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cDNA Cloning and Expression Analysis of *Mest* Gene in the *Bufo gargarizans*

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Abstract：The *Mest*（mesoderm-specific transcript）gene has been considered an imprinting gene in human and mouse, and was also confirmed in other mammals and flowering plants. To investigate the function and evolution of this gene, the cDNA of full length *Mest* gene was obtained using 5'- and 3'-RACE from the Chinese Large Toad (*Bufo gargarizans*). The transcript is 1325 bp in length which contains a complete open reading frame (ORF) encoding a polypeptide of 326 amino acids (GenBank accession number: ABQ10905). There is a typical α/β hydrolase fold domain in the putative gene product, and it shows high similarity to sequence of homologous protein of Xenopus tropicali (86%), mammlian (70% − 80%). RT-PCR (reverse transcriptase-polymerase chain reaction) analysis demonstrated that the *Bufo gargarizans Mest* (*BgMest*) gene is expressed widely in testis, ovary, liver, kidney, spleen, brain, stomach and lung. The conservation of the *BgMest* gene sequences, protein secondary structure of the *BgMest* protein, in addition to the expression pattern of the *BgMest* gene, suggested that the function of *BgMest* was conserved in amphibians. However, the phylogenetic tree of the imprinting gene of the mammals and other vertebrates examined in this study indicated their divergent origins.

Key words: *Bufo gargarizans*; *Mest* gene; Imprinting gene; RACE; RT-PCR

中华大蟾蜍 *Mest* 基因的 cDNA 克隆和表达分析

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摘要：*Mest* 基因是一种印记基因，在人、小鼠以及其他的哺乳动物和有花植物中都有研究报道。为了更好地研究该基因的功能和进化特点，利用 RACE 法获得了中华大蟾蜍 *Mest* 基因(*BgMest*)的 cDNA 全长序列(1325 bp)，它包含一个完整的 ORF，可编码 326 个氨基酸的多肽（GenBank 登陆号：ABQ10905）。多肽链中包含一个典型的α/β 水解酶折叠结构域，其在氨基酸水平上与热带爪蟾和一些哺乳动物分别存在 86%和 70%～80%的相似性。进化树分析显示 *Mest* 基因为单系起源。RT-PCR 显示，*BgMest* 基因在精巢、卵巢、肝、肾、脑、胃和肺中都有表达，并且该基因在序列、表达模式以及蛋白产物的高级结构的高度保守性都说明它在两栖类生物中是保守的。但是在对哺乳动物以及一些脊椎动物的印记基因进行进化分析时，发现它们具有不同的起源。

关键词：中华大蟾蜍；*Mest* 基因；印记基因；RACE；RT-PCR
中图分类号：Q959.53；Q951.3；Q349; Q78 **文献标识码**：A **文章编号**：0254-5853-(2009)04-0369-08

Genomic imprinting refers to the parent-of-origin specific gene modification resulting in monoallelic expression of a gene dependent on its parental origin (Brannan & Bartolomei, 1999). The molecular mechanism of genomic imprinting appears to share aspects of epigenetic regulation with X inactivation in human and mouse, since both phenomena involve DNA methylation and asynchronous replication of DNA (Saitoh & Wada, 2000). This phenomenon has been also observed in mammals and flowering plants (Scott &

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Spielman,2004). To date, there are approximately 100–200 imprinting gens in the mammal genome, such as H19, IGF2, IGF2R, XIST, etc….(Looijenga et al, 1997). These special genes in mammals are imprinted so that only one of the parental alleles is actually expressed in target cells. *Mest* (mesoderm-specific transcript)/*Peg1* (paternally expressed gene 1) is an important maternally imprinted mammalian gene which is predominantly expressed in the mesoderm and its derivatives (Reule et al, 1998; Isles et al, 2006). Only the paternal copy of the gene is expressed in interspecific hybrids. This gene has been assigned to 7q32 in human and mapped to chromosome 6 in mouse (Lefebvre et al,1997; Kosaki et al,2000)

The *Mest* orthologs were studied mainly in mammals, but there were also reports in non-mammalian species like pufferfish (*Fugu rubripes)*, zebrafish, *Xenopus tropicalis* and tammar wallaby (Suzuki et al, 2005). The widespread *Bufo gargarizans* is a representative of Bufonidae, Anura, Amphbia, and it plays an important role in the ecological balance of nature. The amphibians represent a bridge in the evolution of vertebrates from aquatic to terrestrial. They have highly specialized morphological and functional characteristics to adapt to different environments. In this paper, we isolated and analyzed the expression pattern of *BgMest*. To understand the function and evolution of this gene, the phylogenic tree was also constructed.

1 Materials and Methods

1.1 Animals

Two male and two female adult *B. gargarizans* were obtained from the suburb of Wuhu city, Anhui province, China. Tissue from the testis, ovary, liver, kidney, spleen, brain, stomach and lung were dissected from the toads. Samples were snap frozen in liquid nitrogen, and stored at -80℃ until use.

1.2 Cloning of *Mest* cDNA

To amplify the conservative fragment of *BgMest* cDNA (424 bp), a pair of primers (M1: GATTGCCCTGGACTTTAT, M2: TACACCTGTCGGGAGTCC) were designed according to the conserved amino acid sequences that have been found in human (Accession number: NP_803490), and mouse (Accession number: AAM78507). To obtain the complete *BgMest* cDNA, rapid amplification of cDNA ends (RACE) technique was performed (Invitrogen, Tokyo, Japan) according to instructions.

