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Cloning of Chicken Microsomal Glutathione S-transferase 1 Gene (MGST1) and Identification of Its Different Splice Variants

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ABSTRACT : Mammal microsomal glutathione transferase 1 (MGST1) can conjugate many toxic or carcinogenic substances and depress oxidative stress. In this study, Chicken MGST1 and its variants were cloned for the first time and were composed of 956 or 944 nucleotides. The 12 nt deletion in the exon 2 did not alter the GT-AG rule and the ORFs for the two MGST1 variants were the same, which both comprised 465 nucletides and encoded a peptide with 155 amino acids. It was found that the two different splice variants identified using RT-PCR expressed in all three organs investigated of Dwarf Brown Chicken, namely liver, spleen and shell gland. Moreover, the expression level of MGST1 mRNA in the liver of Dwarf Brown chickens was the highest ($p<0.01$), and there were no significant differences between the spleen and the shell gland. These results provide a base for studying the biological function of Chicken MGST1. (**Key Words :** Chicken, MGST1, RACE, Splice Variant)

INTRODUCTION

Microsomal glutathione transferase 1 (MGST1) is a representative of the superfamily of membrane proteins, which are termed membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). The different MAPEG members display distinct or overlapping physiological functions, including reduction of lipid hydroperoxides (glutathione peroxidase), detoxication of reactive electrophiles (glutathione transferase), and production of leukotrienes and prostaglandin E (Schmidt-Krey et al., 2000). Human MGST1 can conjugate many toxic or carcinogenic substances and depress oxidative stress (Schmidt-Krey et al., 2000; Maeda et al., 2005). Chicken MGST1 probably has important roles in physiological processes similar to Human MGST1. Antioxidative defence systems significantly influence the health of animals (Wang et al., 2006; Ma et al., 2007; Jiang et al., 2008), and as a member of the antioxidative defence system, the study of Chicken MGST1 is useful to animal husbandry. So, the experiment was carried out to identify and characterize MGST1 in chickens.

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MATERIALS AND METHODS

Tissue sampling, total RNA isolation and primer design

Six Dwarf Brown hens at 30 wk of age were slaughtered for the collection of blood and tissues of interest, including liver, spleen and shell gland. The tissues were snap-frozen in liquid N_2 and stored at -80°C until the extraction of RNA. Total RNA was prepared using Trizol reagent (Tiangen, China) according to the manufacturer's guideline. Genomic DNA was isolated from blood samples by the phenol-chloroform method (Sambrook et al., 2001). All primers were designed based on the sequence of chicken spliced EST with GenBank Accession no. BU111648 (submitted by Boardman et al., 2002), which was similar to that of Human MGST1, synthesized with ABI 3900 DNA/RNA Synthesizer (Applied Biosystems) and listed in Table 1.

Cloning of chicken MGST1 cDNA

Attempts to obtain the full length of chicken MGST1 mRNA sequences were carried out by the technique of rapid amplification of cDNA ends; 3'-Full RACE Core Set

Table 1. Primers for the cloning, expression and polymorphism analysis of chicken *MGST1*

Primers name	Sequence (5'→3')	Size (bp)	Applications
Oligo dT-3sites adaptor primer	CTGATCTAGAGGTACCGGATCC$(T)_n$	22+n	3' RACE
3sites adaptor primer	CTGATCTAGAGGTACCGGATCC	22	3' RACE
GSP-3` RACE	GCCAACCCAGAAGATACAGC	20	3` RACE
GSP1	CATAACCAACTGCCCAAGAC	20	5' RACE
GSP2	AGTATGCGAAAGTGTGAAGG	20	5' RACE
GSP3	CCACAGAGAGCATACAGCAG	20	5' RACE
AAP	GGCCACGCGTCGACTAGTACGGGII GGGIIGGGIIG	38	5` RACE
AUAP	GGCCACGCGTCGACTAGTAC	20	5` RACE
28S-F	GGAGCCCCGGGGAGAGTTC	19	To amplify 28S gene to construct the standard curve in real-time PCR
28S-R	GGATTTTCACGGGCCAGCGAGAG	23	To amplify 28S gene to construct the standard curve in real-time PCR
GAPDH-F	ATACACAGAGGACCAGGTTG	20	Used to amplify the internal reference gene in real-time PCR
GAPDH-R	AAACTCATTGTCATACCAGG	20	Used to amplify the internal reference gene in real-time PCR
Mgst-F	TCAGAATCACGAGAAAGGC	19	Used to amplify Chicken MGST1 gene in real-time PCR
Mgst-R	CCACAGAGAGCATACAGC	18	Used to amplify Chicken MGST1 gene in real-time PCR
Splice-F	GCGTTCTCCGAGTGG	15	Used to amplify the cDNA sequence of 12 nt deletion/insertion
Splice-R	GCATAGGTAGCATAAGC	17	Used to amplify the cDNA sequence of 12 nt deletion/insertion

