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Structural and Functional Analysis of the Bovine *Mx1* Promoter

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The bovine *Mx1* promoter region was found to contain 4 IFN-stimulated response elements (ISREs), 7 GC boxes, 2 IL-6 responsive elements, 2 NFκB-binding sites and 2 AP-1-binding sites. Among Holstein, Charolai, and Brahman breeds, 5 nucleotide substitutions were detected in the promoter region. After the *Mx1* promoter region from Holstein had been constructed with pGL-basic expression vector, the transfected cells showed promoter activity after IFN induction. Several artificial deletion mutants were prepared to determine the important regulatory elements responsible for the promoter activity, and it was found that ISRE has a key function in IFN response. The proximal ISRE1 showed potential induction by IFN. Furthermore, the proximal GC boxes were found to be essential for IFN response in the bovine *Mx1* promoter with the deletion mutants. In this case, the 2 GC boxes exhibited a synergistic activation in the IFN response. Mithramycin A, an agent that inhibits gene expression selectively by coating GC boxes, was used, and *Mx* mRNA expression in MDBK cells was suppressed by this chemical. Therefore, GC boxes were also shown to be essential for IFN response in the bovine *Mx1* gene.

Introduction

Mx proteins are expressed in cells by infection with RNA viruses or by stimulation with type I interferons (IFNs) (Staeheli 1990; Haller and Kochs 2002). They are antiviral factors for the native defensive immune system. Mx proteins mainly block the replication of single-strand and negative RNA viruses. (Staeheli 1990; Haller and Kochs 2002). However, human MxA is known to have a broad range of antiviral activities against single-strand and positive RNA viruses and also some DNA viruses (Pavlovic and others 1990; Schnorr and others 1993; Frese and others 1996). Mx proteins structurally contain a highly conserved tripartite GTP-binding domain in the amino terminal region, which is involved in antiviral activity as well as GTP binding and GTPase activity (Nakayama and others 1991; Horisberger 1992), and a GTPase effective domain involving two leucine zipper motifs near the carboxy terminal tail, which has a key function in antiviral activity and oligomerization (Melén and others 1992; Schumacher and Staeheli 1998; Jansen and others 2000). The antiviral potential of Mx proteins is also influenced by genetic variations, including amino acid substitutions, deletions, and insertions. For example, most inbred laboratory mouse strains carry a variant type of the *Mx1* gene coding inactive forms because of genomic deletion with nonsense mutation (Staeheli and others 1988; Jin and others 1998). Furthermore, in the chicken, the antiviral activities of Mx protein differ depending on the specific amino acid substitution at position 631 in the GTPase effective domain (Ko and others 2002).

The antiviral potential of the *Mx* gene is also affected by a nucleotide variation in the promoter region. In the human *MxA* promoter, nucleotide variations at positions –88 and –123 near an IFN-stimulated response element (ISRE) showed ~4-fold different promoter activities (Hijikata and others 2000). Variations in the human *MxA* promoter region have been reported to correlate with the efficiency of IFN therapy for patients infected with hepatitis C virus (Hijikata and others 2001).

All *Mx* promoters have been shown to contain one more ISRE, which is the binding site for an IFN-induced heterotrimeric complex, IFN-stimulated gene factor 3 (ISGF3) (Ronni and others 1998). When cells are stimulated with type I IFNs,

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Janus kinase 1 and tyrosine kinase 2 are activated, and then these 2 kinases activate STAT1 and STAT2 transcription factors. Finally, STAT1 and STAT2 couple to IFN regulatory factor 9 (IRF9) composing ISGF3, which migrates to the nucleus from the cytoplasm. Analysis of some *Mx* promoters showed that 1 or 2 proximal ISREs are important for the response to IFN (Ronni and others 1998). In addition, the promoter of IFN-inducible *Pkr* (RNA-dependent protein kinase) gene has been shown to require not only an ISRE but also a GC box for the IFN response (Kuhen and others 1998; Tanaka and others 2000). In human and mouse *Pkr* promoters, GC boxes are located 4 bp upstream from the ISRE and have consensus sequences, and they have therefore been termed kinase consensus sequences (KCSs) (Kuhen and others 1998; Tanaka and others 2000).