Total RNA was extracted from toad testis with TRIzol (Invitrogen) according to the manufacturer provided protocol. Single-stranded cDNA was prepared with SuperScript TM III reverse transcriptase, following the operating instruction. For the 3'-RACE, M1 and anchor primers (5'-CGTCTAGAGGTACCGGATCCAACA-3') were used. The PCR reaction consisted of 30 cycles of 95℃ (30 s), 65℃ (30 s) and 72℃ (3 min), ending with a 7 min extension step at 72℃. After cDNA fragments had been obtained by 3'-RACE, they were inserted into the PCR2.1 vector and sequenced. For the 5'-RACE, single-stranded (ss) cDNA was reverse-transcribed from total RNA according to the manufacturer's protocol (Invitrogen, Tokyo, Japan). Oligo dA was added to the ss cDNA by terminate deoxynucleotidyl transferase according to the protocol (TOYOBO, Tokyo, Japan), which used as a template for the PCR. The PCR was performed by using M2 and oligo dT anchor (5'-CGTCTAGAGGTACCGGATCCAACAT(17)V-3') primers for 30 cycles under the following conditions: denaturation at 95℃ for 30 s, annealing at 65℃ for 30s, extension at 72℃ for 2 min, and ending with an extension step at 72℃ for 7 min. After cDNA fragments had been obtained by this procedure, they were inserted into the PCR2.1 vector and sequenced.

1.3 RT-PCR analysis and tissue expression of *Mest* gene

To analyze the tissue specificity of *Mest* gene expression, total RNA was extracted from several tissues including testis, ovary, liver, kidney, spleen, brain, stomach, lung of male and female specimens. It was prepared for homogenizing the tissue in 100 uL Trizol reagent (Takara, China) and 50 uL chloroform. The aqueous phase was precipitated in 150 uL isopropanol at 4℃ overnight. The RNA pellet was rinsed in 75% ethanol and resuspended in 12 uL DEPC-treated ddH$_2$O and immediately used for RT-PCR. Reverse transcriptase polymerase chain reaction (RT-PCR) amplifications were performed using two degenerated primers (M1 and M2) The nucleotide sequences of the primers used were EFIα, H1: TCCACCACCACCGGCCACCT; H2:CTCCCACACCAGCAGCAACAAT.

RT-PCR was carried out using 3 uL total RNA per reaction, 1×buffer reaction mix and 0.2 μmol/L b-actin primers or 0.3 μmol/L of *Mest* primers in separate tubes, in a total volume of 20 μL. All reactions for *Mest* contained 6% DMSO. cDNA synthesis was performed at 50℃ for 30 min and amplification conditions were: 94℃/5 min for initial denaturation; 94℃/30 s for denaturation, 56℃/60 s for annealing, 72℃/60 s for extension, 35 cycles; 72℃/10 min for final extension. Each PCR product was detected by electrophoresis on 1.2% agarose gel.

1.4 DNA sequencing and bioinformatical analysis

Homology analysis of the amino acid sequence alignments of *BgMest* and other vertebrates including *Homo species* (Accession number: NP_803490), *Mus musculus* (Accession number: AAM78507), *Macaca mulatta* (Accession number: XP_001098320), *Gallus gallus* (Accession number: XP_425258), *Xenopus tropicalis* (Accession number: NP_001005789), *Takifugu rubripes* (CAB96533), and *Danio rerio* (Accession number: CAK04921) was performed with Clustal X1.81 software. Phylogenetic tree construction was achieved using MEGA V2.1 (Molecular Evolutionary Genetic Analysis) program of neighbour-joining (NJ) methods. Analysis of protein secondary structure was finished by predictprotein program.

2 Results

2.1 Sequence analysis of *BgMest* gene

To isolate the *BgMest*, the complete cDNA were amplified by 5' and 3'-RACE technique, a 1325bp *Mest* cDNA encoding a predicted protein of 336 amino acids was obtained. The cDNA sequence included 34 nucleotide acids in 5'-UTR, 310 nucleotide acids in 3'-UTR, and a complete open reading frame (ORF) which conformed to several characteristics that GC content of cDNA sequence is high but the sequence is short in 5'-UTR. Fig.1 showed the full-length *Mest* cDNA of *B. gargarizans*. This *BgMest* sequence was determined by comparison with orthologous proteins of the amino acid sequences using BLAST (Accession number is ABQ10905 in the NCBI database).

The putative amino acid sequences of *BgMest* were aligned with orthologous proteins from *Homo species*, *Mus musculus*, *Macaca mulatta*, *Gallus gallus*, *Xenopus tropicalis*, *Takifugu rubripes* and *Danio rerio*. The *BgMest* showed a significant sequence homology with those of other vertebrate *Mest* proteins. It exhibited the highest similarity to *Xenopus tropicalis Mest* (86% identities) and the *BgMest* is similar to other vertebrates with 70% − 80%nt identities (Fig.2).

Additionally, based on amino acid sequence of the *Mest* protein, they contained a conserved domain named α/β-hydrolase fold domain. It conformed to the 3-D model of the *Mest* protein (Fig. 3). According to analysis via Phyre program, the model belonged to the α/β-hydrolase family. Fig. 2 showed that this domain was located at amino position 88 − 320 in mammal, at 105 − 337 in zerbrafish and at 64 − 296 in *Gallus gallus*. The domain of *BgMest* protein was located at position 107 − 321. This conserved domain was determined to be prominently expressed in mesodermal derivatives during embryogenesis only from the paternally inherited allele, and was detected in a number of proteins of several animals, plants and microorganism (Kamei et al, 2007; Yun et al, 2002).

