Asian-Aust. J. Anim. Sci.
Vol. 23, No. 7 : 855 - 862
July 2010

www.ajas.info

Expression Analysis of the Mx Gene and Its Genome Structure in Chickens*

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ABSTRACT : Among the known interferon-induced antiviral mechanisms, the Mx pathway is one of the most powerful pathways. The Mx protein has direct antiviral activity and inhibits a wide range of viruses by blocking an early stage of the viral replication cycle. Cloning, characterization, and expression of Mx *in vivo* and *in vitro* have been conducted. The chicken Mx gene spans 21 kb and is made up of 14 exons and 13 introns, of which the promoter region was analyzed. The real-time PCR results showed that Mx expression was increased in chicken embryo fibroblasts (CEF) after 12- and 24-h induction with polyI: C. Induction of Mx expression by poly I: C *in vivo* revealed tissue-specific patterns among the chicken tissues tested. A trace expression of Mx was detected in healthy chicken liver tissues from adult chickens without inducement; the expression levels in the liver, heart, and gizzard were higher than in the muscle and kidney. This is the first report to demonstrate the expression of a glutathione-S-transferase-tagged-Mx fusion protein of 75 KDa, as well as the biological activity tested by SDS-PAGE and western blotting. (**Key Words :** Chicken, Mx, Expression)

INTRODUCTION

Mx protein has been found in many organisms, including yeast, fish, birds, and mammals (Aebi et al., 1989; Meier et al., 1990; Rothman et al., 1990; Bazzigher et al., 1993). The Mx protein of different tissues displays different intracellular localization and antiviral activity. The nuclear mouse Mx1 protein primarily inhibits the replication of orthomyxo viruses (including influenza viruses) (Garber et al., 1991; Haller et al., 1995); the cytoplasmic Mx2 protein mainly inhibits the vesicular stomatitis virus (Zurcher et al., 1992b; Jin et al., 1999). However, human MxA demonstrates a wide antiviral spectrum of activities against orthomyxo viruses (including influenza viruses), rhabdoviruses (Pavlovic et al., 1990), Bunyaviridae (Frese et al., 1996), and paramyxoviride (Zhao et al., 1996). The chicken Mx protein has also been reported to exhibit antiviral activity against the influenza virus and the vesicular stomatitic virus (Ko et al., 2002). Mx plays a major role in IFN-induced host defense. Numerous experiments have indicated that recovery from influenza virus infection in mice requires a functional Mx defense system (Staeheli et al., 1986). Mx1 transgenic mouse experiments also proved that the Mx system is very important for recovery from infections with deadly influenza viruses (Arnheiter et al., 1996). Chickens are natural hosts for the influenza virus (Haller et al., 1980), and the study of the chicken Mx protein is therefore important for elucidating mechanisms of host defense against this infection. Although the genomic structure of Mx has been reported (Bazzigher et al., 1993; Li et al., 2007), only the first four exons have been clearly described, necessitating further investigation of the numbers of exons and introns comprising this gene. We cloned the full-length Mx cDNA, reported the genomic structure, and characterized the chicken Mx promoter. Mx gene expression under different conditions *in vivo* and *in vitro* was also conducted. Liver, heart, and gizzard treated with PolyI:C all exhibited higher Mx expression; in contrast, only a trace expression of Mx was detected in healthy liver tissue from adult chickens without PolyI:C stimulation. This is the first report to demonstrate the expression of a glutathione-S-transferase-tagged-Mx fusion protein of 75

* This study was supported by the Beijing National Science Foundation (5051004). The Colleges and Universities Science and Technology Project of Shandong Province (J09LC63) and The National High Technology Research and Development Program ("863"program) of China (2007AA10Z183).

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Received September 6, 2009; Accepted December 29, 2009

KDa, as well as the biological activity tested by SDS-PAGE and western blotting.

MATERIALS AND METHODS

Cell culture

Chicken embryo tissue culture was prepared from 10-day-old embryonated eggs obtained from White Leghorns (Gallus gallus). Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were seeded and incubated at 37°C with 5% CO_2 in 25 cm^2 cell culture flasks (Corning, USA), and the third passage chicken embryo fibroblasts (CEF) were used.

