MDH2. The nucleotide sequence of the *MDH* cDNA is shown on the top line, and its predicted amino acid sequence is shown below in one-letter code. Numbers on the left and right refer to the nucleotide and amino acid residue, respectively. The asterisk indicates the translation termination codon. The poly(A) signal (AATAAA) is underlined. The double underline indicates a putative mitochondrial-targeting signal. **A.** MDH1; **B.** MDH2.

Sequence analyses of active motifs and essential residues for MDH

Organelle-specific MDHs, such as mitochondrial, peroxisomal, chloroplast, and glyoxysomal MDHs, are translated as precursor proteins that possess a specific organellar targeting signal sequence at their amino terminal ends (Gietl, 1992). MitoProt analysis showed that the putative feline MDH2 precursor possesses a mitochondrial targeting signal of 24-aa residues at its amino terminal end, indicating that the mature MDH2 comprises 314-aa residues with a calculated molecular mass of 33.0 kDa.

Computational analysis by the motif-search algorithm confirmed that feline MDH1 and MDH2 have crucial, highly conserved amino acid residues that participate in catalysis, coenzyme binding, and substrate binding. MDH possesses a conserved active motif called the MDH active site signature (PROSITE, PS00068): [LIVM]-T-[TRKMN]-L-D-x(2)-R-[STA]-x(3)-[LIVMFY]. This motif was found in feline MDH1 (residues 155-167) as L-T-R-L-D-hn-R-A-kaq-I and in feline MDH2 (residues 169-181) as V-T-T-L-D-iv-R-A-ntf-I (Figure 2). The motif-search also revealed that several important residues for catalysis and coenzyme binding

are conserved. The aspartate residues at D42 (MDH1) and D57 (MDH2) are essential for coenzyme binding and specificity for the adenosine ribose hydroxyl group (Birktoft et al., 1989; Hall et al., 1992; Kelly et al., 1993). Three arginine residues (R92, R98, and R162 in MDH1, and R104, R110, and R176 in MDH2) are highly conserved among many MDHs and are important for substrate binding and catalysis. MDH enzymes require a histidine/aspartate pair to form a charge relay system (Goward and Nicholls, 1994). The feline MDHs have the corresponding histidine/aspartate pair at H187/D159 (MDH1) and H200/D173 (MDH2).

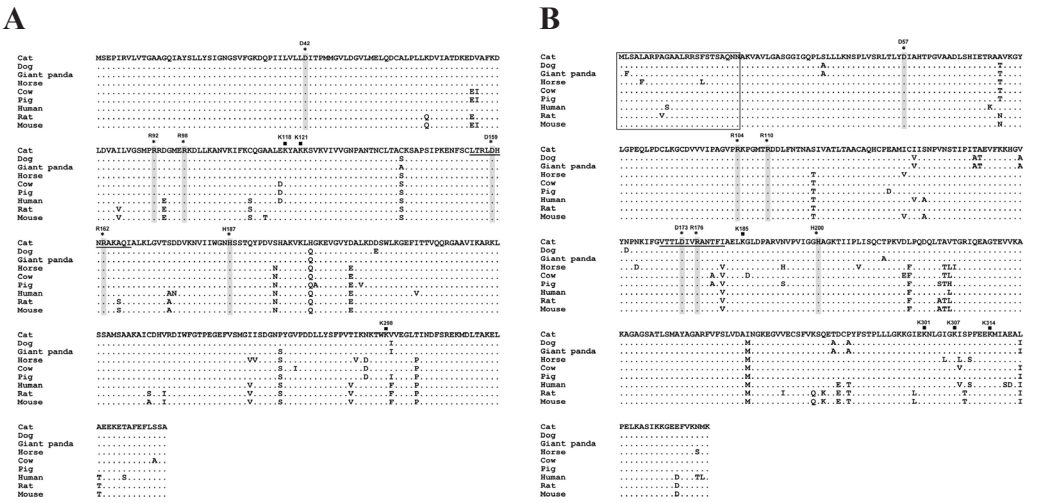

Figure 2. Multiple alignment of the amino acid sequences of feline MDH1 and MDH2. Identical residues across all sequences are represented by dots. The line under the MDH sequence indicates the MDH active motif, while asterisks above the sequence indicate residues that are important for catalysis and coenzyme binding. Solid square indicates a putative lysine acetylation site. Mitochondrial target sequences are boxed. The alignment was produced using ClustalW. **A.** MDH1; **B.** MDH2.

