

Cloning and Characterization of cDNA Encoding the Porcine Mcl-1 Protein

Jyh-Yih Chen, Wei-Tung Huang and Jen-Leih Wu*

Institute of Zoology, Academia Sinica, Taipei, Taiwan 115, R.O.C.

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Jyh-Yih Chen, Wei-Tung Huang and Jen-Leih Wu (2002) Cloning and characterization of cDNA encoding the porcine Mcl-1 protein. *Zoological Studies* 41(2):136-143. The cDNA sequence of the Mcl-1 protein was determined from a porcine model (*Sus scrofa*) and is compared to the corresponding genes of rat, human, and zebrafish. Multispecies comparison of the diversity of the Bcl-2 superfamily demonstrates Mcl-1 homology between the rat, human, and zebrafish. Porcine Mcl-1 cDNA has a size of 2487 nucleotides and encodes a polypeptide of 152 amino acids. Porcine Mcl-1 shares 83.9%, 85.3%, and 50.0% similarity to rat, human, and zebrafish Mcl-1, respectively. Real-time quantitative PCR analysis revealed that Mcl-1 transcripts are highly expressed in the uterus, lung, and liver. Despite these small molecular differences in expression, our results indicate that the structure of porcine Mcl-1 protein appears to have been remarkably conserved, and it may be involved in regulation of apoptosis. <http://www.sinica.edu.tw/zool/zoolstud/41.2/136.pdf>

Key words: Porcine Mcl-1, cDNA, Real-time quantitative PCR, Tissue distribution.

Apoptotic cell death that occurs in cancer, neurodegenerative disorders, autoimmune diseases, and viral infections is critical for the maintenance of tissue balance in a healthy organism, or it may fall ill (Thompson 1995). Apoptosis is characterized morphologically as nuclear fragmentation, chromatin condensation, cytoplasmic membrane blebbing, and mitochondrial disintegration (Wyllie et al. 1980). The molecular programs that control this homeostasis involve identifying genes that influence proliferation and the transition to differentiation and death. During the past several years, significant progress has been made in identifying the molecular determinants of apoptosis (Yang and Korsmeyer 1996, Cryns and Yuan 1998). Specifically antiapoptotic and proapoptotic genes have been identified. Bcl-2, Bcl-xL, A1, Mcl-1, Nr13, and CED9 share antiapoptotic functional homology with other members of the Bcl-2 family, and proapoptotic members include bax, bcl-xShort, bak, and bok/mtd (Vaux et al. 1988,

Cazals-Hatem et al. 1992, Eguchi et al. 1992, Boise et al. 1993, Konopleva et al. 1999, Lee et al. 1999). Comparison of antiapoptotic and proapoptotic Bcl-2 family proteins reveals a common feature of sequence conservation among 1 or more Bcl-2 homologous (BH) domains (BH1 through BH4) that are arranged as α -helices. Bcl-2 and other antiapoptotic family members contain BH1, BH2, and BH3 domains that have been shown to be critical for antiapoptotic function. Some proapoptotic family members contain all 3 of these domains or contain BH3 only. These results suggest that BH3 is a minimum requirement for proapoptotic function (Akgul et al. 2000). Bcl-2 family proteins also contain a putative transmembrane (TM) domain at their C-terminus; however the TM domain is critical for subcellular localization and apoptotic activity (Nguyen et al. 1993 1994, Adams and Cory 1998). Integrating the above findings, it can be suggested that the BH1, BH2, and BH3 domains and the TM anchor

*To whom correspondence and reprint requests should be addressed. Tel: 886-2-27899568. Fax: 886-2-27824595. E-mail: zojlwu@ccvax.sinica.edu.tw

appear to have antiapoptotic activity, and that the BH3 domain is critical for proapoptotic function.

Mcl-1 is an early-induction gene isolated from maturing ML-1 myeloid leukemia cells. Its amino acid sequence is similar to that of Bcl-2, a gene originally found in B-cell lymphomas (Kozopas et al. 1993). Mcl-1 exhibits stage-specific expression in a variety of hematopoietic cells and epithelial tissues (Krajewski et al. 1994 1995) that can be induced by various survival factors, leading to enhanced cell viability (Yang et al. 1996, Chao et al. 1998). The deduced coding region of human Mcl-1 encodes a protein of 37 kDa. Mcl-1 possesses the properties of rapid turnover and transient expression with a feature of the N-terminal region containing 2 PEST sequences. PEST sequences contain proline, glutamic acid, serine, and threonine together with 4 pairs of arginines. Mcl-1 gene expression is up-regulated by cell survival cytokines (macrophage colony-stimulating factor, interleukin(IL)-1 β , and IL-3). Death-inducing signals rapidly down-regulate Mcl-1 expression (Chao et al. 1998, Moulding et al. 1998, Klampfer et al. 1999) with growth factor withdrawal or sodium salicylate treatment. Significantly, gene transfer experiments demonstrate that Mcl-1 overexpression can prolong the survival of cells even with treatment using apoptosis-inducing stimuli (Reynolds et al. 1996).