Genetic variation and antiviral activity of bovine Mx1 protein have already been reported by our group (Nakatsu and others 2004), but bovine Mx1 shows a highly conserved amino acid sequence among various breeds. This means that bovine Mx1 protein has an important function in the mechanism of defense against infectious disease. We therefore tried to analyze the bovine *Mx1* promoter at the molecular level in this study.

Materials and Methods

Breed and cell culture

Blood samples were collected from three cows of Holstein, Charolai, and Brahman breeds. Holstein and Charolai belong to *Bos taurus*, and Brahman is a breed produced in the United States from *Bos indicus* brought from India. Leukocytes were separated from the blood samples by centrifugation at 1800 rpm for 15 min, washed with 8 mL of phosphate-buffered saline (PBS), transferred to a tube containing 2 mL HISTOPAC-1077 (Sigma, St. Louis, MO), and centrifuged at 1800 rpm for 30 min. The obtained pellet of leukocytes was seeded on a 10-cm tissue culture dish (Falcon Labware, Oxnard, CA) containing 10 mL RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL phytohemagglutinin (Sigma), 1 μg/mL pokeweed mitogen (Sigma), 100 IU/mL penicillin (Nacalai Tesque, Kyoto, Japan), and 100 μg/mL streptomycin (Meijiseika, Tokyo, Japan). The cultured leukocytes were incubated for 42 h at 37°C in 5% CO_2.

Cultured Mardin–Darby bovine kidney (MDBK) cells were obtained from Riken BRC (Tsukuba, Japan) and maintained in a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/mL of penicillin and 100 μg/mL of streptomycin.

Nucleotide sequence of the promoter region

Genomic DNAs were prepared from leukocytes of the three breeds. PCR primers of bMx1_F and bMx1_R (Table 1) were used to amplify 994 bp of the bovine *Mx1* promoter region by consulting the sequence published in the database (GenBank accession No. AF525947) (Gérardin and others 2004). The PCR conditions were 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 30 s, 60°C for 30 s, 68°C for 1 min 30 s, followed by 1 cycle at 68°C for 10 min. The PCR products were ligated into the pGEM T-Easy vector (Promega, Madison, WI) by T4-ligase (Promega) and were transformed into *Escherichia coli*–competent JM109 cells (Promega). Recombinant plasmid DNAs were prepared from individual transformed clones, and the nucleotide sequences of the bovine *Mx1* promoter region were determined by using an ABI310 sequencer (Applied Biosystems, Foster City, CA).

5'Rapid amplification of cDNA end

To induce bovine *Mx1* mRNA expression, MDBK cells were treated with human IFN-α-2b (INTRON-A; Schering-Plough Corp, Kenilworth, NJ) at the concentration of 1000 IU/mL for 6 h. Total RNA from MDBK cells was extracted using ISOGEN (Nippon gene, Tokyo, Japan) and was subjected to 5'Rapid amplification of cDNA end (5'RACE) using a GeneRacer kit (Invitrogen, San Diego, CA) according to the manufacturer's recommendation. Then primary and nested secondary PCRs were carried out using the cDNAs synthesized by 5'RACE as templates. Primary PCR was performed using GeneRacer 5' primers and under the conditions of 1 cycle at 94°C for 2 min, 5 cycles at 94°C for 30 s, 72°C for 1 min 30 s, 5 cycles at 94°C for 30 s, 70°C for 1 min 30 s, 25 cycles at 94°C for 30 s, 65°C for 30 s, 68°C for 1 min 30 s, followed by 1 cycle at 68°C for 10 min. Nested PCR was performed using bovine *Mx1*-specific primers bMx1GSP4 and bMx1GSP5 under the conditions of 1 cycle at 94°C for 5 min, 25 cycles at 94°C for 30 s, 65°C for 30 s, 68°C for 1 min 30 s, followed by 1 cycle at 68°C for 10 min. The nested PCR products were ligated into the pGEM T-Easy vector and the transcription start point of bovine *Mx1* gene was identified by sequence analysis.

Plasmid construction for promoter assay

The pGL-basic vector (Promega) containing the firefly luciferase gene was used for the construction of recombinant plasmid for reporter assay of the bovine *Mx1* promoter. Genomic DNA derived from the Holstein cow was used as a template for the PCR, and the PCR was performed with bMx1_F and bMx1_R primers. The PCR conditions were 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 30 s, 60°C for 30 s, 68°C for 1 min 30 s, followed by 1 cycle at 68°C for 10 min. The PCR product was digested with restriction enzymes of *Kpn*I and *Xho*I and was then ligated into *Kpn*I/*Xho*I sites of the pGL-basic vector. We named this vector pGL(1–994).