(Takara, Dalian, CHN) and 5'-RACE kits (Invitrogen, Carlsbad, CA) were used according to the procedures provided by the manufacturer.

For 3`-RACE, reverse transcription was conducted to synthesize RACE-ready cDNA using AMV Reverse Transcriptase XL (Takara) and Oligo dT-3sites Adaptor Primer (Takara). The temperature profile was set as follows: 1 cycle of 30°C for 10 min, 50°C for 30 min, 95°C for 5 min and 5°C for 5 min. All the RACE-ready cDNA products were amplified with the sense primers (GSP-3' RACE) and the anti-sense primers (3sites Adaptor Primer, Takara), and the temperature profile was set as follows: 1 cycle of 95°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 5 min;1 cycle of 72°C for 7 min.

For 5`-RACE, reverse transcription was conducted to synthesize RACE-ready cDNA using SuperScript II (Moloney murine leukemia virus reverse transcriptase, Invitrogen) and the primer GSP1. After the cDNA synthesis, a mixture of RNase H was used to degrade the remainder of the RNA template. The first-strand cDNA was purified with a spin cartridge column. A homopolymeric C tail was then added to the 3` end of the cDNA, using deoxycytidine triphosphate and terminal deoxynucleotide transferase. The first-round PCR amplification was performed with AAP (5` abridged anchor primer) and nested GSP2. The first-round PCR product was diluted and reamplified using AUAP (abridge universal amplification primer) and nested GSP3 in the second-round PCR according to the same thermal profile, which consisted of an initial 94°C denaturation step for 2 min followed by 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 2 min). Finally, a 7-min 72°C step was used before holding at 4°C. The PCR products were separated by electrophoresis in 1.5% agarose, stained with ethidium bromide and visualized with a UV transilluminator gel documentation system (BioRad, Richmond CA). The separated PCR products were purified with the Huitiandongfang DNA purification kit (Huitian, Beijing, China) and ligated into the pMD18-T vector (Takara). Multiple clones for each fragment were sequenced on an Applied Biosystems 3730 DNA analyzer (Applied Biosystems).

Identification of different splice variants and prediction of mRNA secondary structure

Using primers of splice-F and splice-R (listed in Table 1) that were designed to identify the polymorphism of 12 nt deletion-insertion found in the present study, PCR was performed from templates of cDNA that were extracted from liver, spleen and shell gland of Dwarf Brown chicken, and the products were analyzed on a 4% agarose gel at 5 V/cm for 4 h at room temperature in 1×TAE. The mRNA secondary structure was predicted by RNAdraw 1.1b2 (Ole

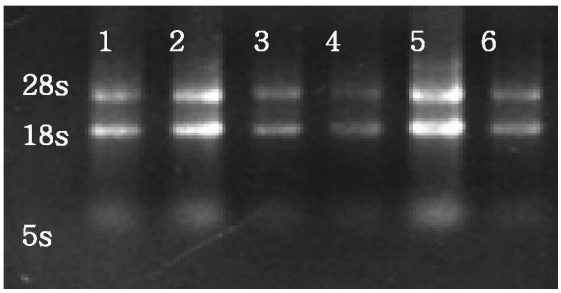

Figure 1. Native 1.2% agarose gel electrophoresis of total RNA from spleen, liver and shell gland. Lane 1 and 4 = Total RNA exacted from spleen; lane 2 and 5 = Total RNA exacted from liver; lane 3 and 6 = Total RNA exacted from shell gland.

et al., 1996).