The porcine model is a good system to study malignant hyperthermia susceptibility (MHS, a skeletal muscle disorder), coronary spasms, and oocyte and follicle growth. Apoptosis appears to play a major role in the developmental process of

these phenomena. Furthermore, using stem cells and gene targeting to study the role Mcl-1 plays in the interaction with disease models or virus infections may provide possible applications to human disease models. As the first step towards this goal, we cloned and examined the tissue distribution of Mcl-1, one of the Bcl-2 family proteins. The results show that Mcl-1 exists in a truncated form in porcine mRNA species.

MATERIALS AND METHODS

Reverse transcription polymerase chain reaction (RT-PCR) for cloning an internal probe

To clone cDNA encoding porcine Bcl-2 family members, one of the members of Mcl-1 was first cloned based on a region of strong nucleotide conservation when compared to many previously published sequences. The 2 designed primers were: Mcl-1 5'-primer (nucleotides 678-703; 5'-TTCTC-GAGTGATGATCCATGTTTTTC) and 3'-primer (nucleotides 893-917; 5'-CCAGCAGCACATTTCA-GATGCCGC) (Fig. 1A). Approximately 20 μ l of porcine testis cDNA library phage solution was used as a PCR template. The PCR reaction was carried out in a final volume of 100 μ l. The reaction constituted the cDNA library liquids, 10 μ l of 10X PCR buffer (HT Biotechnology, Cambridge, UK), 200 μ M of each dNTP, 1 μ g of Mcl-1 forward (nucleotides 678-703) and reverse (nucleotides 893-917) primers, and 2.5 units of *Taq* DNA polymerase. The reaction and process followed previ-

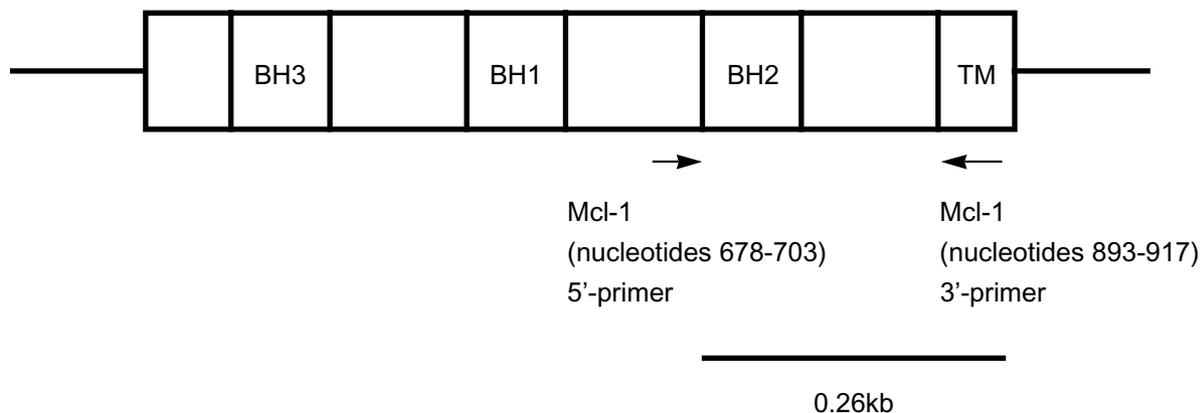


Fig. 1. (A) Diagrammatic presentation of RT-PCR primers for cloning the Mcl-1 internal probe. One 260-bp internal probe was amplified by PCR with Mcl-1 (nucleotides 678-703) 5'-primer (5'-TTCTCGAGTGATGATCCATGTTTTTC) and (nucleotides 893-917) 3'-primer (5'-CCAGCAGCACATTTCA-GATGCCGC).

ously published procedures (Chen et al. 1997 1998).

Isolation of porcine Mcl-1 cDNA clones and sequence analysis

The PCR product, about a 268-bp DNA fragment, was purified by electroelution and was used as a probe for isolating clones from a porcine muscle cDNA library by the plaque hybridization method (Chen et al. 1997 1998). About 1×10^6 recombinant bacteriophages were seeded onto 12 LB plates and transferred to nylon membranes. After denaturing, renaturing, and cross-linking, the membranes were hybridized to the Mcl-1 internal probes generated from the PCR reaction. Hybridization buffer contained SDS (7 g/100 ml), 0.5 M EDTA (pH 8.0) (100 μ l/100 ml), 50% PEG8000 (20 ml/100 ml), and 40% formamide (40 ml/100 ml), and the reaction was carried out at 37 °C for 16 h. After hybridization, filters were washed in 2XSSC, 0.1% SDS, 0.5XSSC, and 0.1% SDS, then in 0.1XSSC and 0.1% SDS at room temperature, 37, and 40 °C. Positive plaques allowed *in vivo* excision of the pBluescript phagemid from the Uni-ZAP vector. Porcine Mcl-1 cDNA was analyzed using the alignment program, GCG (Genetics Computer Group, vers. 7.0). Nucleic acid and deduced amino acid sequences were compared and aligned with other available Mcl-1 sequences using the GAP, PILEUP, DISTANCES, and GROWTREE programs. Distance software was used for calculating the pairwise sequence distances using the Kimura protein model (Trewick et al. 2000).