Plasmid construction for deletion mutants of the promoter

Deletion mutants of 5' end of the bovine *Mx1* promoter region were constructed by an inverse PCR method (Imai and others 1991) using plasmid DNA extracted from the pGL(1–994) vector as a template by PCR with primers bMx1_465F and pGL-Mx1R for pGL(465–994), bMx1_812F and pGL-Mx1R for pGL(812–994), bMx1_833F and pGL-Mx1R for pGL(833–994), bMx1_855F and pGL-Mx1R for pGL(885–994), and bMx1_874F and pGL-Mx1R for pGL(874–994).

Deletion mutants of the ISRE were made (Imai and others 1991) using DNA from pGL(1–994) as a template by PCR with primers bMx1_874F and bMx1_858R for pGL(1–994ΔISRE1), bMx1_833F and bMx1_812R for pGL(1–994ΔISRE2), and bMx1_874F and bMx1_812R for pGL(1–994ΔISRE1+2). Only pGL(1–994ΔISRE1+2) was made using DNAs from

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED FOR PCR IN THIS STUDY

Name	*Sequences (5′ → 3′)*
Primers for 5′ RACE	
GeneRacer1	CGACTGGAGCACGAGGACACTGA
GeneRacer2	GGACACTGACATGGACTGAAGGAGTA
Primers for nested PCR	
bMxl GSP4	CACGTTAGCAGGGACCACCACCAAGT
bMxl GSP5	ACGTGAGGGGAGCTGACCTCCAGACT
Primers for PCR of the promoter region	
bMxl-F	AGAGGTACCGTGGGGAGGACACTTGTTGT
bMxl-R	AGACTCGAGCCGTCCCCAGCGCAGAGA
Primers for PCR of deletion mutants of the promoter	
bMxl465F	ACCTACAGCAGGAGTTTGAT
bMx1-812F	CGTTCGGTTTCGGTTTCCTT
bMx1-833F	CCGATCCAGCAGCCCTGAAA
bMx1-855F	TTTCTGAGTTTCGTTTCTCC
bMx1-874F	CGAGGCTGGGTAGGAGATGA
bMx1-918F	CTAGGGGCGGCGTTAGCG
bMx1-936F	CTGAATAAAGCCGAGGAGGGT
bMx1-812R	GGGTGGCGCACTTCAGCA
bMx1-858R	GAAAGTTTTCAGGGCTGCT
bMx1-880R	AGCCTCGGAGAAACGAAACT
bMx1-898R	GTCCCTCATCTCCTACCCAGC
bMx1-917R	CTCGCTGCCCCGCCTCCCCGT
pGL-Mxl F	ATCTGCGATCTAAGTAATGC
pGL-Mxl R	TATCGATAGAGAAATGTTCT
Primers for semi-quantitative RT-PCR	
bMx1-421 F	AGTGGAAAGGCAAAGTCAGC
bMx1-930R	GATGCTTGATGTCCTGCTGG
bMx2-806F	AAATCCCTCCGAAGTGGAGT
bMx2-1400R	GATGAGCTCGGTGGTAAGTC
Control primer F (G3PDH)	ACCACAGTCCATGCCATCAC
Control primer R (G3PDH)	TCCACCACCCTGTTGCTGTA

pGL(1-994ΔISRE1) as a template. Furthermore, deletion mutants of the GC box or exon1 were constructed using DNA from pGL(1–994) as a template by PCR with primers bMx1-936F and bMx1_898R for pGL(1–994ΔGC1+2) and primers bMx1_F and bMx1_880R for pGL(1–880).

All PCR products for deletion mutants were phosphorylated by T4 polynucleotide kinase (Promega) and self-ligated by T4 DNA ligase after purification of extracts from agarose gel electrophoresis.