Sequence and phylogenetic analysis

Nucleotide sequences of 3'-and 5'-RACE clones were assembled and identified by the NCBI BLAST search program. The ORFs of the overlapped mRNAs were analyzed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the amino acid identity using Blast 2 sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The alignment of multiple peptides of MGST1 was created with the ClustalW multiple sequences alignment program, displayed using the BOXSHADE 3.21 program (http://www.ch.embnet.org) and used to construct the phylogenetic tree using the neighbor-joining methods of MEGA software version 3.0 (Kumar et al., 2004). The reliability of the constructed tree was tested using bootstrap analysis implemented in MEGA software.

Expression profiling by real-time quantitative PCR

The mRNA levels of Chicken MGST1 were measured using the Quant SYBR Green PCR Kit (Tiangen, Beijing, China) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The quantities of MGST1 mRNA from different tissues were normalized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to compensate for variations in input RNA amounts. Seven serial dilutions (from 1×10^1 to 1×10^7 copies per μl) of the 28S RNA gene plasmid were selected as the templates to generate a standard curve. All reactions were carried out in a 20-μl volume containing 10 μl of SYBR Green Master Mix (Tiangen), 7 μl of water, 5 pmol of each primer, 1 μl of serial diluted 28S RNA gene plasmids, or 1 μl of cDNA derived from reverse transcription of each tissue RNA using Moloney murine leukemia virus reverse transcriptase (Tiangen, Beijing, China). Real-time PCR was performed to amplify each cDNA sample with the primers MGST-F and MGST-R in 3 duplicates, each cDNA sample with the primers GAPDH-F and GAPDH-R in 3 duplicates, the negative control and 7 diluted 28S RNA gene plasmids with the primers 28S-F and 28S-R in 3 duplicates within the same 96-well microplates. The amplification program recommended by the manufacturer was used. Specificity of the real-time PCR was evaluated by melting-curve analysis. Normalization was conducted by dividing the average copies of MGST1 by the average copies of GAPDH in each sample. Relative mRNA levels of MGST1 in the three different tissues were subjected to the ANOVA procedure in SAS 8.2. The data were presented as means±SEM.

RESULTS AND DISCUSSION

Molecular cloning of chicken MGST1

The total RNA samples exacted from spleen, liver and shell gland were run on a 1.2% native agarose gel (Figure 1). The 28S and 18S ribosomal RNA bands were clearly visible, which suggested that the total RNA samples were intact.

The fragments of 721 bp for MGST1 were generated by 3'-RACE and 393 or 381 bp by 5'-RACE. The full-length MGST1 cDNA, with 956 or 944 nucleotides, was obtained by assembling the overlapping 3'-RACE and 5'-RACE sequences (Accession number is EF585398 or EU309478). Through alignment with chicken genome in Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway?Org=chicken), it was found that both EF585398 and EU309478 were composed of four exons and the boundaries between the exon and intron conformed to the GT-AG rule, similar to Human MGST1 (Michael et al., 2000). There were two different acceptor splice sites of intron 1 of EF585398 and EU309478, and both were 'AG', which resulted in a 12 nt deletion, TAGTTTTCAAAG, in the exon 2 of EU309478 compared with EF585398 (Figure

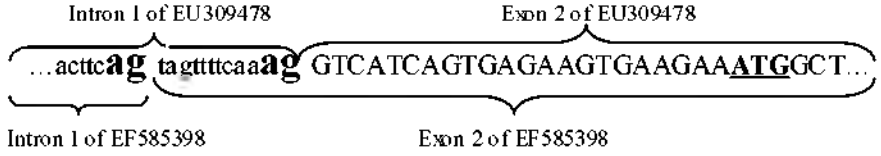

Figure 2. Two different acceptor splice sites of intron 1 of EF585398 and EU309478. The larger bold 'ag' were the two different acceptor splice sites, which resulted in 12 nt deletion, TAGTTTTCAAAG, in the exon 2 of EU309478 compared with EF585398. The bold underlined bases denote the initiation codon.