RNA isolation and quantification of mRNA using real-time RT-PCR assays

Tissue distribution was measured by real-time RT-PCR assay. For tissue distribution, we collected eye, heart, uterus, large intestine, pancreas, lung, brain, ovary, muscle, liver, and spleen to measure the RNA level in each tissue. Total RNA was isolated from each organ of the pig following the manufacturer's protocols (ULTRASPECTM-II RNA isolation system, Biotecx Laboratories Inc., Houston, TX, USA). Every group was replicated 3 times. Before quantification, 4.56 μ g of total RNA was transcribed to 1st-strand cDNA by the random primer method using Superscript II reverse transcriptase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) for the tissue distribution experiment. To run real-time quantitative PCR,

4.56 μ g of total RNA was isolated from each tissue and transcribed to 50 μ l 1st-strand cDNA, then 1 μ l of 1st-strand cDNA and 1 μ g of each primer were used. Nucleotides, *Taq* DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Inc., Rotkreuz, Switzerland). A total mixture with a volume of 18 μ l was loaded into glass microcapillary reaction vessels. The cDNA mixture was denatured by heating to 96 °C for 1 min. The cDNA mixture was amplified by 50 cycles of denaturation for 5 s at 95 °C, annealing of primers at 55 °C for 5 s, and extension at 72 °C for 10 s. Extension periods varied with specific primers depending on the length of the product (~1 s/25 bp). Fluorescence data were acquired during annealing or extension for reactions containing SYBR Green I (Roche Diagnostics Inc.). Thereafter, PCR products were identified by generating a melting curve. Since the melting curve of a product is sequence specific, it can be used to observe the loss of fluorescence at the denaturation temperature. The general melting protocol consisted of heating the samples to 96 °C followed by cooling to 50 °C and slow heating at 0.2 °C/s to 97 °C while monitoring fluorescence. The curve was then redrawn as the negative derivative of fluorescence with respect to temperature to generate a melting peak. The test cDNA values were compared with standard cDNA and quantified. A standard curve was constructed from the products amplified by the porcine Mcl-1 primers and quantified spectrophotometrically. PCR amplification was performed with template dilutions of 575, 57.5, 5.75, 0.575, 0.0575, and 0.00575 μ g/ μ l, and the fluorescence signal was obtained at 85 °C, just below the melting temperature (T_m) of the product and above the T_m of the primer dimers. Total RNA was analyzed by quantitative real-time PCR using a LightCycler-DNA Master SYBR Green I system (Roche Diagnostics Inc.). The following sequence-specific primers for porcine Mcl-1 were designed using Primer Express software (PE Applied Biosystems, Boston, Mass, USA): for Mcl-1 5'-primer (nucleotides 678-703; 5'-TTCTC-GAGTGATGATCCATGTTTTTC) and 3'-primer (nucleotides 893-917; 5'-CCAGCAGCA-CATTTTCAGATGCCGC). Primers were used at a concentration of 1 μ g in each reaction. The RT-PCR parameters followed the protocols of the LightCycler-DNA Master SYBR Green I instrument (Roche Diagnostics Inc.). Relative quantities of mRNA were calculated with a known quantity of PCR fragments of porcine Mcl-1 using the com-

parative threshold cycle number of each sample fitted to a 5-point standard curve. Expression levels were compared to the amount of the same-sized T7 promoter-driven fragment of cDNA from *in vitro* transcription.

RESULTS

Generation of an internal probe of porcine Mcl-1 with RT-PCR

After RT-PCR, we obtained a fragment of the predicted size (268 bp) as shown in figure 1B. This fragment was subcloned into the pCRII-TOPO vector (Invitrogen, Groningen, Netherlands) according to the Invitrogen manual. We selected 5 *E. coli* clones that contained inserts for sequencing analysis. After sequencing, we used the