Transfection and reporter assay

MDBK cells were seeded in 24-well plates (2.5×10^4 cells/well) and were co-transfected with 500 ng of pGL-basic plasmid containing the firefly luciferase gene and 100-ng pRL-TK plasmid with *Renilla* luciferase gene as an internal control. FuGENE-6 (Roche Molecular Biochemicals, Switzerland) was used as a transfection reagent in accordance with the manufacturer's instructions. Namely, 1.8 μL FuGENE-6 diluted in DEME and 0.6 μg plasmid DNA were mixed in a total volume of 100 μL. The mixture was incubated at room temperature for 30 min and added to MDBK cells. After incubation for 42 h at 37°C in 5% CO_2, the cells were treated with IFN and the luciferase activities were measured 6 h later. A dual-luciferase reporter assay system (Promega) was utilized for the measurement of luciferase, and each activity was measured by using an LD 400C Luminescence detector (Beckman Coulter) according to manufacturer's instructions. Relative promoter activities were estimated by dividing the firefly luciferase value by the *Renilla* luciferase value. All experiments for measurement of promoter activities were carried out at least 3 times. All results are expressed as mean values ± standard deviations of the mean (SD). Statistical significance was analyzed using Fisher's protected least significant difference test, and $P \leq 0.05$ was considered to be statistically significant.

Semi-quantitative RT-PCR

After MDBK cells had been cultured with mithramycin A (Wako Ltd, Tokyo, Japan) at the concentration of 10 nM or 50 nM for 12 h, the cells were treated with human IFN-α-2b at the concentration of 1000 IU/mL for 6 h. Total RNA was extracted according to the manufacturer's recommendation. Then cDNAs were synthesized by semi-quantitative RT-PCR using 1 μg total RNA (ReverTra-Plus; Toyobo, Japan) as a template with the primers bMx1_421F and bMx1_930R or bMx2_806F and bMx2_1400R and GoTaq DNA polymerase (Promega). The primers for the RT-PCR were designed on the basis of nucleotide sequences in the database: U88329 for *Mx1* and AF355147 for *Mx2*. Primers for *G3PDH* (glyceraldehyde-3-phosphate dehydrogenase) (Toyobo) were also used as a control experiment of semi-quantitative RT-PCR,