Cell stimulation and detection of chicken Mx mRNA

Cells were treated with poly I:C (Sigma, USA) (100 μg/ml) for 12, 24, 48, and 72 h, to stimulate Mx mRNA expression. Total DNA and RNA were extracted from the cultured cells using a DNA extraction kit (Tiangen, China) and Trizol reagent (Invitrogen, USA), respectively, according to the manufacturers' protocols. RNA samples (5 μg) were used to obtain cDNA via AMV reverse transcription (Promega, USA). The cDNA was then used as a template in PCR reactions employing Taq DNA polymerase (TaKaRA, Japan) and a pair of primers, MxF1: 5'-AAATGGCTCAAGAGGTGGA-3' and MxR1: 5'-TATCGCTGACAGTTGGGTG -3', designed based on an EST contig in the NCBI database, and a PCR product of 420 bp was amplified. A 208 bp fragment of GADPH cDNA was amplified as an internal positive control for RT-PCR using primers MxGF1: 5'-TCACAAGTTTCCCG TTCTCA -3'and MxGR1: 5'-GGAACACTATAAAGG CGAGAT -3'. The PCR conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 20 sec, 72°C for 30 sec, and then one cycle of 72°C for 5 min.

From the results of cell stimulation, the 420 bp cDNA was used as a seed to search the NCBI EST database of *Gallus gallus*, and homologous ESTs were constructed into contigs. The open reading frames (ORF) of these contigs were determined using the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).According to the predicted sequence, two pairs of primers, MxF3: 5'-GGGTAGTAGTGCATTGGAGT-3', MxR3: 5'-CTACCTA CCTCAGAGCTCAA-3' and MxF4:5'- GTAGTGCATT GGAGTGGTTTT-3',MxR4: 5'-TACCCGGATTCAAGC TTTT-3' were designed to amplify the Mx cDNA via nested PCR, including the complete coding region. The PCR program was the same: 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. The amplified cDNA fragment was inserted into a pGEM T-easy vector and sequenced. All primers were designed using Primer Premier 5.0 software.

Genome organization of the chicken Mx gene

The structure of the chicken Mx gene was deciphered by screening a chicken genomic bacterial artificial chromosome (BAC) library followed by sequencing of the specific insert. Gridded array nylon filters containing the library (CHORI, Oakland, CA, USA) were screened under high stringency hybridization conditions using a 32-nt α-32P-tagged probe spanning nt 480 to 511 of the partial Mx sequence and a 423 α-32P-tagged probe spanning nt 821 to 1,243 (PCR product). Thirty BAC clones were positive using the first probe, and 13 using the second, with ten clones positive for both, which were cultured in 100 ml of LB in the presence of 50 μg/ml of chloramphenicol. Plasmid DNA was then extracted using the standard alkaline lysis procedure, precipitated with isopropanol, resuspended in 10 mM Tris-HCl (pH 8), and then cloned into a pUC18 vector. Sequences were joined with the DNAstar software (Lasergene8.0). Sequence data was deposited in GenBank under accession no. GQ390353. The exon-intron structure of the chicken Mx DNA was then confirmed using the UCSC Genome Browser website (http://genome.ucsc.edu) and the BMC Gene Finder program (http://dot.imgen.bcm.tmc.edu:gene-finder). The 5'splice donor and 3'splice acceptor sites within the chicken Mx gene were also identified. The sequence of Mx cDNA (GenBank accession no.EU348752) was also submitted to the UCSC Genome Browser website (http://genome.ucsc.edu) and produced the same result and the exon-intron structure was identical.

Tissue-specific expression analysis by semi quantitative PCR

Six chickens were intramuscularly injected with 100 μg/ml of polyI:C (sigma, USA) in phosphate buffered saline (PBS) and three untreated chickens were maintained as controls. Among the polyI: C-induced chickens, three chickens from each group were selected 24 and 48 h post-injection for RNA extraction.