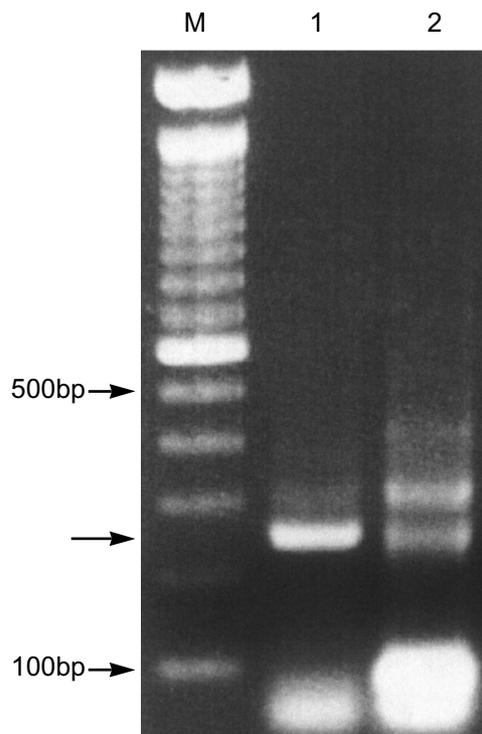


Fig. 1. (B) PCR amplification of Mcl-1 cDNA fragments with Mcl-1 5'-primer (nucleotides 678-703; 5'-TTCTCGAGTGATGATCCATGTTTTTC) and 3'-primer (nucleotides 893-917; 5'-CCAGCAGCACATTTTCAGATGCCGC). Twenty microliters of brain or testis cDNA library as a template was used for PCR, and we obtained the desired band as indicated by the arrow. We used 1.5% agarose gels, which were stained with ethidium bromide and photographed under UV light. Molecular size standards correspond to Gibco BRL 1-kb markers (lane M). Lane 1: testis cDNA library as a template; lane 2: brain cDNA as a template.

BLAST computer program to compare the sequencing data. The resulting nucleotide sequence exhibits 92% identity with the corresponding region of the cDNA sequence of rat Mcl-1 (Leo et al. 1999).

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1   ggcacgaggttaaggagtcggccctccccatttttctccaaggttggtggcggcgccg
#
61   ttgcgATGTTTGGCCTCCAGAGAAACGAGTAATCGGACTCAACCTCTACTGTGGGGGG
    M F G L Q R N A V I G L N L Y C G G A
121  CCGGATTGGGGCTGGAAGCGGCAGCAGCGCTTCCAAGGCATGCTTCGAACTGGACA
    G L G P G S G S S A F Q G M L R K L D I
181  TCAAAAACGAAGACGATGTCAAATCTTGTCTCGAGTGATGGTCCACGTTTAAAGTGAG
    K N E D D V K S L S R V M V H V L S D G
241  GAGTAACAACTGGGGCAGGATTGTGACTTATTCTTTGGTGCCTTTGTGGCCAAC
    V T N W G R I V T L I S F G A F V A K H
301  ACTTGAAGAGTATAAATCAAGAAAGCTGCATCGAACCGTTAGCAGAAAGCATCACAGAT
    L K S I N Q E S C I E P L A E S I T D V
361  TTCTCGTAAGGACAAAACGAGACTGGCTAGTCAAAACAAAGAGGCTGGATGGGTTGTGG
    L V R T K R D W L V K Q R G W D G F V E
421  AGTTCCTCCATGTAGAGACCTAGAAGCGGCATCAGAAATGTGCTGTGGCTTTTCAG
    F F H V E D L E G G I R N V L L A F A G
    *
481  GTGTGCTGGAGTAGGAGCTGGTTTGGCATACTAATAAGATAGccttttaagtgaata
    V A G V G A G L A Y L I R *
541  attgactcttaaccaactccagcccaaaaatcctcactctgtgaaatcaaatgtatttat
601  gaagttggacttcagactgtctagccataatcctcaagaggtctactctagcaacagag
661  aaaaacaagtgccaagaggattatgactgaataaatacctgggaaaagtagtttctctt
721  gaagagtcactgtctgaaagaagcagaattcagtttcagcaagtcacaacttggggaggc
781  ctgaaagactcttttagacttaggtcgatagtagggtaaaagagacttaatactctt
841  gcttagaacaggaagaggccagtagccagcctagcttagagttcattaatatgccca
901  cggacttcattaacttctttagtgcagaagagaagcactaacaacaccactgggtgtg
961  gtaaagcggatttaagctacaggaatagaactatgatctgaagcccaataactgtacaa
1021  cagcatgtgaaggaagccttttctctctataactagcttttcccaaggtttctctctg
1081  aatagaagtccaagtgctcaggacatttatacctgttctgtcttggcctgtggtttgag
1141  ggaactttttagcctcgttttaagacaaaacttgtaaatgtattgtctgtaaaaat
1201  tgtatatttttacagaaaactttcttctcctttggagaaaagtcctcaaatttaca
1261  ttagtttttcataccttttgaatctttaaactctgttagtagaacctgtttcttac
1381  tgtaattgtatgcaccctgttatagtggaacaattctgactcataactatgcagccttta
1441  atttctctgatttgataagtttcttaaggtttttgtgttttttaaacctgggac
1501  tgaaaaattgagaaatgaaaatatacttctcactttatagcgaataggtttgcaataa
1561  ttgagtcagatggggttttaagatttttttgggggtgggacacatggcctgatgac
1621  ttaaaaaaaaataaggggtttgttaccncnaccncaaaggttttttaancgggta
1681  agtaaaaaaaccttgtgtaggggaaccatggtctttgaaagcttcagcctcagaatgt
1741  acctttagtgtgacctccagacaaaaataggcatgaataatagactaagaatgtaat
1801  agggaagaattgcccgctcctccatctccagccataacatcatccagatagagttatt
1861  ttaccctgttattatcctttagcctaaaccacttatttatcatgatctctctc
1921  taaggacctaaaagcactttatgtagtaataatcttaagatcgaattaaggtgactga
1981  aaggcctgtctcccatagccacttgggaaataagccagagatccaattggggagtt
2041  tagggtccccacttcccaacttactgggtgtgactgctgaaatgcagatgagaatcttaa
2101  gtgatatttggggcaggttgggtgatctatagggaaagggagcactgttttaagtc
2161  tgactagctgttagttaggcagctcctcctcaaatggaagggaggagctgttggaaag
2221  atgtctctcagcaacttctgtctgatgtctcctcagggaacacactgcagctcctc
2281  agtgtgtctgtggtctgtgtacattctgttgggggtgaacaccttggttcgtttaa
2341  agctaaaaagctgttgacagctgtgccaggaaggttaggaccaactacaataatagtg
2401  ggctgtaaaaagtagtgttttccctaaccttctgttttctgagaagaaaataataa
2461  tctttattcaaaaaaaaaaaaaaaaa

