Molecular cloning, polymorphism, and functional activity of the bovine and water buffalo Mx2 gene promoter region

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Abstract

**Background**: Bovine Mx2 gene sequences were already reported, but further information about the gene properties is not yet available. The objective of the current study was to elucidate the structural properties of the bovine Mx2 gene mainly the promoter region and its possible functional role. If available, such information would help in assessing the functional properties of the gene, which was reported to confer antiviral action against recombinant VSV.

**Results**: Examinations on the bovine genomic BAC clone—confirmed to contain the Mx2 gene—revealed 883-bp sequences. A computer scan unequivocally identified a 788-bp promoter region containing a typical TATA box, three ISREs and other promoter-specific motifs. Comparative analysis of nine bovine genomic DNA samples showed 19 nucleotide substitutions suggesting the existence of five different genotypes in the promoter region. The water buffalo Mx2 promoter region was determined by using primers based on the bovine Mx2 promoter region disclosing 893-bp, with 56 substitutions, two insertions, 9 and 1 nt at two different sites. A functional analysis of the putative ISRE indicated that ISRE played a synergistic role in the activation of bovine Mx2 gene transcription.

**Conclusion**: Bovine and water buffalo Mx2 promoter region was identified disclosing, the conserved ISRE, located in the proximal end of the promoter region like other members of the antiviral family, suggesting functional activity under interferon stimulation.

Background

The observation that some strains of laboratory mice escaped experimental influenza virus infection was the starting point for the currently known antiviral Mx protein (Lindenmann et al. 1963; Hug et al. 1988). The protein was classified in the dynamin superfamily, members of which include large GTPases (Staeheli et al. 1993). A common feature of this family is the formation of high-molecular-weight oligomers inside cells (Kochs et al. 2002). Furthermore, conserved properties of Mx proteins include the presence of tripartite GTPase domains in the N terminal region, a dynamin signature, and a GTP effector domain (GED) containing a leucine zipper motif in the C terminal region, which plays a key function in the antiviral activity (Melén et al. 1992). Members of type I interferon family are responsible for the induction of Mx proteins in association with the activation of the natural immune system in infections with single-strand RNA viruses (Pavlovic et al. 1993). Different isoforms of the protein have been detected in a variety of vertebrates (Horisberger and Gunst 1991), and at least one isoform showed antiviral activity. An example of this is the human Mx protein in which human MxA (corresponding to the Mouse Mx1) was found to confer antiviral action against infection with single-strand RNA virus, while human MxB was devoid of any antiviral action (Frese et al. 1995).

To date, a uniform mechanism of how Mx proteins induce the antiviral action remains unknown, although the localization of the individual proteins inside the cell is thought to play a role (Lee and Vidal 2002).

The existence of more than one isoform of the Mx gene in farm animals, including poultry (Bazzigher et al. 1993; Schumacher et al. 1994; Ko et al. 2002), porcine...
(Morozumi et al. 2001; Asano et al. 2002), ovine (Charleston and Stewart 1993), and bovine (Ellinwood et al. 1998), strength the probability of implementing genetic selection programs to heighten productivity efficiency in the livestock industry. In addition, association of the Mx protein induction with viral infections highlighted the possibility of using the protein as a marker for acute viral infection to monitor a disease state in livestock (Muller-Dobies et al. 2002, 2004).

The notion that the bovine Mx locus retained an antiviral allele due to long and intensive selective pressure from single-strand RNA viruses has increased the advantages of using the gene to examine the real significance of the antiviral role of Mx proteins and has encouraged the search for further properties of the gene. Studies of the structural properties showed the presence of the splicing isoform of bovine Mx1 and Mx1B (Kojima et al. 2003) and described the fine structure of the bovine Mx1 gene, which was found to consist of 15 exons and a promoter region approximately 1 kb upstream of the 5′-flanking region (Gerardin et al. 2004). A functional analysis of the bovine Mx1 protein revealed the gene’s antiviral action against various viral infections including VSV (Baise et al. 2004). It was also seen to be partially effective against rabies (Leroy et al. 2006) but not against Paramyxoviridae (Leroy et al. 2005). Furthermore, variation between the antiviral role of bovine Mx1 and Mx1a was observed using recombinant VSV (Nakatsu et al. 2004).