Total RNA was isolated from different tissues, such as the heart, liver, muscle, gizzard, and kidney of White Leghorn using Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. Primers were designed based on the sequence obtained to amplify the 150 bp fragment, and GADPH was amplified as a control.

Real-time PCR assay of CEF and tissues

For real-time PCR, total RNAs were extracted from chicken embryo fibroblasts (CEF) and heart, liver, muscle, gizzard, and kidney tissues. An aliquot (0.5 μl) of the cDNA was analyzed with the PCR mixture in a total volume of 20 μl containing 0.5 μM of the primers DLF: 5'-GGACAG

CGAGTAATGACACC -3'and DLR: 5'- AAGAGGAC AAGGATGAGGC -3' in 1×iQ SYBR GREEN SuperMix (Bio-Rad). Amplification was carried out using an iQ5 Real-Time PCR Detection System (Bio-Rad). The cycling protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 20 sec, fluorescence detection at 85°C for 20 sec, and a final extension cycle of 72°C for 5 min. All samples were run in triplicate together with non-template controls in the same plate. The fold changes of the RNA transcripts were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001), and chicken GADPH amplified with primers DLGF: 5'-GCCCAGAACATCATCCCA -3'and DLGR: 5'-CGG CAGGTCAGGTCAACA-3' served as an internal control.

Expression, purification, and detection of glutathione-S-transferase-tagged (GST)-Mx fusion proteins

The ORF (2,118 bp) of the Mx protein was amplified using a plasmid containing full-length cDNA as template by polymerase chain reaction (PCR). The sense primer GTCG ACAACAATCCACGGTCCAACTTC (Sal I site underlined) and antisense primer GCGGCCGCCAGAG ACTTAAAGTCTACCAGG (Not I site underlined) were used to construct the recombinant plasmid. After the recombinant plasmid was verified by digestion with Sal I and Not I, the PCR amplified products were cloned into the pMD 18-T vector, and all DNA constructs were confirmed by DNA sequencing. The GST-Mx fusion proteins were expressed and purified according to the manufacturer's directions for GST expression and purification systems (Amersham Biosciences). After extensive washing with PBS, proteins bound to the resins were eluted with 20 mM glutathione in 50 mM Tris/HCl (pH 8.0) and analyzed by SDS-PAGE (15% (w/v)acrylamide:bisacrylamide (29:1)) and western blotting.

For western blotting, the proteins were transferred electrophoretically to nitrocellulose membranes. Antigen-antibody complexes were visualized using alkaline phosphatase secondary antibodies, according to the manufacturer's protocol (Sigma, USA). After washing with TBS, the membrane was incubated with an anti-GST monoclonal antibody (Sigma, USA) which was added at a concentration of 1 μg/ml in blocking buffer for 2 h, followed by a POD-labeled anti-mouse monoclonal antibody (1 μg/ml in blocking buffer) for 1.5 h at room temperature and further washing with TBS. Mx binding was quantified using a chemi-luminescence substrate and the Lumi-Imager TM instrument (Roche Diagnostics). To exclude false-positive results in the Mx incubation experiment, the membrane was first examined with GST/anti-GST and anti-mouse monoclonal antibodies.

RESULTS

Genomic organization of the chicken Mx gene

The complete sequence of the chicken Mx cDNA was longer than the sequences available thus far, such as 2,118 bp and 2,545 bp. Thus, the predicted mRNA was 2,729 bp long (GenBank accession no: EU348752). The cDNA consisted of an open reading frame beginning with the ATG start codon at base 325 and ending with the TAG stop codon at base 2,442, encoding 705 amino acid residues. The protein sequence was deposited in GenBank under accession No ABY60967.

The exon/intron junctions were identified by reference to the consensus sequences of the donor/acceptor splice sites (Mount, 1982). The exon/intron splice sites of 14 exons and 13 introns followed the ag/gt rule. Therefore, we designed the exon-specific primer sets according to the intronic flanking regions to further study the allelic polymorphism. According to the predicted region, primers were designed to amplify 14 exons and the promoter, all of which were obtained and sequenced (GenBank accession nos. GQ390353 and GQ411205).