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Fig. 2. Nucleotide sequence of porcine Mcl-1 cDNA and deduced aa sequence of the porcine Mcl-1 protein (EMBL accession no. AJ307006). Capital letters in the nucleotide sequence represent the coding region of 456 bp; lowercase letters represent the non-coding region. The coding sequence, and start (#) and stop (*) codons have been identified by aligning the porcine nucleotide sequence with the known Mcl-1 sequences of rat, zebrafish, and human. Numbering of the nucleotide sequence is shown on the left. Capital letters represent the deduced Mcl-1 protein sequence. The 2 underlined segments are the potential polyadenylation signals (AATAAA).

Isolation and characterization of the porcine Mcl-1 cDNA gene

Rat, chicken (Lee et al. 1999), and sheep Mcl-1 cDNA genes used in previous analyses were cloned from the ovary and bursa. But here, we screened about 1×10^6 recombinant bacteriophages from a porcine muscle cDNA library (Stratagene, La Jolla, CA, USA), and finally

	1	50
h1Mc1-1	MFGLKRNNAVIGLNLYCGGAGLGAGSGGATRPGGRLLATEKEASARREI	GG
hsMc1-1	MFGLKRNNAVIGLNLYCGGAGLGAGSGGATRPGGRLLATEKEASARREI	GG
pMc1-1	MFGLQRNAVIGLNLYCGGAGLGPGSGS-----	
rMc1-1	MFGLRRNAVIGLNLYCGGASLGAGGGS---PAGTRLAEEAKARRE--GG	
zMc1-1-1a	M-----A---L-----SL-----	
Mammalian conserved	MFGL RNAVIGLNLYCGGA LG G G	
	51	100
h1Mc1-1	GEAGAVIGGSAGASPPSTLTPDSRRVARRPPPIGAEPVDTATPARLLFFA	
hsMc1-1	GEAGAVIGGSAGASPPSTLTPDSRRVARRPPPIGAEPVDTATPARLLFFA	
pMc1-1	-----	
rMc1-1	GEA-----ALLPGARVVARPPVGAEDPDTASERRLLKS	
zMc1-1-1a	-----	
	101	150
h1Mc1-1	PTRRRAAPLEEMEAPADAIMSP--EEELDGY--EPEPLGK-RPAVLPLL	
hsMc1-1	PTRRRAAPLEEMEAPADAIMSP--EEELDGY--EPEPLGK-RPAVLPLL	
pMc1-1	-----	
rMc1-1	PGLLAVPPEEMAASAA--AIMSP--EEELDGC--EPEVLSK-RPAVLPLL	
zMc1-1-1a	DFRRRTATMSFFAQGVQPTLTKTCVEDELDTGTEEEAPLKRLRPGTNGLK	
	151	200
h1Mc1-1	ELVGESGNNTSDGLSPSTPPPAEEEEDELYRQSLIISRYLREQATGAK	
hsMc1-1	ELVGESGNNTSDGLSPSTPPPAEEEEDELYRQSLIISRYLREQATGAK	
pMc1-1	-----	
rMc1-1	ERVSEAAKSSGADSLPSTPPPEEEDDELYHQSLIISRYLREQATGSK	
zMc1-1-1a	GLQLDGRFVYATDGLSPSTPPPEELDYAELEDRTRQLLDFYRHTTGMP	
	201	250
h1Mc1-1	DTKPMGRSGATSRKALETLLRRV--GDGV-QRNHETAFQGM-----RKL	
hsMc1-1	DTKPMGRSGATSRKALETLLRRV--GDGV-QRNHETAFQGMVCGWLPCRGP	
pMc1-1	-----AFQGM-----RKL	
rMc1-1	DAKPLGEAGAARRALETLLRRV--GDGV-QRNHETAFQGM-----RKL	
zMc1-1-1a	VDRKL-----HHAIPMKRVV--DNILVK-HQIAYKGM-----RQL	
Mammalian conserved	AFQG R	
	251	300
h1Mc1-1	D1KNEDDVKLSRVMIH-VFSDGVTNWGRIVTLISFGAFVAKH-----	
hsMc1-1	RRWHQECAGFCRCWVS-RSWFGISN--KIALL-----	
pMc1-1	D1KNEDDVKLSRVMIH-VFSDGVTNWGRIVTLISFGAFVAKHTTCGTAG	
rMc1-1	D1KNEDDVKSFSRVMTH-VFKDGTNWGRIVTLISFGAFVAKH-----	
zMc1-1-1a	QLDSQPASLDFIRCIASMTFKDGTNWGRIASLVAFGAVVCSR-----	
Mammalian conserved	R G N I L	
	301	350
h1Mc1-1	-----LKTINQESCIEPLAESITDVLVRTKRDWLKQKRGWDFVEFFH	