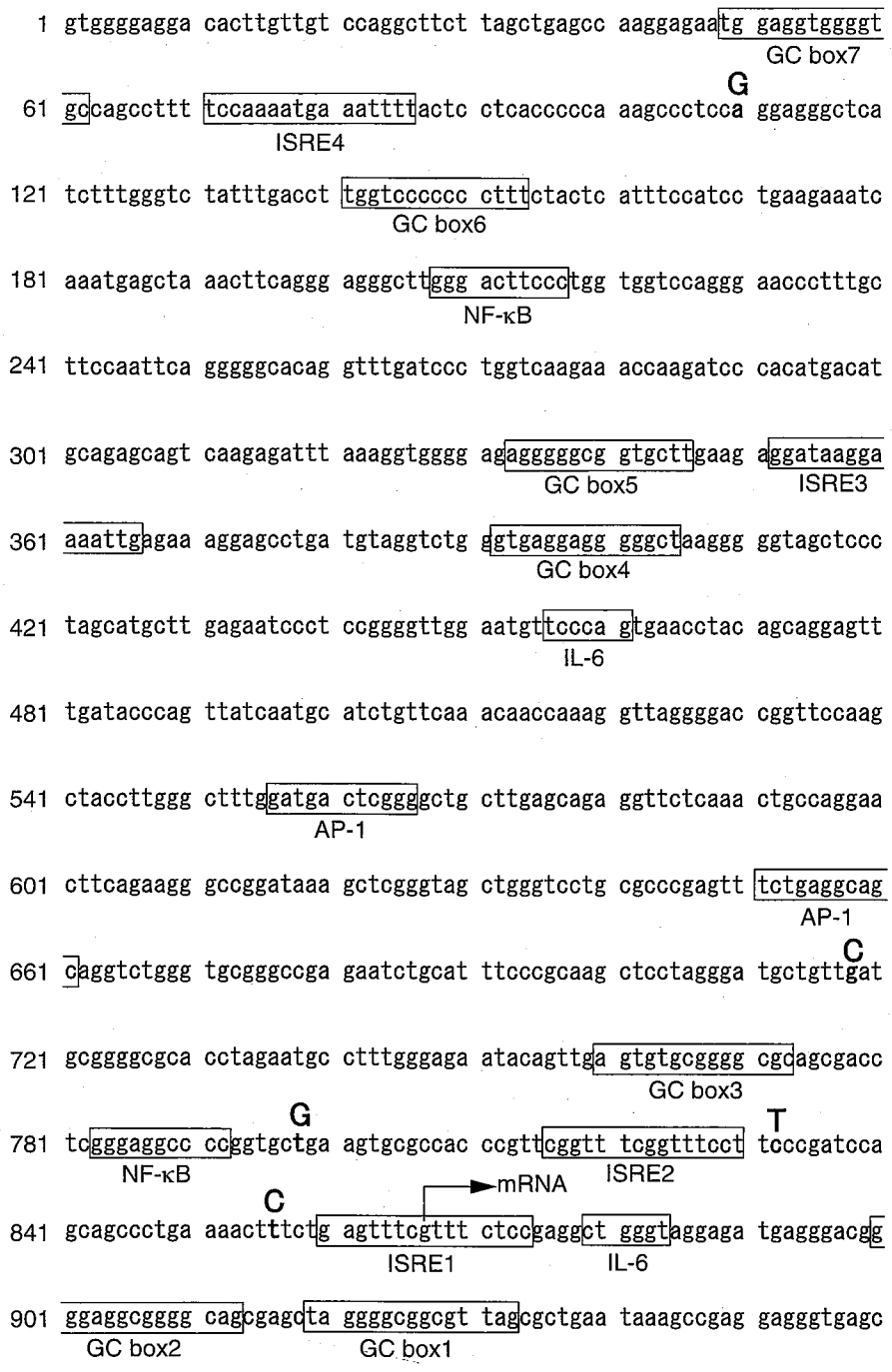

FIG. 1. Nucleotide sequence in the bovine *Mx1* promoter from a Holstein breed examined in this study (accession No. AB443582). The putative functional elements of potential transcription factor–binding sites are indicated as open boxes. ISREs and GC boxes are numbered in the order from downstream. A transcription start point is indicated by an arrow. Nucleotide substitutions among Holestein, Charolai, and Brahman breeds found in the present study are shown by bold capital letters above the sequence.

because the expression of *G3PDH* was constant with or without mithramycin A (Fibach and others 2003). The semi-quantitative RT-PCR conditions were 1 cycle at 94°C for 5 min, 25 cycles at 94°C for 30 s, 60°C for 30 s, followed by 1 cycle at 72°C for 5 min. The RT-PCR products were confirmed by determination of the nucleotide sequences.

Results

Nucleotide sequence of bovine Mx1 promoter

The nucleotide sequence between positions 21 and 976 in the bovine *Mx1* promoter region was first determined by using PCR products from a Holstein genomic DNA amplified with bMx1_F and bMx1_R primers, as shown in Figure 1. Computer analysis showed that the bovine *Mx1* promoter contained four ISRE elements, 7 GC boxes, two IL-6 responsive elements, two NFκB-binding sites and two AP-1-binding sites. The sequences of ISRE1 and ISRE2 completely corresponded to the ISRE consensus sequence. The transcription start point was identified by sequencing a 5'RACE product using total RNA from MDBK cultured cells. The start point of exon1 was located in ISRE1, of which the sequence was spread over the promoter region and exon1. We have registered the sequence of the bovine *Mx1*

promoter region in the database as GenBank accession No. AB443582.

The nucleotide sequences of this *Mx1* promoter region were also determined using genomic DNAs from Charolai and Brahman breeds besides Holstein (AB443582) and compared with that reported previously (AF525947). As shown in Table 2, only 5 nucleotide substitutions were detected in 956 bp among the promoter regions of Holstein, Charolai, and Brahman; however, many variations including 26 substitutions, 2 insertions, and 5 deletions were observed in the same sequences between the above three breeds and that reported previously (AF525947).

TABLE 2. NUCLEOTIDE SUBSTITUTIONS IN THE BOVINE *Mx1* PROMOTER REGION

	Breed			
Nucleotide position	*Holstein (AB443582)*	*Charolai*	*Brahman*	*Database (AF525947)*
29	C	•	•	T
65	G	•	•	A
83	T	•	•	A
110	A	G	G	G
114	G	•	•	A
158	C	•	•	A
225	C	•	•	T
368	G	•	•	A
388	C	•	•	T
426	T	•	•	A
448	T	•	•	-
462	T	•	•	G
463	G	•	•	A
465	A	•	•	C
514	A	•	•	G
ins 514/515	-	•	•	G
ins 539/540	-	•	•	C
571	C	•	•	A
576	G	•	•	A
598	G	•	•	C
672	G	•	•	C
676	G	•	•	C
718	G	C	•	•
752	T	•	•	C
759	G	•	•	T
798	T	G	•	G
799	G	•	•	T
806	G	•	•	A
826	T	•	•	G
832	C	T	T	T
853	A	•	•	-
856	T	C	•	•
920	A	•	•	-
932	A	•	•	-
944	A	•	•	-

Numbers in the nucleotide positions were based on Accession No. AB443582.