Bovine Mx2 gene sequences (accession no. AF355147) have recently been reported, but further information about the gene properties is not yet available. The aim of our study was to learn more about the structural properties of the bovine Mx2 gene by focusing on the promoter region and its possible functional role. If available, such information would help in assessing the functional properties of the gene, which was described as conferring antiviral action against recombinant VSV (Babiker et al. 2007).

It become clear that the promoter region of type I interferon-induced genes such as mouse Mx1 and Mx2, human MxA, bovine Mx1, and porcine Mx1 contain cytokine binding sites and regulatory factors, mainly the interferon stimulation-responsive element ISRE, GAAA elements, and GC-rich boxes. However, the classical TATA box consensus was reported only in mouse Mx1 and Mx2 (Hug et al. 1988; Asano et al. 2002; Chang et al. 1991; Gerardin et al. 2004; Thomas et al. 2006). The presence of at least one ISRE is essential to initiate the transcription of interferon proteins. Previous reports indicated that a conserved proximal ISRE has been detected in almost all the promoter regions of every species possessing the gene as a characteristic feature (Reid et al. 1989; Ronni et al. 1998; Asano et al. 2002; Yap et al. 2003; Altmann et al. 2004). It was noticed that the activity of ISRE in human MxA was affected by a single nucleotide polymorphism (Hijikata et al. 2001). Moreover, multiple ISRE have also been seen to participate in activation of the promoter region under interferon stimulation (Hijikata et al. 2000).

In this study we have identified the promoter region of bovine and water buffalo Mx2 genes. We have also demonstrated the variation between the two species, thus providing evidence about the adaptational modifications that took place in the gene. The presence of the conserved ISRE detected in both genes confirms the gene’s relatedness to members of the antiviral Mx protein family induced by type 1 interferon. Moreover, the presence of the typical TATA box places the gene in the top position among other corresponding genes detected in domestic animals. Expression analysis results suggested that the entire putative ISRE participate synergistically in the activation of the promoter.

**Methods**

**Characterization of the BAC clone containing the bovine Mx2 gene**

The bovine Mx2 gene, was obtained by scanning a bovine genomic bacterial artificial chromosome library (BAC) using a 240-nt α-32P-tagged probe generated by a pair of primers, forward primer bMx2 216F (5′-AGAGGACCAGAGGCACTGAG) and reverse primer bMx2 470R (5′-CAAATTCAGCTGATTGAAGTTGT), based upon sequences of putative exon 2 and 3 of bovine Mx2 cDNA (accession no. AF355147). Clones positive to the hybridization were examined for the presence of the Mx2 gene by sequencing using M13 primers with Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI-301 Genetic Analyzer (Applied Biosystems). The BAC clone identified to contain the Mx2 gene was transformed into E. coli cells JM 109 (Promega, Madison, WI, USA) that were cultured overnight at 37 °C in the presence of 25 µg/ml chloramphenicol. The recombinant plasmid DNA was then extracted from these cells following the standard alkaline lysis method, then resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C, ready for use as stock.