hsMc1-1	-----	
pMc1-1	TGTCTACKLSINQESCIEPLAESITDVLVRTKRDWLKQKRGWDFVEFFH	
rMc1-1	-----LKSINQESCIEPLAESITDVLVRTKRDWLKQKRGWDFVEFFH	
zMc1-1-1a	-----LKELQQDQCVERVAEQISSYLTSEQQDILKNKSWHGFVEFFH	
	351	380
h1Mc1-1	VEDLEGGIRNVLLAFAGVAGVAGLAYLIR	
hsMc1-1	-----	
pMc1-1	VEDLEGGIRNVLLAFAGVAGVAGLAYLIR	
rMc1-1	VQDLEGGIRNVLLAFAGVAGVAGLAYLIR	
zMc1-1-1a	QEDVESVVRHGLLALVGCAGIAGLAFILR	

Fig. 3. (A) Alignment of the amino acid sequences of the available Mcl-1 sequences. Sequences start at the 1st methionine peptide amino acid residue; the Mcl-1 protein contains the BH3, BH1, BH2, and TM domains. A dash (-) represents a gap/deletion. A hypothetical conserved mammalian Mcl-1 sequence is shown below. GeneBank accession nos. are AF203373, AF162676, AF162677, AF231016, AF302805, and AF115380.

obtained 5 positive colonies. The recombinant plasmids of each of these clones were excised and extracted *in vivo*, and sized by 1% agarose gel electrophoresis. One of the 5 clones, designated Mcl-1-1, was chosen for further study. The size of the cDNA appeared to be about 2.5 kb, and its sequences suggest that it should be porcine Mcl-1. The nucleotide sequences were originally cloned into the EcoRI site of the phage ZAP vector. Recombinant DNA was used for sequence analysis of porcine Mcl-1 as shown in figure 2 (clone number: Mcl-1-4-9044). The porcine Mcl-1 cDNA contains 65 bp in the 5' UTR and 1963 bp in the 3' UTR, while the coding region has a length of 459 bp. Porcine Mcl-1 cDNA can be translated into a mature peptide of 153-amino acid residues. Mcl-1 amino acids from different animals are compared in figure 3A. Porcine Mcl-1 clones are about 2.5 kb in transcript length. Originally after screening, we had obtained 3 clones, namely Mcl-1-1-9044, Mcl-1-4-9044, and Mcl-1-5-9044 with nucleotide lengths of 1.6, 2.5, and 1.7 kb, respectively. Mcl-1-4-9044 and Mcl-1-5-9044 are the same clone but have dissimilar 5' sequences as shown in figure 3B. We continued to analyze Mcl-1-1-9044. Porcine Mcl-1 shares 83.89%, 85.24%, and 50.00% similarity with rat, human, and zebrafish Mcl-1, respectively. With deduced amino acid sequence comparison between mammalian species, we inferred that ancestral mammalian Mcl-1 was highly conserved in the N-terminal region. Porcine Mcl-1 has a deletion in the BH3 domain. Figure 3 shows that porcine Mcl-1 has a 219-amino-acid deletion when compared to the 4 other published Mcl-1 peptides. These phenomena are first described in this report. However, we sequenced 3 other clones, of which 2 clones differing in 5' sequences also exhibit this deletion (clone numbers Mcl-1-4-9044 and Mcl-1-5-9044, Fig. 3B). It may possibly be a muscle-specific expression type.