Tissue-specific expression analysis

Semi quantitative RT-PCR analysis was performed to detect the expression of Mx in the heart, liver, muscle, gizzard, and kidney 24 h and 48 h after injection of polyI: C (Figure 1A). The liver, heart, and gizzard demonstrated higher expression levels than the muscle and kidney, and trace expression levels of Mx were found in liver of healthy adult chicken untreated with PolyI:C (control). PolyI:C treatment enhanced the Mx mRNA expression levels, especially in the liver, heart, and gizzard as clearly apparent at 24 h, with only a slight difference observed between 24 h and 48 h. Real-time PCR results of the expression level of Mx gene in different tissues was identical to semi-quantitative RT PCR (Figure 1B).

Real-time PCR analysis of CEF

Poly I:C was used at concentrations from 0.1 μg/ml to 100 μg/ml in DMEM culture medium to induce Mx expression (Figure 2). Mx expression was assessed by real-time PCR at 12 to 72 h following polyI:C administration, and no expression was detected in the controls. All polyI:C doses resulted in significantly increased Mx gene expression compared with the untreated control. The results indicated that 12 h of treatment with polyI: C (100 μg/ml) was sufficient.

Expression of fusion proteins

In order to further detect Mx protein expression and activity, the complete coding cDNA was cloned into a

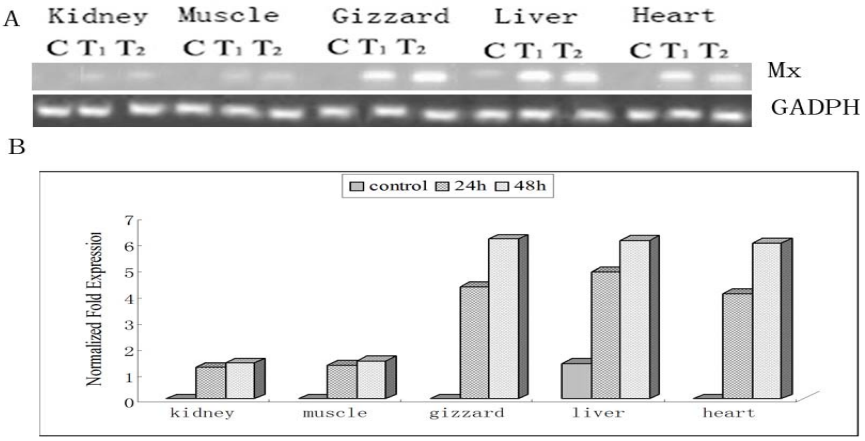

Figure 1. Expression of *Mx* mRNA in chicken tissues by semi-quantitave PCR and real-time PCR. (A) Time course of *Mx* expression in chicken liver, heart, gizzard, muscle, kidney tissues and GADPH amplification for the respective PCR reaction. C: control; T_1: 24 h after injection of polyI: C (100.0 μg/ml); T_2: 48 h after injection of polyI:C (100.0 μg/ml). (B) Mx gene expression detection by real-time PCR.Mx gene expression in different tissues at 48 h is slightly higher than at 24 h. Normalized fold expression of Mx in gizzard, liver and heart which was 6.1256, 6.0731 and 5.9738, respectively, showed no significant differences; the expression level in liver at 48 h is 4.43-fold that of kidney and 4.17-fold that of muscle.

pGEX 4T-2 plasmid (Amersham, USA) and expressed in *Escherichia coli* as a GST-recombinant protein. Aliquots of the sonicated bacterial cell lysate and supernatant were analyzed by SDS-PAGE after IPTG induction. A 75-kDa band was observed only in bacterial cells incubated with IPTG. Expression of the GST fusion protein was observed in bacterial cells transformed with GST-Mx (Figure 3). An anti-GST antibody was tested for reactivity with the fusion proteins by immunoblot analysis, yielding positive results.