Dot • means the same as AB443582.

Hyphens and ins indicate the deletion and insertion against AB443582, respectively.

Bovine Mx1 promoter activity after IFN induction

To determine the appropriate IFN concentration for the induction of bovine *Mx1* promoter activity, the dose dependency of IFN was first examined. A plasmid containing the *Mx1* promoter region from 1 to 994 of the Holstein promoter sequence (Fig. 1) was constructed with pGL-basic vector and was named pGL(1–994). pGL(1–994) was transfected into MDBK cells, and the transfected cells were treated with IFN at three concentrations, 100, 1000, and 10,000 IU/mL. When the promoter activities were measured by the reporter assay using firefly luciferase in the transfected cells at 6 h after addition of IFN, the activity reached a plateau at 1000 IU/mL IFN (Fig. 2).

In order to determine the important regulatory elements responsible for the bovine *Mx1* promoter activity, several deletion mutants were prepared by an inverse PCR method (Imai and others 1991) using DNA extracted from pGL(1–994) as a template. First, deletion mutants of the 5′ end in the promoter region were constructed as shown in Figure 3 and were transfected into MDBK cells. The promoter activities in the transfected cells were measured at 6 h after 1000 IU/mL IFN treatment. Deletion mutants pGL(465–994), pGL(812–994), pGL(833–994), and pGL(855–994) showed the same IFN induction level in the transfected cells as that of pGL(1–994) as a control (Fig. 3). Only pGL(874–994), which contains no ISRE, showed no IFN response in the transfected cells.

In agreement with the results shown in Figure 3, an internal deletion mutant of ISRE1, pGL(1–994ΔISRE1), showed a lower level of promoter activity than that of the control pGL(1–994) after IFN induction in transfected MDBK cells (Fig. 4). However, the promoter activity of pGL(1–994ΔISRE2), an internal deletion mutant of ISRE2, in transfected cells was

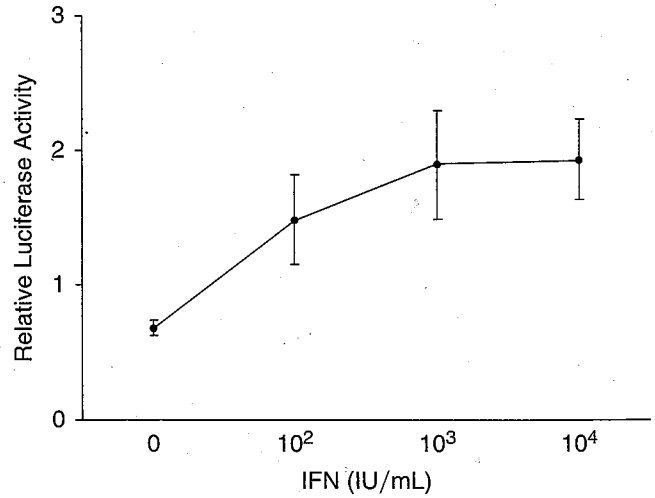

FIG. 2. Dose dependency of IFN in the promoter activity of bovine *Mx1*. A plasmid containing the *Mx1* promoter region from Holstein constructed with the pGL-basic vector, pGL(1–994), was transfected into MDBK cells, and the transfected cells were treated with IFN at 3 concentrations, 100, 1000, and 10,000 IU/mL. The promoter activities were measured by a reporter assay using firefly luciferase in the transfected cells at 6 h after addition of IFN.