Analysis of protein secondary structure (Fig. 4) and 3-D model of *BgMest* protein can understand its function and recognize the interactions within proteins, which is important in biological and medical research. Protein secondary structure of *BgMest* gene is composed of helix (h), strand (s) and coil (c) like the *Mest* gene of mammals. It includes six helixes, six strands and sixteen coils within the *BgMest* protein (Fig. 4). It was shown that the *BgMest* protein also contains a conserved α/β-hydrolase fold domain by analysis 3-D model of *BgMest* protein (Fig.3).

2.2 Phylogenetic tree of *Mest* gene

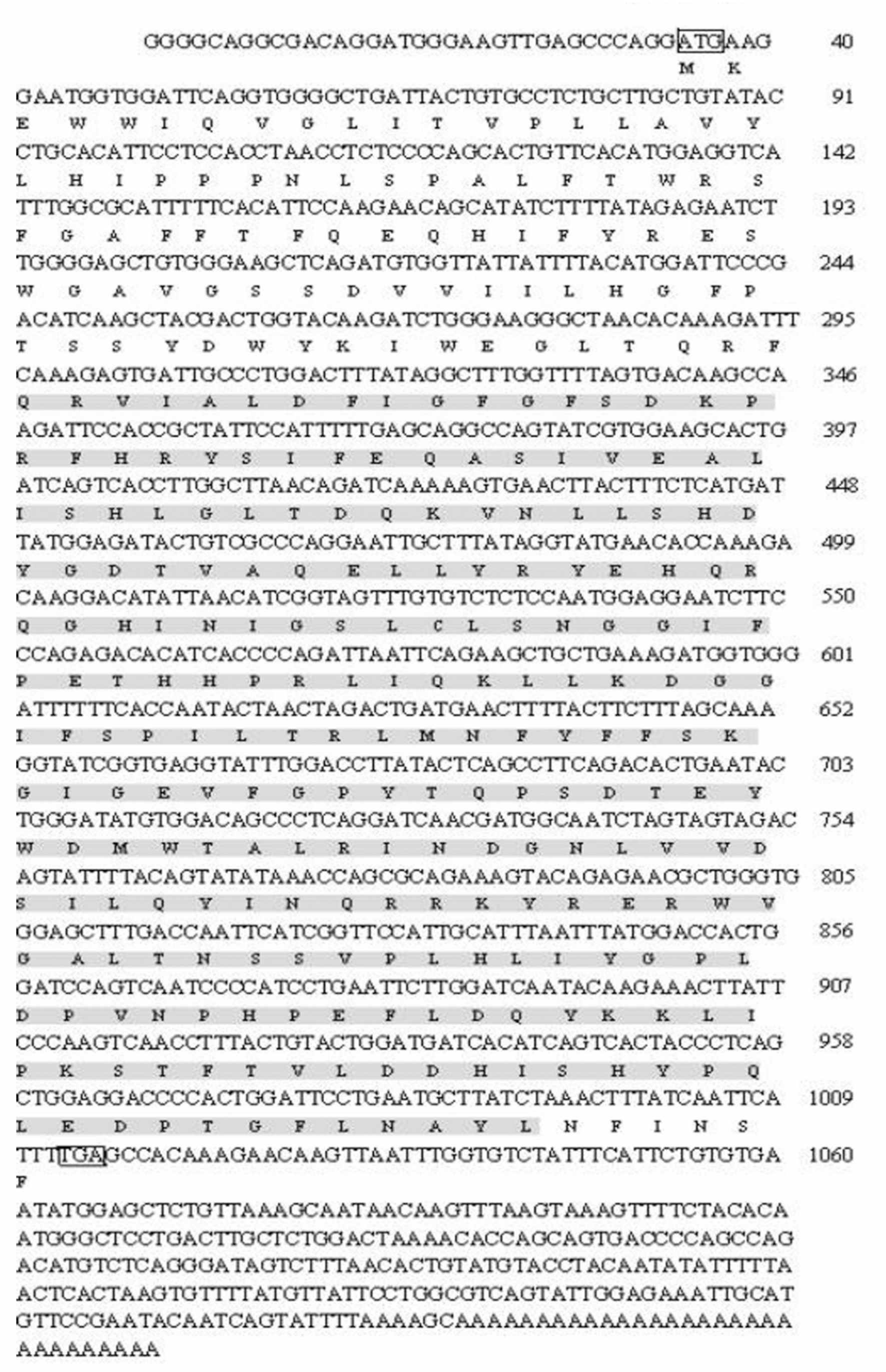
The relationships between the proteins encoded by *BgMest* gene and the corresponding proteins from other species were analyzed using neighbour-joining (NJ) method (Fig. 5). The tree showed that all mammalian proteins cluster together and the fish and amphibian cluster into another branch. This may suggest the divergent origins separating mammalians from the other vertebrates including teleosts, avian and amphibians. *Mycobacterium tuberculosis C* belonged to an actinomyces, which was separated from the vertebrate. The results were identical with traditional classification, which supports the *Mest* protein monophyly.

2.3 Expression of *BgMest*

To determine the multiple tissue expression of this gene, RT-PCR was carried out on various tissues (including testis, ovary, liver, kidney, spleen, brain, stomach and lung) in adult *B. gargarizans*. As a control, cytoplasmic EFI-*a* gene from *B. gargarizans* was used. As seen in Fig. 6, all the aforementioned tissues were expressed.