DISCUSSION

According to the functional analysis of Mx, variants of the GTPase effector domain (GED) play a key role in GTPase activity, polymer formation, and antiviral activity (Melen et al., 1992; Lee and Vidal, 2002). The GED sequences of avian and mammalian Mx, including human, mouse, rat, cow, pig, sheep, horse, chicken, and duck were well aligned. The GenBank accession numbers for these are NP002450, NP0024520, P18588, P79135, NP_999226,

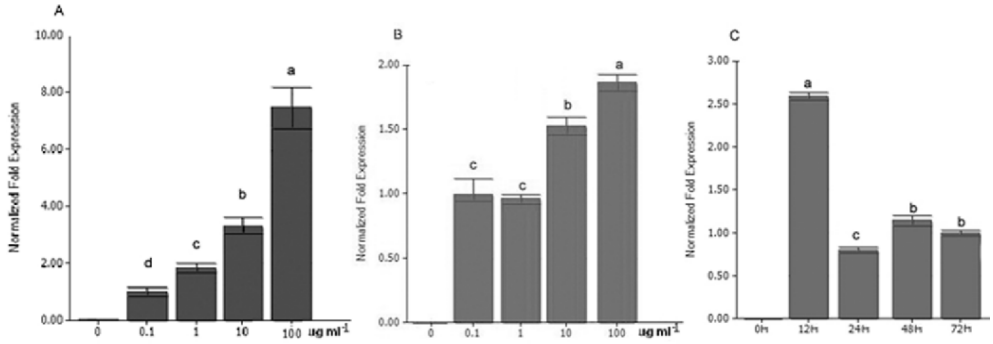

Figure 2. Detection of CEF Mx expression by real-time PCR. (A) Expression after induction for 12 h with different doses of polyI:C (0.1, 1.0, 10.0, 100.0 μg/ml) demonstrating an increase in activity in a dose-dependent manner. The resultant increase in activity was 1.87-fold, 3.33-fold, and 7.54-fold higher for the three concentrations (1, 10, 100 μg/ml, respectively) of poly I:C used as compared with 0.1 μg/ml; The means obtained for the 100 μg/ml and 10 μg/ml treatment were significantly ($p<0.01$) greater than the treatment with low concentrations (0.1 μg m-1 l and 1 μg/ml), and the difference between 100 μg/ml and 10 μg/ml was also markedly and significantly ($p< 0.01$) different, whereas no significant difference ($p>0.05$) was observed between the 0.1 μg/ml and 1.0 μg/ml treatments. (B) Mx protein expression in response to treatment with different doses of polyI:C (0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml, respectively) for 24 h. Incubation for 24 h with different doses also resulted in significant increases in expression of the Mx gene ($p<0.05$). (C) Mx protein expression after 12, 24, 48, and 72 h treatment with polyI:C (100 μg/ml). After 12, 24, 48, and72 h of incubation with polyI:C (100 μg/ml), 2.59-fold, 0.80-fold, and 1.15-fold increases in expression were detected, respectively (compared with the untreated control). The results were significant ($p<0.05$). The difference between the 12 h and 24 h treatment was also markedly significant ($p<0.01$). Error bars indicate standard deviations. Column bars with different letters were significantly different at $p<0.05$ (N = 3).

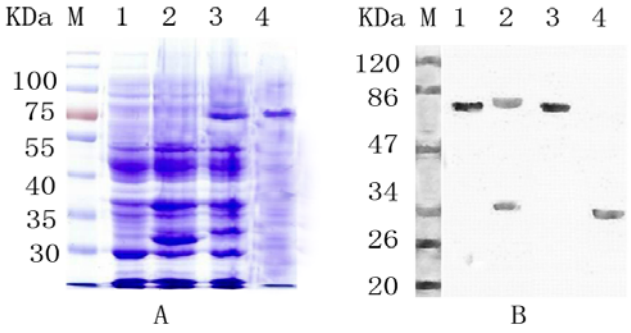

Figure 3. (A) SDS-PAGE of the fusion protein. M, protein marker; lane 1, protein from the *E. coli* Rosetta strain induced by IPTG; lane 2, protein from the *E. coli* Rosetta strain carrying pET28a induced by IPTG; Lane 3, protein from the *E. coli* Rosetta strain carrying pET28a/Mx induced by IPTG; lane 4, purified fusion protein. (B) Western blotting analysis of the Mx protein expressed in *E. coli*. M, protein marker; lanes 1, 3, purified GST-Mx protein, lane 2, GST-Mx; lane 4, pGEX-GST, (control GST).