MDH activity by lysine-acetylation has been well studied, and several recent reports have shown that many metabolic enzymes are regulated by lysine acetylation (Kim et al., 2006; Choudhary et al., 2009; Wang et al., 2010; Zhao et al., 2010). Putative lysine acetylation residues are conserved in both MDH1 and MDH2: at K118, K121, and K298 in feline MDH1 (Figure 2A) and K185, K301, K307, and K314 in MDH2 (Figure 2B).

Comparison of MDH amino acid sequences from cats and other mammals

To search for species-specific differences, we compared the amino acid sequences of feline MDH1 and MDH2 with those of selected mammalian species. As shown in Table 2, feline MDH1 shares 98.8-93.7% sequence homology with MDH1 from other mammals. Protein sequence alignment revealed that feline MDH1 shares the highest homology (98.8%) with canine MDH1 and giant panda MDH1, while it shares the lowest homology with murine MDH1 (93.7%). Feline MDH2 shares the highest and lowest homology with canine (96.7%) and human (94.4%) MDH2, respectively (Table 2). In contrast, the sequence homology between feline MDH1 and MDH2 was found to be only 16.8%.

Table 2. Amino acid sequence comparisons between feline MDHs and those of other mammals.

	Cat	Dog	Giant panda	Horse	Cow	Pig	Human	Rat	Mouse
MDH1	100	98.8	98.8	97.0	96.4	96.1	94.9	94.3	93.7
MDH2	100	96.7	96.4	94.7	96.4	96.4	94.4	94.7	94.7
MDH1 *vs* MDH2	16.8	15.6	15.6	16.5	15.6	15.9	15.3	15.6	15.3

Data are reported as percent.

Determination of tissue distribution by qPCR

We performed qPCR to determine the expression of *MDH1* and *MDH2* mRNA in different feline tissues. The constitutively expressed *CYPA* gene was used as an internal control. The results indicated that *MDH1* and *MDH2* mRNAs are expressed ubiquitously in all tissues tested (Figure 3). In normal cats, *MDH1* mRNA expression levels, normalized to liver (1.0-fold), are the highest in skeletal muscle (39.9-fold) and heart (25.4-fold) (Figure 3A); moderate in kidney (6.3-fold), stomach (4.2-fold), pancreas (2.6-fold), and ileum (1.4-fold); and the lowest in spleen (0.32-fold), colon (0.70-fold), lung (0.72-fold), and adipose tissue (0.78-fold) (Figure 3B). *MDH2* mRNA expression levels are the highest in skeletal muscle (76.7-fold) and heart (9.9-fold) (Figure 3A), moderate in ileum (2.4-fold), kidney (1.8-fold), adipose tissue (1.4-fold), and colon (1.1-fold), and the lowest in lung (0.26-fold), spleen (0.51-fold), pancreas (0.45-fold), and stomach (0.72-fold) (Figure 3B).