3 Discussion

The genomic structure of *Mest* gene has been shown to include a C_PG island in human, mouse and zerbrafish (Reule et al, 1998; Kosaki et al, 2000; Hahn et al, 2005). A characteristic of imprinted genes is that maternal and paternal alleles show differences in methylation position



```
              GGGGCAGGCGACAGGATGGGAAGTTGAGCCCAGGATGAAG      40
                                                M  K
GAATGGTGGATTCAGGTGGGGCTGATTACTGTGCCTCTGCTTGCTGTATAC   91
E  W  W  I  Q  V  G  L  I  T  V  P  L  L  A  V  Y
CTGCACATTCCTCCACCTAACCTCTCCCCAGCACTGTTCACATGGAGGTCA  142
L  H  I  P  P  P  N  L  S  P  A  L  F  T  W  R  S
TTTGGCGCATTTTTCACATTCCAAGAACAGCATATCTTTTATAGAGAATCT  193
F  G  A  F  F  T  F  Q  E  Q  H  I  F  Y  R  E  S
TGGGGAGCTGTGGGAAGCTCAGATGTGGTTATTATTTTACATGGATTCCCG  244
W  G  A  V  G  S  S  D  V  V  I  I  L  H  G  F  P
ACATCAAGCTACGACTGGTACAAGATCTGGGAAGGGCTAACACAAAGATTT  295
T  S  S  Y  D  W  Y  K  I  W  E  G  L  T  Q  R  F
CAAAGAGTGATTGCCCTGGACTTTATAGGCTTTGGTTTTAGTGACAAGCCA  346
Q  R  V  I  A  L  D  F  I  G  F  G  F  S  D  K  P
AGATTCCACCGCTATTCCATTTTTGAGCAGGCCAGTATCGTGGAAGCACTG  397
R  F  H  R  Y  S  I  F  E  Q  A  S  I  V  E  A  L
ATCAGTCACCTTGGCTTAACAGATCAAAAAGTGAACTTACTTTCTCATGAT  448
I  S  H  L  G  L  T  D  Q  K  V  N  L  L  S  H  D
TATGGAGATACTGTCGCCCAGGAATTGCTTTATAGGTATGAACACCAAAGA  499
Y  G  D  T  V  A  Q  E  L  L  Y  R  Y  E  H  Q  R
CAAGGACATATTAACATCGGTAGTTTGTGTCTCTCCAATGGAGGAATCTTC  550
Q  G  H  I  N  I  G  S  L  C  L  S  N  G  G  I  F
CCAGAGACACATCACCCCAGATTAATTCAGAAGCTGCTGAAAGATGGTGGG  601
P  E  T  H  H  P  R  L  I  Q  K  L  L  K  D  G  G
ATTTTTTCACCAATACTAACTAGACTGATGAACTTTTACTTCTTTAGCAAA  652
I  F  S  P  I  L  T  R  L  M  N  F  Y  F  F  S  K
GGTATCGGTGAGGTATTTGGACCTTATACTCAGCCTTCAGACACTGAATAC  703
G  I  G  E  V  F  G  P  Y  T  Q  P  S  D  T  E  Y
TGGGATATGTGGACAGCCCTCAGGATCAACGATGGCAATCTAGTAGTAGAC  754
W  D  M  W  T  A  L  R  I  N  D  G  N  L  V  V  D
AGTATTTTACAGTATATAAACCAGCGCAGAAAGTACAGAGAACGCTGGGTG  805
S  I  L  Q  Y  I  N  Q  R  R  K  Y  R  E  R  W  V
GGAGCTTTGACCAATTCATCGGTTCCATTGCATTTAATTTATGGACCACTG  856
G  A  L  T  N  S  S  V  P  L  H  L  I  Y  G  P  L
GATCCAGTCAATCCCCATCCTGAATTCTTGGATCAATACAAGAAACTTATT  907
D  P  V  N  P  H  P  E  F  L  D  Q  Y  K  K  L  I
CCCAAGTCAACCTTTACTGTACTGGATGATCACATCAGTCACTACCCTCAG  958
P  K  S  T  F  T  V  L  D  D  H  I  S  H  Y  P  Q