**Identification of the promoter region and transcription start site of the bovine Mx2 gene**

The gene walking technique was followed to identify the predicted promoter region of bovine Mx2. The plasmid DNA was sequenced using reverse primer bMx2 25R (5′-AGAGGACCAGAGGCACTGAG) based upon the sequences of bovine Mx2 cDNA (accession no. AF355147). Sequencing was performed using Big Dye terminator chemistry (Applied Biosystems) with an ABI
Prism 310 Genetic Analyzer (Applied Biosystems) in accordance with the manufacturer’s instructions. The sequencing PCR reaction mix used included 100 ng of plasmid DNA as template, 2 µl of ready-mix reagent, and 1 µl 10x buffer. It was then completed to a total volume of 10 µl with autoclaved distilled water. Cycling was done on Thermal Cycler (Perkin-Elmer, Norwalk, CO, USA), with a protocol consisted of an initial denaturing step at 96 °C for 1 min followed by 25 cycles at 96 °C for 30 s, 50 °C for 30 s, and 66 °C for 4 min. The sequencing results identified 500 bp sequences, the computer-assisted scanning of which identified 21 bp similar to those of the putative exon 1 which was followed by an upstream area of 405 bp containing promoter-specific motifs including three ISRE at nt −85, −111, and −134 upstream. Downstream of exon 1 were 74 bp not specific to bovine Mx2 gene suggesting the 5′ end of intron 1. Two separate sequencing procedures were carried out using a specific reverse primer bMx2 pro788F (5′-TGCTGGAG) and forward primer bMx2 p444F (5′-GGAGAACCTGTTAGAC) and using the gene-specific reverse primers bMx2 5′-CGACTGGAGCAGGAGCAT and GeneRacer 5′-CGACTGGAGCACTGACATG with the entire three ISRE deleted. These constructs included pGL3-ΔISRE1, containing the entire ISRE as wild type; pGL3-ΔISRE2 which was generated from the pGL-ΔISRE1 construct as a template using primers without restriction end, while reverse primers included the KpnI restriction site in the 5′ end, while reverse primers included the XhoI restriction site in the 5′ end. The

Amplification of the Mx2 promoter region using bovine and water buffalo genomic DNA

A genomic DNA library derived from one water buffalo and nine bovine blood samples were used to identify the promoter region of the water buffalo Mx2 gene and to demonstrate the allelic polymorphisms of the bovine Mx2 promoter region, respectively. The Mx2 promoter region was amplified from the genomic DNA by PCR using forward primer bMx2 pro788F 5′-AATTTGCCCGAAGTCAGG and reverse primer bMx2 95R 5′-AGACTGGAGCAGCAGGTTCACAGGACG designed based upon the promoter sequences, partial 5′ UTR, and intron 1, which was identified using the BAC clone, as previously described. The amplicons were electrophoresed in 1% agarose for purification of the target cDNA. The obtained PCR product was then T/A cloned into pGEM easy vector (Promega), and the recombinant plasmid was incorporated into E. coli JM 109 (Promega) competent cells. The purified recombinant plasmid was sequenced using promoter-specific primers on an ABI gene analyzer as described before. The sequencing results obtained were subjected to a computer-assisted comparative analysis to examine the promoter-specific motifs and polymorphic properties of the bovine and water buffalo Mx2 promoter region, respectively, using the website-based tools.

Characterization of the role of ISRE in promoter region activity

The role of the putative ISRE was examined using the dual-luciferase reporter system (Promega), which contains promoter-less pGL3 vector expressing the firefly luciferase and internal control plasmid pRL-TK, expressing Renilla luciferase. The test was conducted using two types of pGL3 constructs—deletion- and truncation-generated constructs—in accordance with the template used. The deletion-generated constructs included pGL3-(−422), a fragment that include the proximal interferon-sensitive regulatory factors: pGL3-(−154), containing the entire ISRE as wild type; pGL3-(−132) with the distal ISRE deleted; pGL3-(−109), that the distal and intermediate ISRE deleted; and pGL3-(−76), where the entire three ISRE deleted. These constructs were established by the T/A cloning of fragments generated from the genomic DNA by progressive deletion relative to the transcription start point using primers (Table 1) for the pGL3 vectors. Truncation-generated constructs included pGL-AISRE1 + 2, pGL-AISRE1, and pGL-AISRE2 which was generated from the pGL-(−154) construct as a template using primers without restriction sites (Table 1). Forward primers used included the KpnI restriction site in the 5′ end, while reverse primers included the XhoI restriction site in the 5′ end. The
PCR mix reaction included 100 ng template, 10 pmol primer, 75 mM MgSO₄, 5 µl 10× buffer, 10 mM dNTP mix, and 5 units Taq polymerase. The reaction was completed to 24 µl with autoclaved distilled water (DW). The cycling protocol started with initial denaturing at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. The PCR product of fragments generated by progressive deletion was checked by agarose gel electrophoresis.