P33237, AAC23906, ABY60967, and P33238, respectively. The GED of chicken Mx displayed the highest homology with duck Mx (64.1% identity), followed by bovine and sheep Mx (35.9% identity). Near position 631 (624-651) of the Mx protein sequence, chicken showed the highest homology (96%) when compared with other species. Ser was observed in duck Mx, human MxA, cow Mx1, and sheep Mx1; Gly was observed in rat Mx1, horse Mx1, and pig Mx (Figure 4). Thr was found only in mouse Mx1, and chicken Asn. The resulting amino acid differences may account for the variations in the antiviral activities of the allelic gene products (Haller et al., 2007a;b; Li et al., 2007). A number of single-point mutations can result in the loss of antiviral activity, such as that observed for chicken Mx protein. Only the N631S (Asn to Ser) mutation in the GED resulted in the loss of antiviral function (Ko et al., 2002; Ko et al., 2004) .The variation observed among the different GEDs might also reflect the fact that the carboxyl region plays a critical role in the antiviral activity. When blood samples from White Leghorns (70 chickens) were used to detect the GED region, the sequence results revealed that the amino acid present at position 631 was most commonly Asn (68/70) (Yin, unpublished), which is predicted to have positive antiviral activity according to Ko et al. (2002, 2004), although Shamo (SHK) possessing Asn (631) was found to be devoid of activity against H5N1 strain at the cellular level by Camilla et al. (2008). Since all of the data reported to date have been acquired *in vitro*, the antiviral function and spectrum of the Mx protein still needs to be clarified *in vivo*.

The chicken Mx gene was found to extend over more than 21 kb, including 14 exons and 13 introns. All splice sites followed the ag/gt rule. The coding exons varied in length between 48 bp and 329 bp, with the last exon comprising more than 481 bp and encoding 78 amino acids. The first exon was found to be non-coding, identical to those of the human and mouse Mx genes. Human Mx1 has 17 exons, of which the first four exons are non-coding (Tazi-Ahnini et al., 2000). Mouse Mx1 contains 14 exons, with the first exon again being non-coding (Hug et al., 1988). The length of the chicken Mx exons 1-4 were 48 bp, 329 bp, 193 bp, and 138 bp, respectively, identical to the results reported by Schumacher et al. (1994). The first exon was found to be very small (48 bp) as previously reported (Schumacher et al., 1994). Comparisons of the chicken Mx gene with those of human and mouse revealed a high level of conservation in size and sequence between human exons 6-16 and mouse exons 3-8 (Figure 5). The similar region with antiviral function may have been eliminated in response to conservative selection pressures (Arnheiter et al., 1996; Frese et al., 1996). So we can predict that these similar exons are important for the structure and function of Mx protein; the polymorphism of these regions may relate to the function of Mx protein although these exons are not in the carboxyl end. According to methods of the Human Genome Project, SNPs found in different regions can work collaboratively on the antiviral activity. So the study of relativities between typical SNP such as S631N and new SNP found in different exons in effecting the antiviral activity of Mx protein is very important. We have cloned 1-14 exons of Mx gene and have found new SNPs in the detection process; the relativities still need to be studied.