CTGGAGGACCCCACTGGATTCCTGAATGCTTATCTAAACTTTATCAATTCA 1009
L  E  D  P  T  G  F  L  N  A  Y  L  N  F  I  N  S
TTTTGAGCCACAAAGAACAAGTTAATTTGGTGTCTATTTCATTCTGTGTGA 1060
F
ATATGGAGCTCTGTTAAAGCAATAACAAGTTTAAGTAAAGTTTTCTACACA
ATGGGCTCCTGACTTGCTCTGGACTAAAACACCAGCAGTGACCCCAGCCAG
ACATGTCTCAGGGATAGTCTTTAACACTGTATGTACCTACAATATATTTTTA
ACTCACTAAGTGTTTTATGTTATTCCTGGCGTCAGTATTGGAGAAATTGCAT
GTTCCGAATACAATCAGTATTTTAAAAGCAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAA
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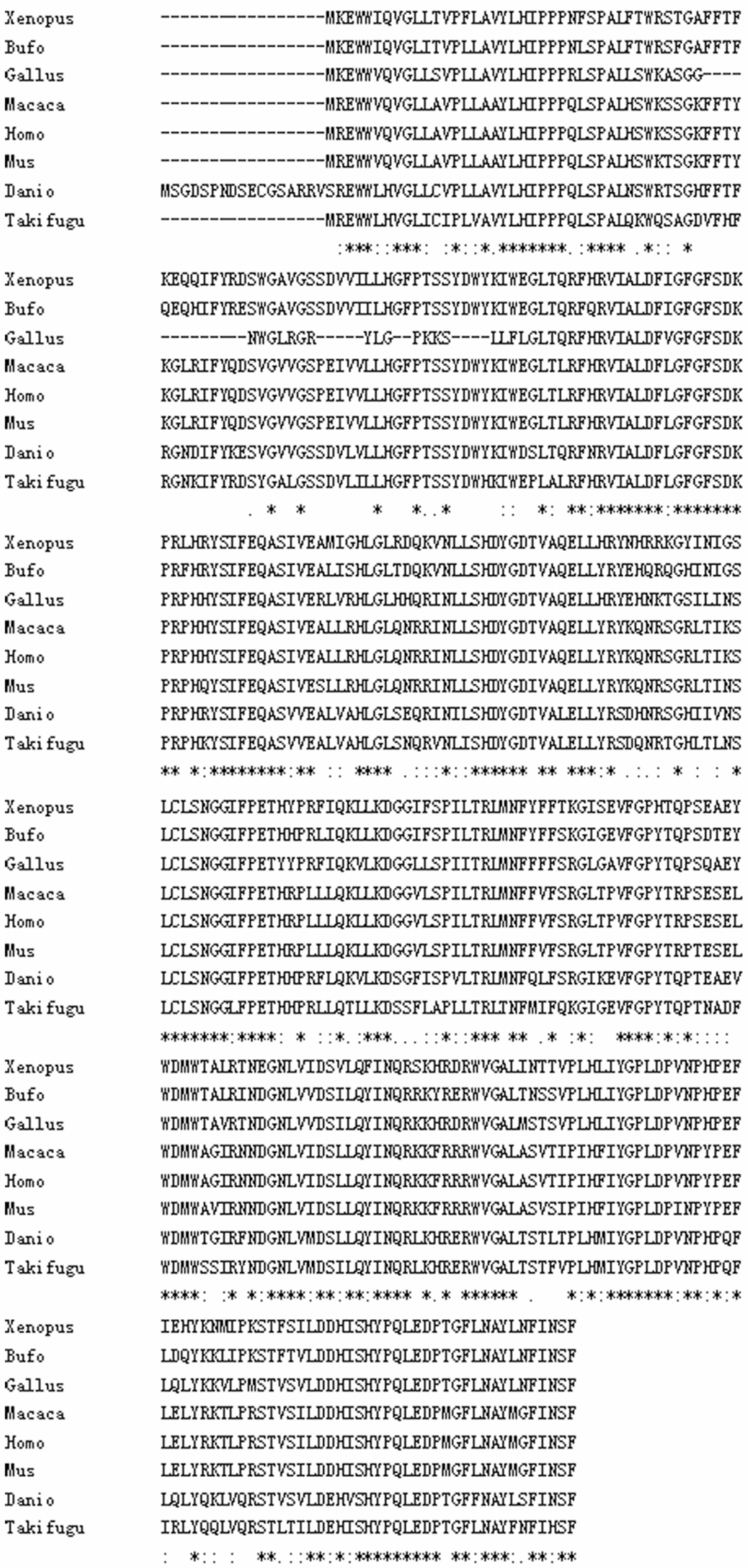
Fig. 1 *Mest* gene nucleotide and deduced amino acid sequence of *Bufo gargarizans*

Nucleotides are numbered up and deduced amino acids are below. ATG: initiation codon; TAG: termination codon.

(Georgiades et al, 2001; Edwards et al, 2007). In the researches of huamn and mouse, imprinted role of *Mest* gene was related to the methylation of C_PG island (Reule et al, 1998; Kosaki et al, 2000). However, unlike the C_PG island of normal and biallelically expressed genes, the C_PG island of *Mest* is exclusively unmethylated on the expressed paternal allele, but fully methylated on the silenced maternal allele (Reule et al, 1998). In human and mouse, *Mest* gene was expressed in amnion, brain, heart, lung, stomach, kidney and liver. Mammalian *Mest* gene is widely expressed throughout the embryo, mostly in mesodermal tissues (Reule et al, 1998; Kosaki et al, 2000). In our study, the *Mest* gene was also expressed in testis, ovary, liver, kidney, spleen, brain, stomach and lung of the adult *B. gargarizans*. And there is high similarity of the protein sequences and evolutionary



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Xenopus         -----------------MKEWWIQVGLLTVPFLAVYLHIPPPNFSPALFTWRSTGAFFTF
Bufo            -----------------MKEWWIQVGLITVPLLAVYLHIPPPNLSPALFTWRSFGAFFTF
Gallus          -----------------MKEWWVQVGLLSVPLLAVYLHIPPPRLSPALLSWKASGG----
Macaca          -----------------MREWWVQVGLLAVPLLAAYLHIPPPQLSPALHSWKSSGKFFTY
Homo            -----------------MREWWVQVGLLAVPLLAAYLHIPPPQLSPALHSWKSSGKFFTY
Mus             -----------------MREWWVQVGLLAVPLLAAYLHIPPPQLSPALHSWKTSGKFFTY
Danio           MSGDSPNDSECGSARRVSREWWLHVGLLCVPLLAVYLHIPPPQLSPALNSWRTSGHFFTF
Takifugu        -----------------MREWWLHVGLICIPLVAVYLHIPPPQLSPALQKWQSAGDVFHF
                                  :***::***: :*::*.*******.:**** .*:: *