Table 1 Primers and templates used in generation of deletion and truncated mutants for luciferase activity test

| Construct | Primer name | Primer type | Template |
|-----------|-------------|-------------|----------|
| pGL3−(−422) | bMx2 p 422 + KpnI | Forward | Genomic DNA |
| bMx2 p 20 + XhoI | Reverse |
| pGL−(−154) | bMx2 p 154 + KpnI | Forward | Genomic DNA |
| bMx2 p 20 + XhoI | Reverse |
| pGL−(−132) | bMx2 p 132 KpnI | Forward | Genomic DNA |
| bMx2 p 20 + XhoI | Reverse |
| pGL−(−109) | bMx2 p 109 + KpnI | Forward | Genomic DNA |
| bMx2 p 20 + XhoI | Reverse |
| pGL−(−76) | bMx2 p 76 + KpnI | Forward | pGL−(−154) |
| bMx2 p 20 + XhoI | Reverse |
| pGL−ΔISRE1 + 2 | bMx2 p84F | Forward | pGL−(−154) |
| bMx2 p127 | Reverse |
| pGL−ΔISRE1 | bMx2 p84F | Forward | pGL−(−154) |
| bMx2 p99 | Reverse |
| pGL−ΔISRE2 | bMx2 p109 | Forward | pGL−(−154) |
| bMx2 p127 | Reverse |

80–90% confluence. A transfection mixture including 100 µl of serum-free medium, 1.8 µl of FuGENE 6 (Roche), a total concentration 0.5 µg pGL3 constructs, and 0.1 µg internal control pRL-TK (Promega) with a ratio of 5:1, test construct:internal control was incorporated into the cell culture by dropping to secure equal distribution. The cell cultures were incubated for 41 h post transfection in a CO2 incubator at 37 °C. Recombinant human interferon-α 2b at a concentration of 1000 units/ml was then added into the wells of one set of the duplicates, and the incubation continued for 48 h for both treated and non-treated cells. At the end of the incubation time, the cell cultures were removed from the incubator, washed with PBS, and subjected to lysis with 100 µl of 1× passive lysing buffer. The plate was then rocked on a shaker for 15 min and lysates were removed to 0.5 ml micro tubes, kept on ice for 1 min, and centrifuged for 2 min at 4 °C at maximum speed (15,000 rpm). Finally, 20 µl of the lysates was added to a 96-well plate to run the luciferase assay.

The test was carried out in an automated LD 400C Luminescence detector (Beckman Coulter, CA, USA). A volume of 100 µl Luciferase assay reagent II (Promega) was added to each well and the luciferase activity was measured for 10 s after a 3 s delay. Then 100 µl of Stop&Glo Solution was added to the wells to quench the luminescence of the test constructs and to activate the luminescence of the internal control constructs. The cumulative light emitted was expressed as relative light units (RLU).
Comparative and computer assisted analysis and biometrics

The similarity between sequences and allelic polymorphisms was investigated using the BLAST programs at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi/ (Altschul et al. 1990), while the MOTIF program available at http://motif.genome.jp/ was used to search for a promoter-specific motif.

The data obtained from the luciferase activity test was analyzed using STATVIEW software (Abacus Concepts Inc. CA, USA). The statistical significance was analyzed using Fisher’s protected least significant difference test. All the results were expressed as mean values ± standard errors of the mean (SE).

Results

Promoter region and transcription start point of the bovine Mx2 gene

The results of the first sequence using the plasmid DNA and reverse primer designed from the putative exon 1 of bovine Mx2 cDNA sequences (accession no. AF355147) disclosed a 500-bp amplicon. Computer-assisted scanning of the amplicon identified a 21-bp sequence typical to those of the putative exon 1 of bovine Mx2 cDNA (accession no. AF355147). Moreover, exon 1 was followed by an upstream area of 405-bp including a typical TATA box at nt −39 upstream of the putative transcription start point and other promoter specific motifs characterizing the promoter of type I interferon-induced antiviral Mx genes, unequivocally identifying the predicted promoter region. Downstream of exon 1 were 74-bp, expected to belong to intron 1. The result of the second sequencing using primers designed based on the partial sequences of the identified promoter region added about 383 nt. This brought the promoter sequences to 788-bp upstream of the putative transcription start site of the bovine Mx2 gene. As shown in Fig. 1, computer-assisted scanning of the obtained sequences revealed a typical TATA box at nt −39 upstream, three ISRE at nt −85, −111, and −134 upstream, AP-1 at nt −310 upstream, and 11 GC-rich boxes at nt −22, −234, −249, −282, −325, −364, −411, −553, −580, −656, and −683 upstream of the putative transcription start site. One of these GC rich boxes was found at nt +35 downstream of the 5′ end of intron 1.