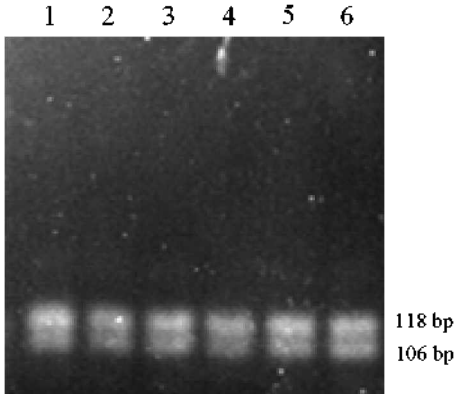

Figure 3. RT-PCR products in shell gland, spleen, and liver of Dwarf Brown chickens. Lane 1 and 4 = RT-PCR products in shell gland, lane 2 and 5 = RT-PCR products in spleen; lane 3 and 6 = RT-PCR products in liver.

2). The ORFs for the two kinds of MGST1 cDNA were the same, which comprised 465 nucleotides and encoded a peptide with 155 amino acids and resembled Human MGST1, of which four transcript variants encode one protein isoform (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=4257).

The MGST1 peptide was predicted to have an isoelectric point of 9.67 and a molecular weight of 17.24 kDa by using the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). The ORF was flanked by a 5'-UTR, which was 101 or 89 nucleotides long and a 3'-UTR, which was 363 nucleotides long. The first exon contained only 5'-UTR, in agreement with a report on the human MGST1 gene (Michael et al., 2000).

Analysis with Blast for 2 sequences (http://www.ncbi.nlm.nih.gov/blast/) revealed that the identities and positives of amino acid sequence were 69 and 80%, 69 and 80%, 67 and 80%, 67 and 81%, 43 and 59%, 42 and 58% when Dwarf Brown chicken MGST1 peptide was compared with rat, mouse, cow, human, drosophila-ra and drosophila-rb, respectively. Dwarf Brown chicken MGST1 peptides were predicted to have no N-terminal signal peptide by the PSORT II program (http://psort.ims.u-tokyo.ac.jp), which was similar to the MGST1 proteins of the rat, mouse, cow, human and drosophila.

KKXX-like motif, KALY, a probable ER (endoplasmic reticulum) Membrane Retention Signal, was found in the C-terminus of Chicken MGST1 peptides. Human MGST1 protein can catalyze the conjugation of glutathione to electrophiles and the reduction of lipid hydroperoxides and is localized to the endoplasmic reticulum and outer mitochondrial membrane where it is thought to protect these membranes from oxidative stress (Iida et al., 2001). It is very probable that Chicken MGST1 protein possesses a similar location and function.

Distribution of splice variants and function prediction of the 12 nt deletion/insertion

The electrophoresis results (Figure 3) showed that two bands, 118 bp and 106 bp, appeared in each lane, which suggested that both splice variants expressed in the three organs of Dwarf Brown chicken. The 12 bp deletion/insertion polymorphism was not found in genomic DNA (data not shown), which indicated that the 12 nt deletion-insertion in chicken MGST1 mRNA was not due to the mutation of DNA and it must have resulted from other factors. What constitutes these "other factors" needs further investigation.

The 12 nt insertion, which lay at -23nt to -34nt upstream of a translation initiation site (TIS), could cause obvious change of mRNA secondary structure predicted by RNAdraw 1.1b2 (Panel A, B of Figure 4), and a little change of free energy value at 41.5°C (From -149.29 to -149.33 kcal; the body temperature of Chicken is 41-42°C). Rousseau et al. (2003) reported that a 14 bp deletion/insertion polymorphism in the 3' UTR of the HLA-G gene influenced HLA-G mRNA stability. In further study, it would be interesting to determine if the 12 nt deletion/insertion polymorphism in the 5' UTR of the MGST1 gene functions similarly.