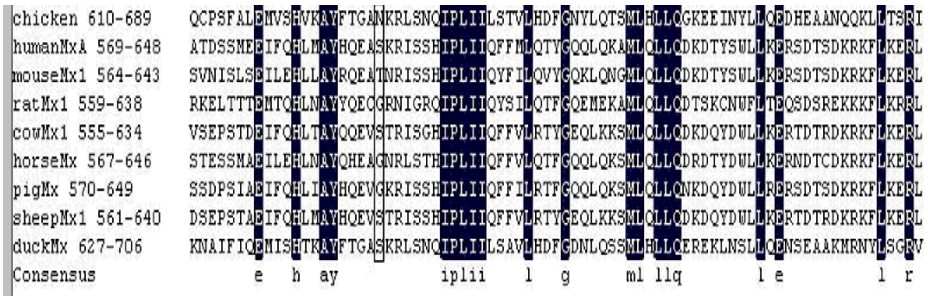

Figure 4. Sequence alignment of the GED of the chicken Mx protein with the available mammalian and duck Mx proteins. Conserved amino acids are indicated in black boxes. Position 631 in the chicken Mx gene and the corresponding position in those of mammals and duck are boxed.

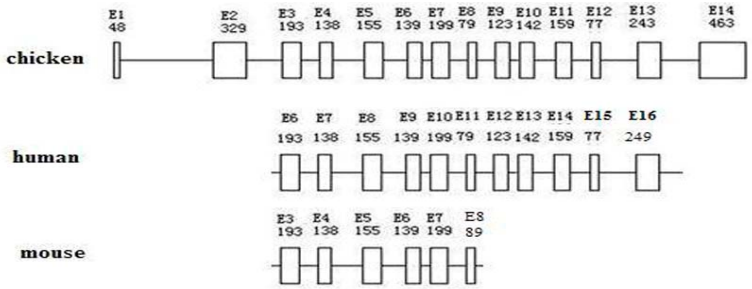

Figure 5. The similar length of exons in Mx gene from chicken, human and mouse. The numbered exons (E) were indicated as boxes with the length in nucleotides above the box. The length of human exons 6-16 and mouse exons 3-8 are similar to chicken exons 3-13 and 3-8, respectively.

Concerning the promoter (Figure 6), the transcriptional response to IFN-α/β results from a sequence that conforms to the consensus 5'A/GGTTTCN (1-2) TTTCC/T3'or its reverse complement (Hug et al., 1988). The chicken interferon stimulating response (ISRE) motif has been reported, shown to be conserved, and found to regulate the expression of IFN-inducible genes in both mammals and avians (Schumacher et al., 1994). One distal ISRE-like (ISRE2) element was also found in the chicken Mx gene promoter, similar to the human MxA promoter. Human distal ISRE has been shown to be nonfunctional (Ronni et al., 1998), and the roles of ISRE-like motifs in the chicken remain to be tested. The ISRE core sequence GAAA was found to be present three times (GAAACC,GAAAGC, GAAACA) in the promoter region, as also detected in the IFN-inducible promoters of mammalian genes (Hug et al., 1988; Chang et al., 1990), suggesting that these regions are functional in response to inducers. The putative sp1 binding site in the Mx chicken promoter aligns with 8 of 10 bases of sequence (G/T) GGGCGG (G/A) (G/A) (C/A). The TATA-like box sequence TATAGAAA is uncommon, although it is present in the human adenovirus IVa2 promoter (Carcamo et al., 1991; Kasai et al., 1992). The functions of the ISRE2, GAAA, TATA-like box remain to be verified.

PolyI:C is an artificial double-stranded RNA that induces Mx expression *in vivo* and *in vitro*. In the present study, Poly I:C was used to induce chicken embryo fibroblast Mx expression under different conditions, as well as in adult chickens. Mx mRNA expression was enhanced *in vitro* in response to poly I:C after 12 h, 24 h, and 48 h, with the clearest results observed at 12 h in chicken CEF in our study.