The transcription start site was determined by sequencing a total RNA-derived cDNA fragment including a synthetic polynucleotide adaptor in the 5′ end. Observation of the nucleotide sequences obtained identified the nucleotide “C” directly next to the last adaptor-specific nucleotide. Furthermore, computer-assisted scanning of the nucleotide sequences obtained identified exon 1 compared with the promoter and partial 5′UTR sequences obtained using the BAC clone. Therefore, as indicated in Fig. 1, nucleotide “C” was identified as the bovine Mx2 gene transcription start site.

Bovine Mx2 promoter region polymorphism

Sequence analysis of the nine samples of genomic DNA from five bovine breeds using primers based on the promoter region identified in this study revealed an amplicon of 883-bp. Further comparative analysis revealed that these amplicons were highly similar (99%) to the bovine Mx2 promoter identified using the BAC clone. Moreover, several nucleotide substitutions were detected among the breeds examined with a total number of 19 different nucleotide substitutions. Seventeen of these nucleotide substitutions were located in the promoter and two were detected in intron 1 (Table 2). Similarly, four of these nucleotide substitutions were found in all the samples examined: insertion of adenine at position −509, cytosine to thymine at −230, cytosine to guanine at −190, and insertion of adenine at 23 (Table 2). According to the nucleotide substitution detected, the genomic DNA samples examined were classified into five genotypes (Fig. 2). However, none of these nucleotide substitutions were detected in the putative ISRE or in the exon 1 sequences.

Promoter region of the water buffalo Mx2 gene

The sequence result of the amplicon generated from water buffalo genomic DNA using primers designed based upon the bovine Mx2 promoter region revealed 893-bp to be highly homologous (93%) to the bovine sequences. Computer-assisted scanning of the obtained sequences clearly identified the presence of the TATA box and other promoter-specific motifs that characterize the promoter region of type I interferon-induced genes. These genes include the antiviral Mx gene, confirming the water buffalo Mx2 gene promoter region. Moreover, as shown in Table 2, the scanning result also identified 56 nucleotide substitutions compared with the bovine Mx2 promoter region, in addition to a 9 nt insertion at position −546 and a 1 nt insertion at position −227. None of these substitutions were detected in the putative ISRE.

Role of the putative ISRE in the activation of the promoter

The role of the putative ISRE was determined by measuring the luciferase activity using lysates of interferon-treated or non-treated MDBK cells transfected with the different pGL3 constructs individually. As shown in Fig. 3, the highest expression level was detected in interferon-treated cells transfected with pGL(−154) construct, which was 6.6-fold higher than the non-treated cells transfected with the same construct. The expression was suppressed dramatically when all or two of the ISRE were deleted [pGL(−154ΔISRE1 + 2), pGL(−109),
Fig. 1 Sequences of bovine *Mx2* gene promoter region Compared to those of water buffalo *Mx2* gene promoter region (accession number will be provided after final acceptance) displaying nucleotide substitutions encountered. Dot symbol means same as the bovine's one. Boxes indicate the putative transcription and regulatory. Dashed boxes with arrows indicate the nucleotide sequences and the position of insertions in water buffalo *Mx2* gene promoter region. Letters in small cases indicates sequences of intron 1.
| Animals          | −742 | −704 | −614 | −582 | −542 | −532 | 509 | −265 | −261 | −255 | −253 | −222 | −209 | −169 | −100 | −20 | 23 | 53 | Alleles |
|------------------|------|------|------|------|------|------|-----|------|------|------|------|------|------|------|------|-----|----|----|---------|
| BAC clone        | C    | A    | A    | C    | T    | A    | −   | A    | G    | C    | C    | G    | A    | A    | A    | T   | −  | A | 1       |
| JaBla, Brah 2-1,3| −    | −    | −    | C    | −    | −    | A   | −    | −    | −    | G    | −    | −    | C    | −    | −   | A  | −  | 2       |
| Charolait        | −    | −    | −    | T    | −    | −    | A   | −    | −    | −    | −    | A    | −    | −    | −    | A   | A  | −  | 3       |
| Hol 1, 2, 3, Here, Brah 2-2 | T | G    | −    | T    | G    | −    | A    | C    | A    | G    | G    | A    | G    | C    | G    | C   | A  | T | 4       |
| Brah 1           | T    | G    | G    | T    | G    | G    | A    | C    | A    | G    | G    | A    | G    | C    | G    | C   | A  | T | 5       |