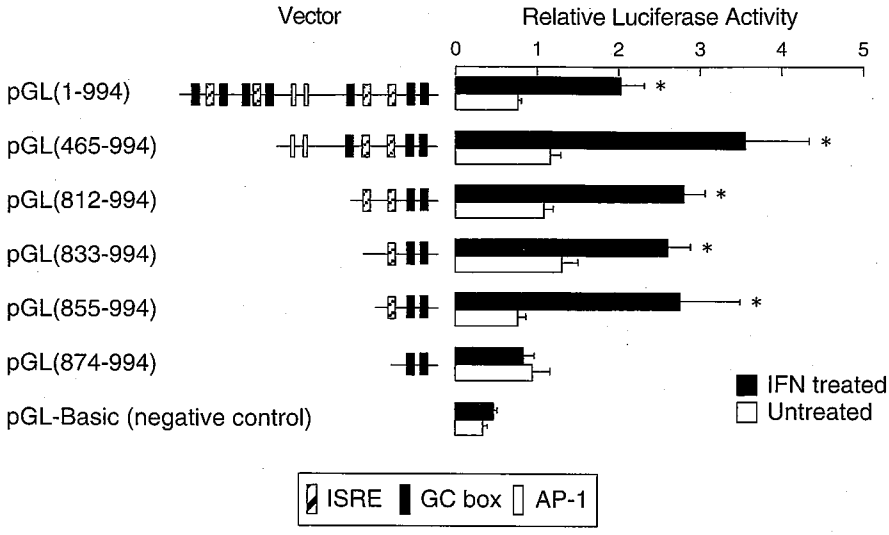

FIG. 3. Promoter activities of bovine *Mx1* with 5′-deleted constructs. The schema on the left shows a representation of the 5′-deleted constructs of bovine *Mx1* promoters, and that on the right shows the promoter activities assayed in MDBK cells transfected with plasmids after induction of 1000 IU IFN for 6 h. Experiments were carried out independently at least 3 times and the results are expressed as means ± SD. *$P < 0.05$ was considered statistically significant when the IFN-treated cells were compared with corresponding untreated cells.

the same as that in the control cells. A mutant with deletions of both ISRE1 and ISRE2, pGL(1–994ΔISRE1+2), also showed a lower level of activity in the transfected cells than in the control cells.

Subsequently, we prepared deletion mutants in the 3′ end of the promoter region (Fig. 5), focusing on the two proximal GC boxes, because pGL(1–800), in which both GC boxes 1 and 2 are deleted, showed a much lower level of promoter activity than that of the control pGL(1–994) after IFN induction in transfected MDBK cells (Fig. 5). pGL(1–994ΔGC1+2) did not respond to IFN in the transfected cells. Furthermore, pGL(1–994ΔGC2) showed a remarkably lower level of activity after IFN induction in the transfected cells than that of pGL(1–994), whereas pGL(1-994ΔGC1) showed a relatively high level of activity.

GC box for basal transcription and IFN response

To confirm whether IFN induction in the bovine *Mx1* promoter requires not only an ISRE but also a GC box, we treated MDBK cells transfected with pGL(1–994) and with an ISRE1-deletion mutant, pGL(874–994), with mithramycin A. Mithramycin A is an agent that inhibits gene expression selectively by coating GC boxes in the promoters bound by a transcription factor such as Sp1 (Fibach and others 2003). When cells were treated with mithramycin A at concentrations of 10 and 50 nM, there was no IFN response in either pGL(1–994)-transfected or pGL(874–994)-transfected cells after IFN induction as shown in Figure 6. Therefore, both or either of GC boxes 1 and 2 would be essential for IFN response in the bovine *Mx1* gene.