Xenopus         KEQQIFYRDSWGAVGSSDVVILLHGFPTSSYDWYKIWEGLTQRFHRVIALDFIGFGFSDK
Bufo            QEQHIFYRESWGAVGSSDVVIILHGFPTSSYDWYKIWEGLTQRFQRVIALDFIGFGFSDK
Gallus          ---------NWGLRGR-----YLG--PKKS----LLFLGLTQRFHRVIALDFVGFGFSDK
Macaca          KGLRIFYQDSVGVVGSPEIVVLLHGFPTSSYDWYKIWEGLTLRFHRVIALDFLGFGFSDK
Homo            KGLRIFYQDSVGVVGSPEIVVLLHGFPTSSYDWYKIWEGLTLRFHRVIALDFLGFGFSDK
Mus             KGLRIFYQDSVGVVGSPEIVVLLHGFPTSSYDWYKIWEGLTLRFHRVIALDFLGFGFSDK
Danio           RGNDIFYKESVGVVGSSDVLVLLHGFPTSSYDWYKIWDSLTQRFNRVIALDFLGFGFSDK
Takifugu        RGNKIFYRDSYGALGSSDVLILLHGFPTSSYDWHKIWEPLALRFHRVIALDFLGFGFSDK
                         . *  *       *   *..*     ::  *: **:*******:*******

Xenopus         PRLHRYSIFEQASIVEAMIGHLGLRDQKVNLLSHDYGDTVAQELLHRYNHRRKGYINIGS
Bufo            PRFHRYSIFEQASIVEALISHLGLTDQKVNLLSHDYGDTVAQELLYRYEHQRQGHINIGS
Gallus          PRPHHYSIFEQASIVERLVRHLGLHHQRINLLSHDYGDTVAQELLHRYEHNKTGSILINS
Macaca          PRPHHYSIFEQASIVEALLRHLGLQNRRINLLSHDYGDTVAQELLYRYKQNRSGRLTIKS
Homo            PRPHHYSIFEQASIVEALLRHLGLQNRRINLLSHDYGDIVAQELLYRYKQNRSGRLTIKS
Mus             PRPHQYSIFEQASIVESLLRHLGLQNRRINLLSHDYGDIVAQELLYRYKQNRSGRLTINS
Danio           PRPHRYSIFEQASVVEALVAHLGLSEQRINILSHDYGDTVALELLYRSDHNRSGHIIVNS
Takifugu        PRPHKYSIFEQASVVEALVAHLGLSNQRVNLISHDYGDTVALELLYRSDQNRTGHLTLNS
                ** *:********:** :: **** .:::*::****** ** ***:* .:.: * : : *

Xenopus         LCLSNGGIFPETHYPRFIQKLLKDGGIFSPILTRLMNFYFFTKGISEVFGPHTQPSEAEY
Bufo            LCLSNGGIFPETHHPRLIQKLLKDGGIFSPILTRLMNFYFFSKGIGEVFGPYTQPSDTEY
Gallus          LCLSNGGIFPETYYPRFIQKVLKDGGLLSPIITRLMNFFFFSRGLGAVFGPYTQPSQAEY
Macaca          LCLSNGGIFPETHRPLLLQKLLKDGGVLSPILTRLMNFFVFSRGLTPVFGPYTRPSESEL
Homo            LCLSNGGIFPETHRPLLLQKLLKDGGVLSPILTRLMNFFVFSRGLTPVFGPYTRPSESEL
Mus             LCLSNGGIFPETHRPLLLQKLLKDGGVLSPILTRLMNFFVFSRGLTPVFGPYTRPTESEL
Danio           LCLSNGGIFPETHHPRFLQKVLKDSGFISPVLTRLMNFQLFSRGIKEVFGPYTQPTEAEV
Takifugu        LCLSNGGLFPETHHPRLLQTLLKDSSFLAPLLTRLTNFMIFQKGIGEVFGPYTQPTNADF
                *******:****: * ::*.:***...::*::*** ** .* :*:  ****:*:*::::

Xenopus         WDMWTALRTNEGNLVIDSVLQFINQRSKHRDRWVGALINTTVPLHLIYGPLDPVNPHPEF
Bufo            WDMWTALRINDGNLVVDSILQYINQRRKYRERWVGALTNSSVPLHLIYGPLDPVNPHPEF
Gallus          WDMWTAVRTNDGNLVVDSILQYINQRKKHRDRWVGALMSTSVPLHLIYGPLDPVNPHPEF