Construction of phylogenetic tree of the orthologous peptides for the MGST1

A phylogenetic tree of the orthologous peptides for MGST1 was constructed based on their homology in amino acid sequence using two kinds of drosophila MGST1 as the outgroup (Figure 5). The MGST1 protein of chicken was clustered into the same orthologous group with those published for other vertebrates, rat, mouse, bovine, human, respectively, all of which were mammals and these mammal MGST1 proteins were further clustered into another orthologous group. The phylogenetic tree based on MGST1 was consistent with the species phylogenetic tree, which indicated that the MGST1 gene can be regarded as a useful gene for the study of animal phylogenesis.

Expression analysis of MGST1 mRNA in Dwarf chickens

The expression profiles of the chicken MGST1 genes were examined by real-time PCR. As shown in Figure 6, MGST1 expressed in all 3 tissues investigated, liver, spleen and shell gland. The expression level of MGST1 mRNA in was higher in liver than in spleen and shell gland ($p<0.01$), and there were no significant differences between spleen and shell gland (Figure 6).

DeJong et al. (1998) and Michael et al. (2004) reported that the expression of human and murine MGST1 mRNA was also the highest in liver. From this observation, it could

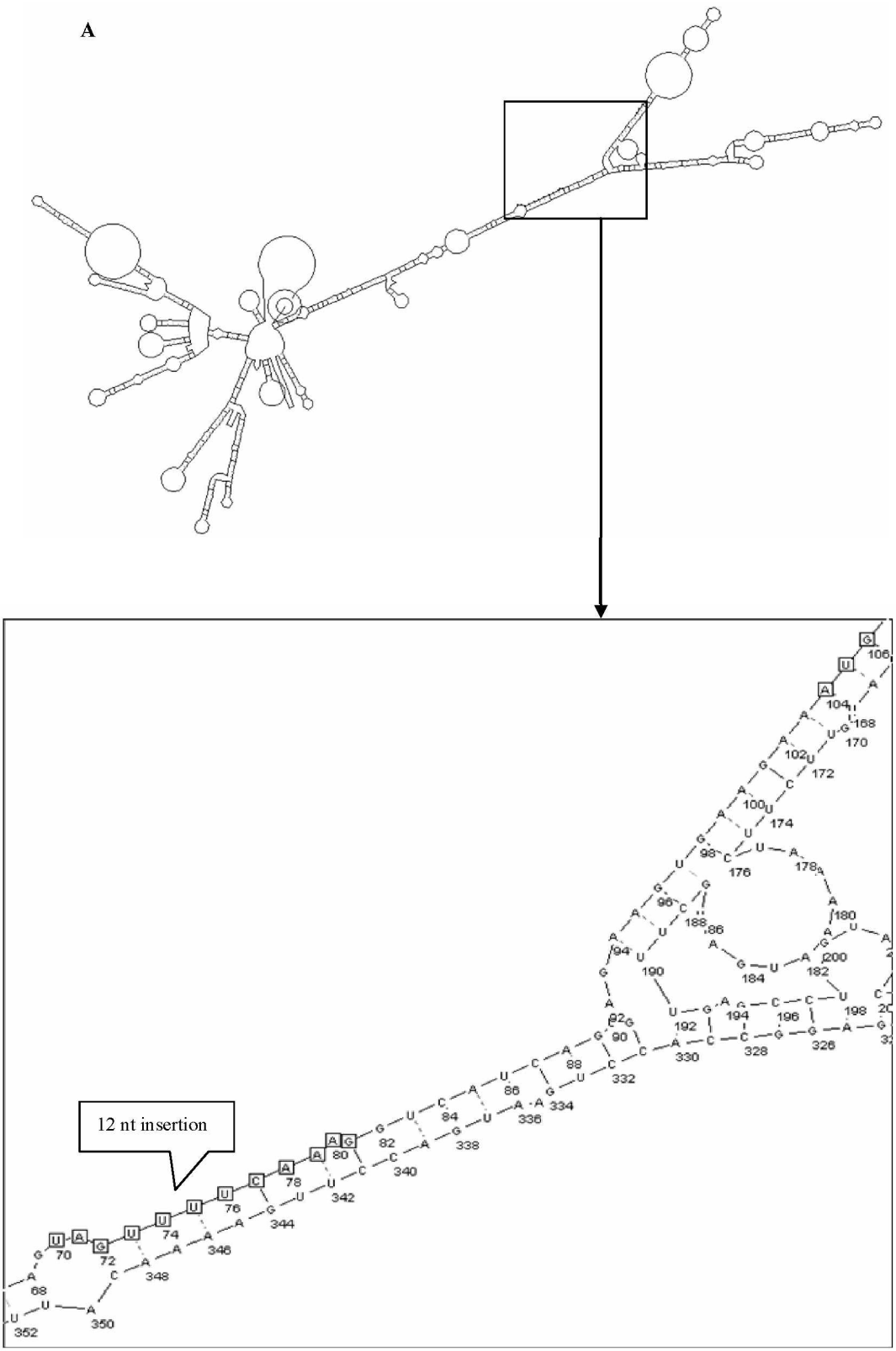

Figure 4. (A) The change of mRNA secondary structure caused by the 12 nt deletion. Panel A denoted the predicted secondary structure of mRNA EF585398 (12 nt insertion at -23nt to -34nt upstream of translation initiation site of AUG).