Interestingly, this is the first study to show a basal level of constitutive Mx expression in healthy liver tissues of adult chicken. Literature reviews of Mx expression revealed that a basal level of expression was common in many fish species (Lee et al., 2000). Furthermore, induction of Mx expression by polyI:C demonstrated tissue-specific patterns among the chicken tissues examined. The liver, heart, and gizzard showed higher expression levels than did muscle and kidney. In adult chicken, the study of regulation factors related to poly I:C up-regulation of Mx expression is critical

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-307  TTTCCTGATATCCAACCTGAAAAGGAAAGGGCTGTTGCTCATAAGCAGTA
                      ISRE2
-257  TTACCTAGCACCTGTGCCATCTGCCCGCTGATTCCTCTGCAGGAGAAGGG

-207  AGAAACCACAGGACAAGGAGGGTAGTAGTGCATTGGAGTGGTTTTGTTAC

-157  AGGGTTCTGCAAAGAACTGGGACGAAAATTGGCTAATGATGAGGAATTTG
                      Sp1
-107  AGTGAAACACACATCAGGATACTGTTTTCAATAATGAAAGCATTTTAGTT
                                                    ISRE1
-57   TCGTTTCTCCTTGTTTATGTCATGTAGGTGGAGTCTGTGTATAGAAAAGC
                                              TATA-like box
-7    ATTCAGAGCGGCTGAATGTAGTTAATTGTTTCTCCTTGCTGTGTGACTCT
           +1      exon1
44    GGCAGAGGCTGTCAAGAGTGGTCGGTGTCGATAATAATCACTGCTCGGTG

94   CAGTACATGCAGACAAGCCATAGAGCAAGCCAGAAGAACAGCAGAACATG
                                                    +141
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Figure 6. Sequence of the chicken Mx promoter region. The Sp1-binding site, interferon stimulating response ISRE1 and ISRE2 regions, and exon1 are underlined. The GAAA and TATA-like box are boxed. The start of the ORF is boxed and in bold font. Position +1 of the nucleotide sequence was defined as the most upstream nucleotide transcribed.

for determining expression levels during this process. Thus, time course and dose-dependent analyses of poly I:C regulation of Mx expression would be useful.

In order to detect the expression of Mx protein, a GST-Mx fusion protein was expressed in *E. coli* and detected by SDS-PAGE and western-blotting. The purified GET-Mx fusion protein of 75 kDa was demonstrated. To measure the biological activity of the recombinant protein, the hemagglutination test and preliminary animal experiments were conducted. One hundred microliters of ND antigen (AD, provided By Harbin veterinary research institute) was injected into the allantoic cavity of each 10-day old chicken embryo. One hundred microliters of purified Mx protein at different concentrations was subsequently injected. Seventy-two hours following that injection, allantoic fluid was collected under sterile conditions and the antiviral titer of the expression product was analyzed. The activity unit for the recombinant protein was calculated according to the Reed-Muench method. Two hundred microliters of the original allantoic fluid from 15-day-old chickens diluted 1,000-fold was infected with the ND virus using the eye dropping method. The results indicated that the effects of various doses of Mx protein were significantly different. Mx protein plays a significant role in resisting the ND virus. However, its role in resistance to H1N1, H5N1, and H5N2 requires further investigation. Influenza spreads around the world in seasonal epidemics. Avian influenza (AI) is a violent infectious disease which affects fostering birds seriously and threatens the health of mankind. Since chicken is the natural host of AI, research on Mx protein production and antiviral activity and spectrum of chicken Mx protein is significant in treatment of both human and poultry disease.

In conclusion, the present study reports the characterization and expression of Mx *in vivo* and *in vitro*. Sequence analysis of the Mx gene organization confirmed that the chicken Mx gene consists of 14 exons and 13 introns. The results obtained by real-time PCR after induction with polyI:C under different conditions indicated that Mx expression increases after 12 h and 24 h CEF induction by polyI:C (100 μg/ml), with 12 h demonstrating the most marked increase in Mx expression. Furthermore, induction of Mx expression by polyI:C revealed tissue-specific patterns among the chicken tissues evaluated. A trace Mx expression was detected in healthy liver tissues of adult chickens without being treated with poly I:C. Furthermore, a GST-Mx fusion protein was also assessed in the present study.

Our results provide support for the further study of the properties of the chicken Mx protein. Future research of chicken Mx expression and host protection against different virus strains using time course and dose dependent activity studies, as well as characterization of the promoter region, would be useful in understanding the antiviral activity of this gene. Furthermore, novel substitutions in the GED region should also be analyzed.

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