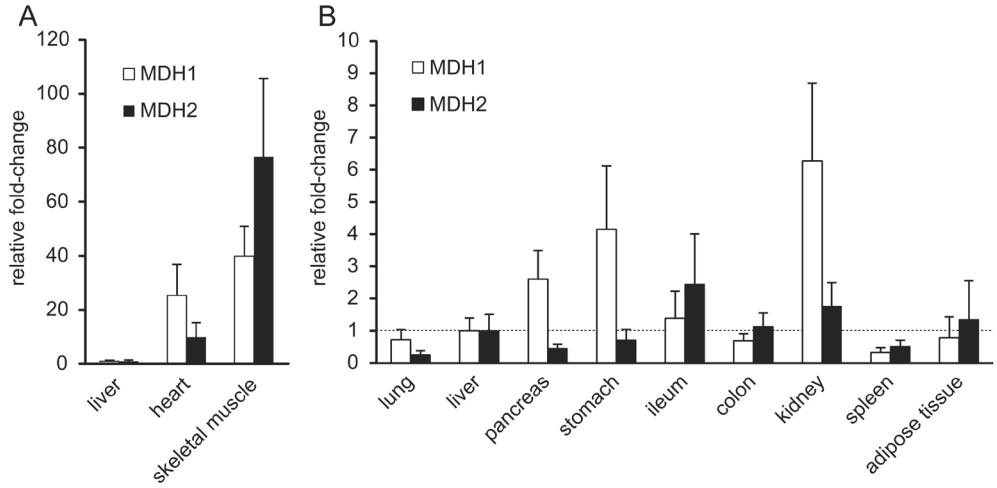

Figure 3. Expression of malate dehydrogenase mRNAs in tissues by quantitative RT-PCR. *MDH1* mRNA (open columns) and *MDH2* mRNA (closed columns) expression levels in each tissue are plotted as averages of the fold-changes. The expression level of cyclophilin A (*CYPA*) mRNA was used as an internal control. The ratio of *MDH* mRNA to *CYPA* mRNA (expressed as arbitrary units) from each tissue was normalized to liver. The columns represent the mean and standard deviation calculated from results of three independent qPCRs. **A.** High MDH expression levels in heart and skeletal muscle. **B.** Moderate or low MDH expression levels in tissues.

DISCUSSION

MDH is one of the most well-studied enzymes because of its crucial roles in the cell me-

tabolism. However, the structure, regulatory mechanisms, and distribution of feline MDHs have not been reported. In this study, we cloned two full-length *MDH* cDNAs from cats by RACE and determined their primary structure.

The cDNAs obtained were identified as feline *MDH1* and *MDH2* cDNA according to their homology to *MDH* genes of other species. The deduced polypeptide sequences of feline MDH1 and MDH2 show high homology with their counterpart in other mammals, including dog, giant panda, horse, cow, pig, human, mouse, and rat.

All active motifs and amino acid residues, which are conserved in both MDHs from other species, are important for catalysis, coenzyme binding, and substrate binding (Musrati et al., 1998). As shown in Table 2, the results indicate that the primary structures of MDHs are highly conserved among mammals; furthermore, they lack specific substitutions or deletions. The sequence homology between feline MDH1 and MDH2 is only 16.8%; this finding is consistent with that of other mammals (Table 2) from previous reports (Joh et al., 1987; McAlister-Henn, 1988). Our results show that feline MDH1 is more closely related to MDH1 from other mammals than feline MDH2. We presume that feline MDH1 and MDH2 have similar activities and are expressed in the cytoplasm and mitochondria, respectively.

More recently, Zhao et al. (2010) reported that lysine acetylation plays a major role in metabolic regulation in humans. They reported that MDH is activated by acetylation of lysine residues, and found the presence of four acetylated lysine residues (K185, K301, K307, and K314) in human MDH2. Our findings showed that the corresponding lysine residues are conserved in feline MDH2 at the same positions (Figure 2B). Kim et al. (2012) reported that the acetylation level of MDH1 increases during adipogenesis in adipocytes. When putative lysine residues are replaced with arginine, decreased activity of the mutated enzyme results in the inhibition of adipogenesis. On the basis of the feline MDH1 sequence, we determined that putative lysine acetylation residues are conserved and located at K118, K121, and K298 in feline MDH1 (Figure 2A). These findings suggest that the activity of feline MDH is similarly regulated by lysine acetylation and deacetylation.

In humans, MDH1 mRNA is expressed at relatively high levels in heart, skeletal muscle and brain (Lo et al., 2005). Similarly, Mori et al. (2009) reported that MDH1 mRNA is highly expressed in skeletal muscle in cats. We confirmed the high expression level of MDH1 in feline heart and skeletal muscle and determined that MDH2 is also expressed at high levels in heart and skeletal muscle. High expression levels of MDH1 and MDH2 in heart and skeletal muscle are reasonable because these tissues have a high metabolic rate and mainly use the malate/aspartate shuttle, which uses MDH1 and MDH2 as key enzymes. Both MDH1 and MDH2 expression levels in lung and spleen are low compared to liver. The lowest level of MDH1 expression was observed in human lung and bone marrow (Lo et al., 2005).

In conclusion, the differences in MDH activity between species are independent of the structure of the enzyme itself; however, these differences may depend on the regulation of expression, activity, or both. The distribution study showed that the level of MDH transcription correlates with the cellular energy requirement of the tissue. In addition, the enzyme activities are presumably regulated by lysine acetylation in the short term. Additional studies are required to clarify the mechanisms by which hormones and nutritional status regulate the activities, expression, and acetylation levels of MDHs in cats.

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