Macaca          WDMWAGIRNNDGNLVIDSLLQYINQRKKFRRRWVGALASVTIPIHFIYGPLDPVNPYPEF
Homo            WDMWAGIRNNDGNLVIDSLLQYINQRKKFRRRWVGALASVTIPIHFIYGPLDPVNPYPEF
Mus             WDMWAVIRNNDGNLVIDSLLQYINQRKKFRRRWVGALASVSIPIHFIYGPLDPINPYPEF
Danio           WDMWTGIRFNDGNLVMDSLLQYINQRLKHRERWVGALTSTLTPLHMIYGPLDPVNPHPQF
Takifugu        WDMWSSIRYNDGNLVMDSILQYINQRLKHRERWVGALTSTFVPLHMIYGPLDPVNPHPQF
                ****: :* *:****:**:**:**** *.* ****** .   *:*:*******:**:*:*

Xenopus         IEHYKNMIPKSTFSILDDHISHYPQLEDPTGFLNAYLNFINSF
Bufo            LDQYKKLIPKSTFTVLDDHISHYPQLEDPTGFLNAYLNFINSF
Gallus          LQLYKKVLPMSTVSVLDDHISHYPQLEDPTGFLNAYLNFINSF
Macaca          LELYRKTLPRSTVSILDDHISHYPQLEDPMGFLNAYMGFINSF
Homo            LELYRKTLPRSTVSILDDHISHYPQLEDPMGFLNAYMGFINSF
Mus             LELYRKTLPRSTVSILDDHISHYPQLEDPMGFLNAYMGFINSF
Danio           LQLYQKLVQRSTVSVLDEHVSHYPQLEDPTGFFNAYLSFINSF
Takifugu        IRLYQQLVQRSTLTILDEHISHYPQLEDPTGFLNAYFNFIHSF
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Fig. 2 *Mest* amino acid alignment of the *Bufo gargarizans* (GenBank accession number: ABQ10905)

The identical amino acid residues are indicated (*), the α/β-hydrolase fold domain was boxed by straight line. Accession numbers of other vertebrates are *Homo species* (NP_803490), *Mus musculus* (AAM78507), *Macaca mulatta* (XP_001098320), *Gallus gallus* (XP_425258), *Xenopus tropicalis* (NP_001005789), *Takifugu rubripes* (CAB96533) and *Danio rerio* (CAK04921).

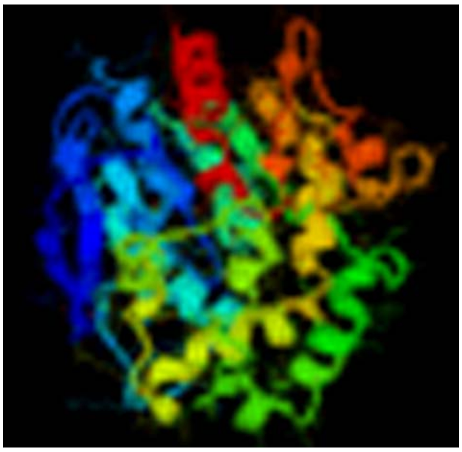
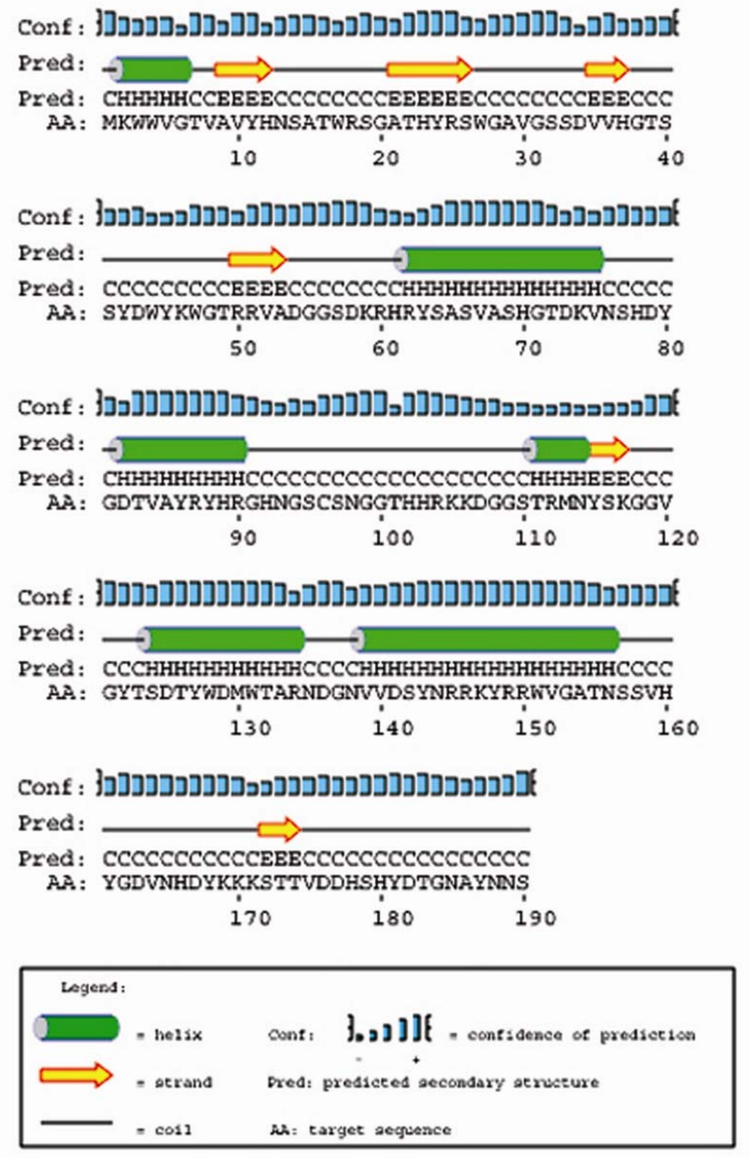


Fig. 3 3-D model of the *BgMest* protein

conservation between *BgMest* and the *Mest* genes of other vertebrates. But according to the analysis of development and allelic expression of the zebrafish *Mest*, Hahn et al (2005) concluded that zebrafish *Mest* gene is not imprinted, at least in the larval stage. Thus, whether the genomic structure of *BgMest* contains a typical CpG island and structure of DNA methylation needs to be further dertermined.

According to Fig. 5, the tree may indicate the evolutionary relationships of the potential imprinting genes of mammals and other vertebrates examined in the study. The classical *Mendel's* law of inheritance suggested that both alleles are actively transcribed and functionally equivalent. However, imprinted genes represent an exception to this rule, as they are expressed