(−) symbol means same as the BAC clone’s one

Ja black Japanese Black, Hol Holstein, Brah Brahman
pGL(−76), with RLU 1.0, 0.8, and 0.8, respectively]. A relative drop in the expression level was detected when only one ISRE was deleted [pGL(−154ΔISRE2), pGL(−132), pGL(−154ΔISRE1) with RLU 4.0, 3.2, and 3.1 respectively]. The negative control pGL3basic transfected/ treated or non-treated and non-treated/transfected cells were devoid of significant luciferase activity.

**Discussion**

The promoter region of type I interferon-induced proteins, including the Mx protein, were demonstrated to contain ISRE, GAAA, and GC-rich box elements related to promoter activity under interferon stimulation in many species (Gerardin et al. 2004). Consistent with this findings, our present analysis of the bovine Mx2 gene

| Constructs | AP-1 GC box | ISRE | ISRE2 | ISRE1 | TATA box |
|------------|-------------|------|-------|-------|----------|
| pGL(−422)  |             |      |       |       |          |
| pGL(−154)  |             |      |       |       |          |
| pGL(−132)  |             |      |       |       |          |
| pGL(−109)  |             |      |       |       |          |
| pGL(−76)   |             |      |       |       |          |
| pGL(−154ΔISRE1+2) |      |       |       |       |          |
| pGL(−154ΔISRE1) |      |       |       |       |          |
| pGL(−154ΔISRE2) |      |       |       |       |          |
| pGL3-basic |             |      |       |       |          |

| Constructs | AP-1 GC box | ISRE | ISRE2 | ISRE1 | TATA box |
|------------|-------------|------|-------|-------|----------|
| pGL(−422)  |             |      |       |       |          |
| pGL(−154)  |             |      |       |       |          |
| pGL(−132)  |             |      |       |       |          |
| pGL(−109)  |             |      |       |       |          |
| pGL(−76)   |             |      |       |       |          |
| pGL(−154ΔISRE1+2) |      |       |       |       |          |
| pGL(−154ΔISRE1) |      |       |       |       |          |
| pGL(−154ΔISRE2) |      |       |       |       |          |
| pGL3-basic |             |      |       |       |          |

**Fig. 2** A diagram displaying nucleotide substitutions of the genotypes detected at the promoter region of bovine Mx2 gene from the breeds examined. Arrows indicate the genetic variations.