Mcl-1-4-9044	51	CGGCGGCGGCTTGCATGTTTGGCTCCAGAGAAACGCGAGTATCGGACT
Mcl-1-5-9044	1	GGCAGAGGGCAGGCGGAAT
Mcl-1-4-9044	101	CAACCTCTACTGTGGGGGGCCGGATTGGGGCTGGAAGCGGCAGCAGCGG
Mcl-1-5-9044	20	CGTGCCCTGGT.TTAGACAAAGAGGGCGGTGAGAACCTGCATGCCCTTTT
Mcl-1-4-9044	151	CCTTCCAAGGCATGCTTCGGAACTGGACATCAAAAACGAAGCAGATGTC
Mcl-1-5-9044	69	TCCTCTCAGGCATGCTTCGGAACTGGACATCAAAAACGAAGCAGATGTC

Fig. 3. (B) Comparison of deduced nucleotide sequences for Mcl-1-4-9044 and Mcl-1-5-9044. The predicted ATG amino acid in Mcl-1-4-9044 is in number 66.

Tissue distribution of porcine Mcl-1 gene expression

To facilitate studies of porcine Mcl-1 regulation and function in porcine, we first used real-time RE-PCR to analyze tissue distribution of expression. Real-time RT-PCR analysis fragments are located in the translated region in Mcl-1. Figure 4 shows the results of expression levels in various tissues. These findings suggest that Mcl-1 mRNA is relatively abundant in the liver, lung, and uterus. Expression of Mcl-1 mRNA suggests the possible involvement of apoptosis in a variety of cell types, especially in the liver. The pattern of expression of porcine Mcl-1 thus appears distinct from that of other known human Mcl-1 forms suggesting specific function(s) for this novel porcine Mcl-1.

DISCUSSION

The work described was aimed at identifying, cloning, and determining mRNA tissue distribution of porcine Mcl-1. We were able to isolate Mcl-1 which suggests the existence of an Mcl-1 gene in pigs as one of the Bcl-2 family of proteins. In retrospect, it is surprising that a porcine cDNA clone

was found in muscle, and it represents a truncated form. Usually, Mcl-1 is expressed early in myeloid differentiation and proves to have sequence similarity to Bcl-2; the gene is also very important in lymphoid development. Different transcripts of Mcl-1 in various organs are possibly regulated by an alternative promoter, alternative splicing, or alternative polyadenylation (Bae et al. 2000). Porcine Mcl-1 contains a longer 3' UTR than those of other mammals. A common feature of mRNAs in vertebrates is that AATAAA is present upstream of the poly (A) tail. Two putative polyadenylation signal sequences, AATAAA, were found in the 3' UTR (690-696, 2455-2460) of porcine Mcl-1. This indicates that porcine Mcl-1 may use a different polyadenylation mechanism.

The Mcl-1 protein is an important cellular regulator of antiapoptosis, with structural and biological actions attributed to the Bcl-2 family. The nucleotide sequence, 228-287, of porcine Mcl-1 is homologous to that of the human BH1 domain. Furthermore, the porcine BH2 domain (nucleotide sequence 384-431) that is analogous to the human BH2 domain. The nucleotide sequence, 465-524, of porcine Mcl-1 is homologous to that of the human TM domain. No BH3 domain was found in the porcine Mcl-1 nucleotide sequences. We designed ATG and TGA PCR primers to run an RT-PCR check on the length of the RT-PCR products. Unfortunately, we obtained no product in this experiment and are thus unable to say what the normal length of porcine Mcl-1 cDNA fragment is compared to the human Mcl-1 cDNA fragment. There are 14-amino acid insertions between the BH1 and BH2 domains. Among mammals, Mcl-1 comparisons (Fig. 3A) show that these regions are very distinctive. It is not clear whether these regions function in antiapoptosis. In humans, a splicing variant of Mcl-1 encoding a proapoptotic protein possessing only the BH3 domain was found (Bae et al. 2000). These transcripts are now designated Mcl-1S and Mcl-1L in humans. The 2 splicing variants of the human Mcl-1 protein are capable of forming dimers, and the proapoptotic action of Mcl-1S can be antagonized by Mcl-1L. From its structural and functional attributes, the BH3 domain is a proapoptotic ligand which is essential for binding activity. Generally, the antiapoptotic channel-forming Bcl-2 proteins have 3 or 4 BH domains (BH1 to BH3 or BH4) and a TM anchor sequence. However, porcine Mcl-1 contains no BH3 domain. The BH3 domain occurs only in the Bcl-2 subfamily proteins and can be further divided into 2 groups based on whether or not