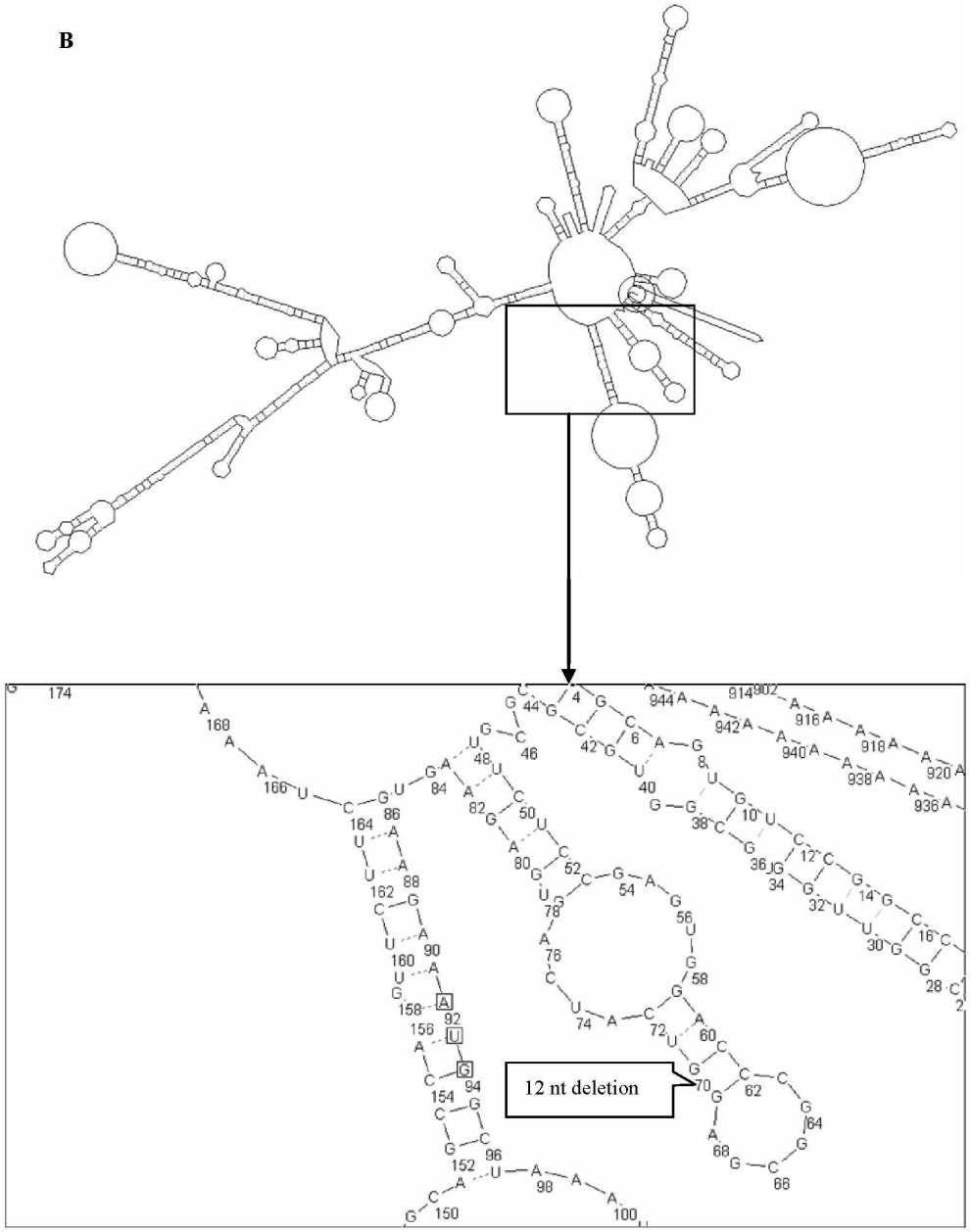

Figure 4. (B) The change of mRNA secondary structure caused by the 12 nt deletion. Panel B denoted the predicted secondary structure of mRNA EU30947 (12 nt deletion between -22nt and -23nt upstream of translation initiation site of AUG).

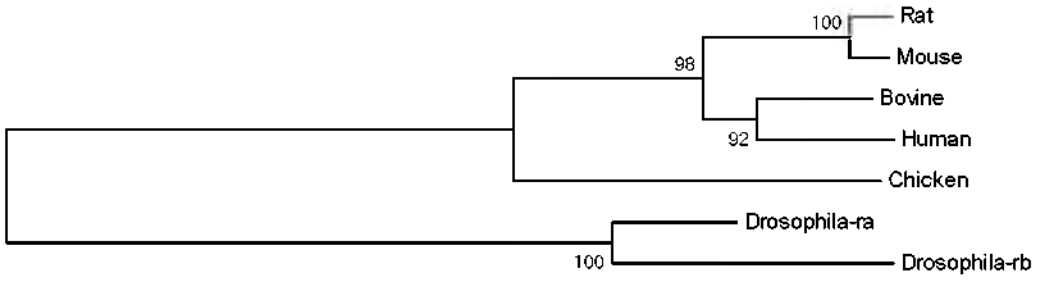

Figure 5. The phylogenetic tree of the orthologous peptides for MGST1. This phylogenetic tree was constructed by the neighbor-joining method. Numbers adjacent to the branch point indicate the bootstrap values.

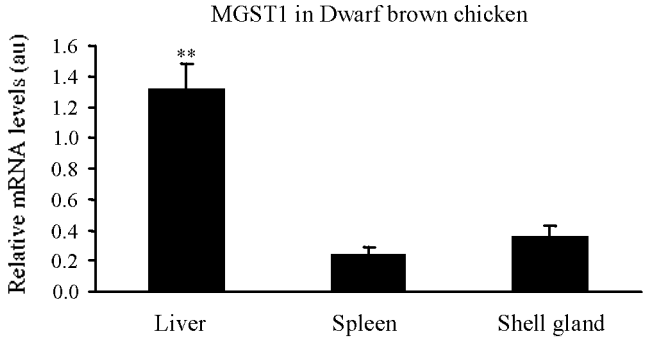

Figure 6. Expression analysis of MGST1 mRNA in the organs investigated of Dwarf Brown chickens. ** $p<0.01$. The internal standard for real-time quantitative PCR is GAPDH. The relative mRNA levels of MGST1 in one of the 3 organs are represented by the averages of ratio values, which were calculated by dividing the average copies of MGST1 by the average copies of GAPDH in each sample (n = 6). au = arbitrary units.

be deduced that chicken MGST1 probably played important physiological functions in liver similar to mammals, such as glutathione-dependent protection against lipid peroxidation (Mosialou, 1993).

In summary, the current study carried out the molecular cloning of chicken MGST1, the identification of its splice variants and the expression analysis of MGST1 mRNA in Dwarf Brown chickens for the first time and provided a base for the study of its biological function.

ACKNOWLEDGMENTS

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