**Fig. 3** A histogram of activity examinations of the putative ISREs detected in the bovine Mx2 gene promoter region (right), with schematic diagram depicting the putative regulatory factors of each used construct correspondingly (left). The luciferase activity test was performed using lysates of interferon treated (black square) or non-treated (white square) MDBK cell transfected with pGL3 constructs including pGL3(−422), pGL3(−154), pGL3(−132) pGL3(−109), pGL3(−76), pGL(−154ΔISRE1+2), pGL(−154ΔISRE1), pGL(−154ΔISRE2), and pGL3-basic were transfected simultaneously with pRL-TK. Twenty ml of each lysate was used to measure the luciferase activity on an automated LD 400C Luminescence detector using the specified reagents and the results are expressed as mean values ± standard errors of the mean (SE).
promoter region revealed 788-bp complete sequences of the promoter region with a typical TATA box and three ISRE with several GC-rich boxes and GAAA series characterizing the promoter of type I interferon-induced antiviral Mx genes (Fig. 1). This is in accordance with results obtained in a functional analysis of bovine Mx2 cDNA, which was found to confer antiviral activity against recombinant VSV as a new member of the antiviral Mx protein family (Babiker et al. 2007). The presence of a TATA box was reported in the promoter region of both mouse Mx1 (Hug et al. 1988) and Mx2 (Asano et al. 2002), rainbow trout Mx1 (Trobridge and Leong 1995) and in the Japanese flounder Mx gene (Ooi et al. 2006) (Fig. 4), though no functional role was detected for these elements concerning promoter region activity in relation to stimulation with interferon. The detected TATA box placed the bovine Mx2 protein promoter region at the top among the promoter regions of Mx proteins of other mammals, such as human MxA (Chang et al. 1991), bovine Mx1 and porcine Mx1; all of which were reported to lack a typical TATA box (Gerardin et al. 2004; Thomas et al. 2006). However, the presence of three proximal ISRE puts the bovine promoter region in the same group with the human MxA and porcine Mx1 promoter region, apart from the “standard” Mx promoter of some fish, chicken, and bovine Mx1, as claimed by Thomas et al. (2006) (Fig. 4). The sequences of the three ISRE detected in the bovine Mx2 promoter region currently was in accordance with the conserved sequences found in promoter regions of other species such as bovine Mx1 and porcine Mx1 1(GAAA1-2GAAA(G/C)). No ISRE was found in the intron of the bovine Mx2 gene. GC-rich boxes are the binding site for Sp1 [a ubiquitous transcription factor in mammals (Husmann et al. 2000)]. The presence of GC-rich boxes in the promoter regions of Mx proteins lacking a TATA box, such as bovine Mx1 and porcine Mx1, was thought to replace the TATA box functionally leading to the transcription suggesting multiple transcription start sites (Gerardin et al. 2004; Thomas et al. 2006).

Interestingly, an activator protein element was found in the promoter region of bovine Mx2, which is a member of influenza virus-activated cytokine binding sites (Julkunen et al. 2001). Another member of this family is NFκB; was detected in the bovine Mx1 gene promoter region (Gerardin et al. 2004) and found to be more sensitive to activation by the virus (Zamanian-Daryoush et al. 2000).

Results obtained comparing the bovine Mx2 gene promoter region in nine animals representing five different breeds with those of the BAC clone demonstrated the promoter region’s polymorphism with 19 nucleotide substitutions (Table 2). Accordingly, the bovine Mx2 promoter region is expected to host one of five different genotypes, as suggested according to these nucleotide substitutions (Fig. 2). However, absence of nucleotide substitution in exon 1 was consistent with a previous study on the polymorphic properties of bovine Mx2 cDNA (Babiker et al. 2007) as well as bovine Mx1 (Nakatsu et al. 2004). In addition, although the number of nucleotide substitutions is relatively large compared to those detected in the gene’s cDNA, the gene conservation was evident, that is due to the absence of nucleotide substitution in the transcription regulatory elements characterizing the promoter of the antiviral Mx proteins such as ISRE. The same result was obtained in analysis conducted using the bovine Mx1 protein promoter region (Yamada et al. 2009).