```
Pred: CHHHHHCCEEEECCCCCCCCEEEEEECCCCCCCCEEECCC
  AA: MKWWVGTVAVYHNSATWRSGATHYRSWGAVGSSDVVHGTS
                10        20        30        40

Pred: CCCCCCCCCEEEECCCCCCCCHHHHHHHHHHHHHHCCCCC
  AA: SYDWYKWGTRRVADGGSDKRHRYSASVASHGTDKVNSHDY
                50        60        70        80

Pred: CHHHHHHHHHCCCCCCCCCCCCCCCCCCCCHHHHEEECCC
  AA: GDTVAYRYHRGHNGSCSNGGTHHRKKDGGSTRMNYSKGGV
                90       100       110       120

Pred: CCCHHHHHHHHHHHCCCCHHHHHHHHHHHHHHHHHHCCCC
  AA: GYTSDTYWDMWTARNDGNVVDSYNRRKYRRWVGATNSSVH
               130       140       150       160

Pred: CCCCCCCCCCCEEECCCCCCCCCCCCCCCC
  AA: YGDVNHDYKKKSTTVDDHSHYDTGNAYNNS
               170       180       190
```

Fig. 4 Protein secondary structure of the *BgMest* gene

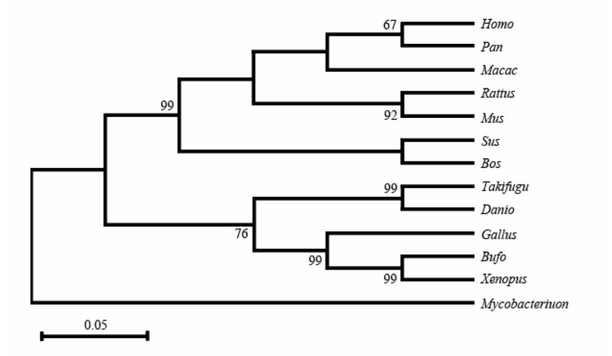


Fig. 5 Neighbour joining tree of the *BgMest* and *Mest* from other organisms based on amino acid sequence

Explanations of a part of sequences used for this tree are given in Fig. 2. Branches with less than 50% support have been collapsed. *Bos taurus* (*Bos*, AAI33640); *Sus scrofa* (*Sus*, NP_001121943); *Rattus norvegicus* (*Rattus*, NP_001009617); *Pan troglodytes* (*Pan*, XP_519382); *Mycobacterium tuberculosis C* (*Mycobacterium*, YP_002076879).

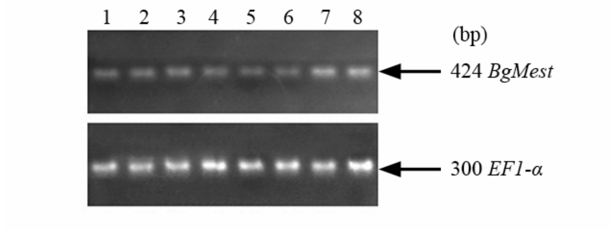


Fig. 6 RT-PCR expression of Mest in various tissues of *Bufo gargarizans*

1: Testis; 2: Ovary; 3: Liver; 4: Kidney; 5: Spleen; 6: Brain; 7: Stomach; 8: Lung. *Mest*-specific bands of 424 bp were seen in above tissue.

from one of the two parentally inherited chromosome homologues and repressed on the other (Kaneko et al, 2002; Hou et al, 2005). The *Mest* genes of human and mouse were considered as maternal imprinted gene, and imprinting role was for methylation of C$_{P}$G island (Reule et al, 1998; Kosaki et al, 2000). In mice, normal expression of *Mest* gene was very important for development. Knocking out and low expression of this gene may result in restraining growth and decreasing prenatal and postnatal survival (Reule et al, 1998). Nishita et al(1996)suggested that human *Mest* gene, the first imprinted gene to be identified on chromosome 7, was involved in the causation of Silver-Russell syndrome (SRS) (Nishita et al, 1996). Frequent loss of imprinting of the *Mest* gene in humans may cause cancer tissues, such as breast cancer or lung cancer and additionally, this gene regulates placental and fetal growth (Pedersen et al, 1999; Kosaki et al, 2000; Nakanashi et al, 2004; Suzuki et al, 2005). The zebrafish *Mest* has been found to be biallelic expressed (Hahn et al, 2005). In addition, ASCL2/CASH4 and INS were imprinted genes in mammals, but shown biallelic expression in the chicken (Yokomine et al, 2005). In the opossum (marsupial) IGF2 and M6P were imprinted as in placental mammals, but these genes are biallelically expressed in monotremes and birds (Suzuki et al, 2005). These observations may suggest that the gene imprinting occurs in mammals except monotremes (Hahn et al, 2005). Hahn et al (2005) considered that species, or clade, specific imprinted genes may have survived the selection for diluted imprinting in the evolution of non-mammalian species. Does the *BgMest* gene conform to the opinion of Hahn et al (2005)? Is *BgMest* an imprinted gene? Further research is required to uncover the structure and function of this gene.

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