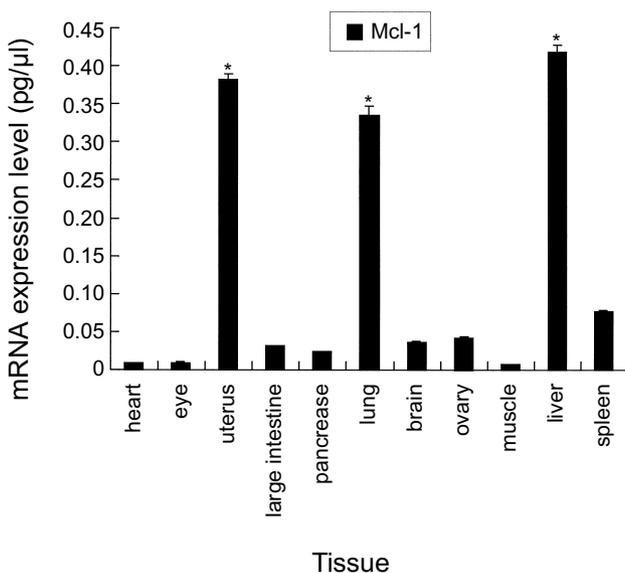


Fig. 4. Quantitative analysis of changes in Mcl-1 expression. Total RNA extracted from different tissues was analyzed using real-time RT-PCR assays. The data are expressed as explained in "Materials and Methods". The measured RT-PCR values represent 3 repeat experiments. Measured values are in pg/μl. * $p < 0.05$.

they have the TM anchor region. For example, Mcl-1S, BAD, and BID have the BH3 domain but lack the TM region. Most genes only have the BH3 domain which interacts with antiapoptotic Bcl-2 family proteins. It may be reasonable to suggest that the porcine Mcl-1 gene product that interacts with antiapoptotic Bcl-2 family proteins does not require the BH3 domain. But, these variable regions should be considered and investigated separately in future experiments.

The tissue distribution expression pattern of the porcine Mcl-1 transcript was analyzed by real-time quantitative RT-PCR. As shown in figure 4, porcine Mcl-1 was highly expressed in the liver, lung, and uterus. Relative and absolute quantitative PCR are tools that can be used to understand levels of expressed genes. Absolute quantification relates the PCR signal to the input copy number using a standard curve (Winer et al. 1999), while relative quantification measures the relative change in mRNA expression. Real-time PCR is more precise and displays a greater dynamic range than does endpoint PCR. Using real-time quantitative PCR, we determined that muscle tissue has lower expression compared to all other tissues we measured. Expression in liver compared to that in muscle showed a significant difference of about 60 fold. Liver is an important endocrine organ. Expression of the Bcl-2 family during liver regeneration and identification of Bcl-x as a delayed early response gene have been reported (Tzung et al. 1997). Induction of Bcl-2 and Bcl-x has been demonstrated in mitogen-stimulated lymphocytes *in vitro*, suggesting that these 2 apoptosis modulators may also play roles during proliferation. After a 60% partial hepatectomy in C3H/HeN mice, the levels of the 2 Mcl-1 mRNA species remained low without significant changes; inversely in normal liver, Mcl-1 mRNA species may exhibit high-level expression to protect the liver. Immunohistochemical analysis of the Mcl-1 protein in human tissues detected little or no Mcl-1 in neurons in the brain and spinal cord. Conversely, strong Mcl-1 protein immunostaining was found in cardiac and skeletal muscle, which contain comparatively less Bcl-2 (Krajewski et al. 1995).

In conclusion, we have cloned and characterized a novel member of the Mcl-1 class of the Bcl-2 family from the pig, which has a truncated form like those of other Bcl-2 family proteins. These findings demonstrate that Mcl-1 expression is widespread in tissues and that Mcl-1 proteins fulfill different roles in the overall physiology of cell death regulation. The above analyses suggest

that porcine muscle expressed Mcl-1 is a novel member of the Mcl-1 protein class.

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豬 Mcl-1 cDNA 之選殖與組織表現之研究

陳志毅 黃尉東 吳金冽

從豬之肌肉互補核苷酸基因庫中選殖出 Mcl-1 互補核苷酸序列，其由 2487 個核苷酸組成，並可轉譯出 152 個胺基酸。以豬之 Mcl-1 胺基酸序列比對大鼠、人及斑馬魚之已發表序列顯示，其相似性分別為 83.9%、85.3% 及 50.0%。另以定量聚合酶連鎖反應儀器分析顯示，Mcl-1 於豬之子宮、肺及肝臟中其核糖核酸之表現量較高。綜合上述，豬之 Mcl-1 蛋白質結構保守性高，似與細胞凋亡之調節具有相關性。

關鍵詞：豬 Mcl-1，互補核苷酸，定量聚合酶連鎖反應，組織表現。

中央研究院動物研究所