![Fig. 4](image-url) Schematic diagram displaying the variation in the fine structure of promoter regions of bovine Mx2 (accession number will be provided after final acceptance), Japanese flounder fish (Ooi et al. 2006), rainbow trout Mx1 (Trobridge and Leong 1995), mouse Mx1 (Hug et al. 1988), mouse Mx2 (Chang et al. 1991), and bovine Mx1 (Kojima et al. 2003). The transcription start site is indicated by +1. ISRE interferon stimulation responsive element, GC GC-rich box, Sp1 specificity protein 1(transcription factor), AP-1 activator protein 1, NF-xb nuclear factor kappa-light-chain-enhancer of activated B cells.
Sequence results of PCR products from water buffalo genomic DNA using primers designed from the sequences of bovine Mx2 promoter disclosed a homologous (93%) promoter region, an analysis of which revealed the presence of 56 nucleotide substitutions in addition to two inserts at two dependent sites (Table 2). The same result was found in previous studies when cDNA sequences of water buffalo Mx1 and Mx2 were compared to bovine Mx1 and Mx2 cDNA sequences, respectively (Nakatsu et al. 2004; Babiker et al. 2007). Based upon the bovine Mx1 gene, these findings substantiate what had already been demonstrated that the Mx protein was a target for adaptational changes (Gerardin et al. 2004).

Previous reports indicated that a proximal ISRE is essential for Mx promoter region activity and that ISRE detected in the distal area are non-functional if on their own and may provide support to the proximal ISRE if several are present at the same time (Ronni et al. 1998; Asano et al. 2002; Yap et al. 2003; Altmann et al. 2004). No distal ISRE or ISRE-like elements were detected in the bovine Mx2 gene promoter region. Therefore, fragments cloned in the pGL3 constructs used in the expression analysis were generated to contain the putative elements found in the proximal end of the promoter region (Fig. 3). The selection of these fragments was also based upon observation driven from similar analysis conducted previously using the bovine Mx1 promoter region, which suggested that those distal transcription regulatory elements, if available, are almost nonfunctional (Yamada et al. 2009). The results of a luciferase activity test conducted in this study clearly agreed with these findings (Fig. 3). The expression level in constructs containing the three ISRE was the highest among the expression levels obtained using other constructs, even those including all the ISRE, with other regulatory factors near the distal end of the promoter region. It was obvious that the three ISRE participate synergistically in the activation of the promoter region under interferon stimulation. Moreover, in accordance with previous reports, the constructs containing the distal and the proximal ISRE—but not the intermediate—gave 60% activity compared to other combinations.

**Conclusion**

In conclusion, we have identified the bovine and water buffalo Mx2 promoter region. Although conservativeness of the genes was demonstrated, based upon results obtained by observations of promoter regions of the animals and breeds examined, we assume that adaptational changes took place between the species. Moreover, like other members of the antiviral family, the bovine Mx2 promoter region disclosed the conserved ISRE, located in the proximal end of the promoter region, suggesting functional activity under interferon stimulation.

**Abbreviations**

AGPC: acid guanidinium phenol chloroform; cDNA: complementary deoxyribonucleic acid; DMEM: Dulbecco’s modified Eagle’s medium; DNA: deoxyribonucleic acid; dNTP: deoxyribonucleotides triphosphate; FBS: fetal bovine serum; GTPase: guanosine 5′-triphosphatase; IFN: interferon; ISRE: interferon stimulated response sequence; PCR: polymerase chain reaction; MDBK: Madin–Darby bovine kidney (MDBK) cells; mRNA: messenger ribonucleic acid; RNA: ribonucleic acid; RT: reverse transcription; TE: tri ethylenediamine tetracetic acid; VSV: vesicular stomatitis virus.

**Authors’ contributions**

HAEB conducted all the tough work including selection of primers, preparation of samples for sequencing, sequencing process, collection and analysis of the data, and preparing the article for publication. TS, ran the first sequencing using the BAC clone and helped with primers selection. YN prepared bovine genomic DNA samples used all over the study, and co-supervised on bench work. ST, MM, and YS worked on chromosome library (BAC) using a 240-nt 454-tagged probe and provided the recombinant plasmid DNA used for the identification of the promoter region. JU, co-supervisor and helped on the preparation of all the materials and reagents used during the experimental work. TW, supervisor and the leader of the working team, checking the work progress step-by-step providing advices and experience and help on the preparation and revision of the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

Nucleotide sequence of bovine and water buffalo Mx2 promoter region will be deposited at GenBank (National Center for Biotechnology Information NCBI).

**Consent for publication**

All the co-authors in this report know and agree with all the information presented, and a written informed consent was obtained from the patient for publication of this case report and any accompanying images.

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