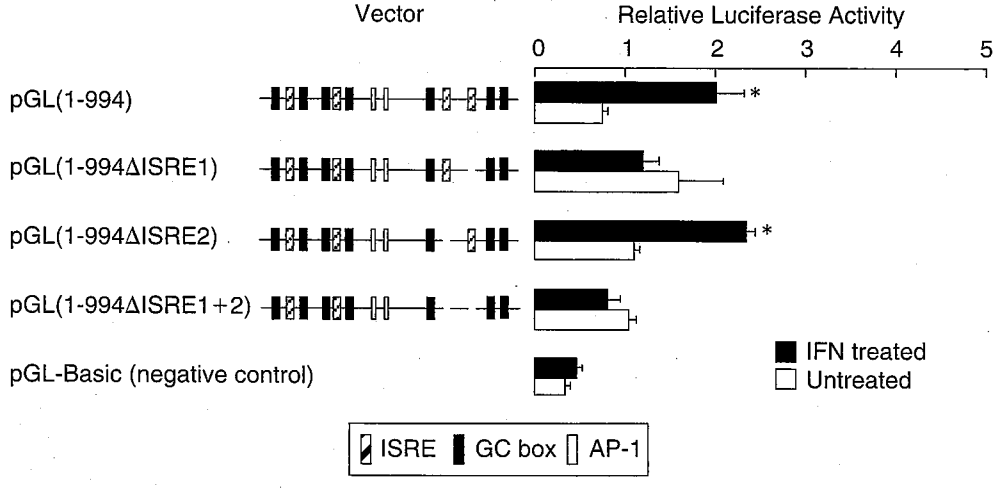

FIG. 4. Promoter activities of bovine *Mx1* with internal ISRE-deleted constructs. The schema on the left shows a representation of the internal ISRE-deleted constructs of bovine *Mx1* promoters, and that on the right shows the promoter activities assayed in MDBK cells transfected with plasmids after induction of 1000 IU IFN for 6 h. Experiments were carried out independently at least 3 times, and the results are expressed as means ± SD. *$P < 0.05$ was considered statistically significant when the IFN-treated cells were compared with corresponding untreated cells.

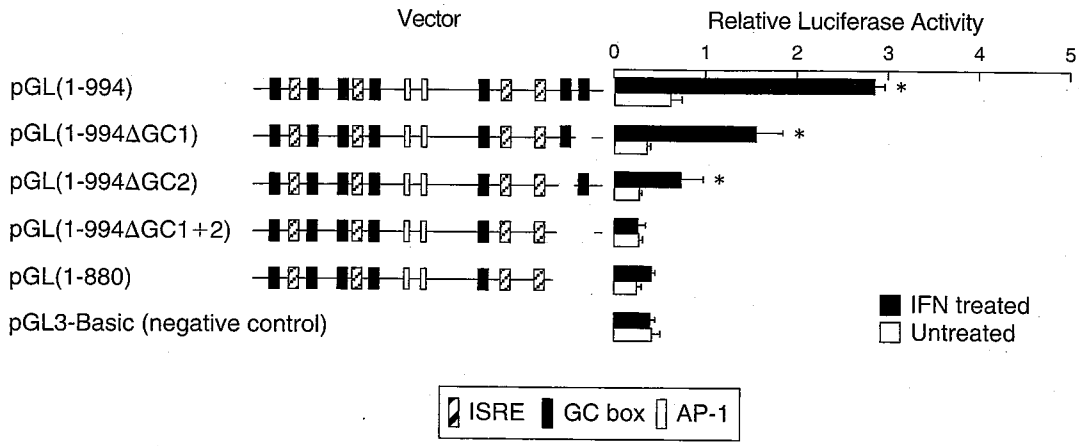

FIG. 5. Promoter activities of bovine *Mx1* with proximal KCS-like GC boxes and 3′-deleted constructs. The schema on the left shows a representation of the proximal KCS-like GC boxes and 3′-deleted constructs of bovine *Mx1* promoters, and that on the right shows the promoter activities assayed in MDBK cells transfected with plasmids after induction of 1000 IU IFN for 6 h. Experiments were carried out independently at least 3 times and the results are expressed as means ± SD. $^*P < 0.05$ was considered statistically significant when the IFN-treated cells were compared with corresponding untreated cells.

Furthermore, we ascertained by semi-quantitative RT-PCR that bovine *Mx* mRNA expression was suppressed by mithramycin A. Mithramycin A clearly decreased both *Mx1* and *Mx2* mRNA expression levels (Fig. 7), although the expression of *G3PDH* used as a control was not changed.

Discussion

The bovine *Mx1* promoter sequence has already been reported (AF525947). We determined again the *Mx1* promoter sequences using genomic DNAs from Holstein (AB443582), Charolai, and Brahman cows in this study. The nucleotide sequences of the *Mx1* promoter region in the three breeds examined in this study showed only 5 substitutions among them (Table 2), but many variations, including 26 substitutions, 2 insertions, and 5 deletions, were observed between the three breeds and the database (AF525947). We consider our results to be reliable because even the Brahman breed showed very few variations in the *Mx1* promoter sequence. Putative-binding sites of transcription factors, two ISREs, five GC boxes, two IL-6 responsive elements, an NFκB-binding site, two Sp1-binding sites and no TATA box, in the bovine *Mx1* promoter (Gérardin and others 2004) have been reported, but we obtained slightly different results of four ISREs, seven GC boxes, two IL-6 responsive elements, two NFκB-binding sites, two AP-1-binding sites and no TATA

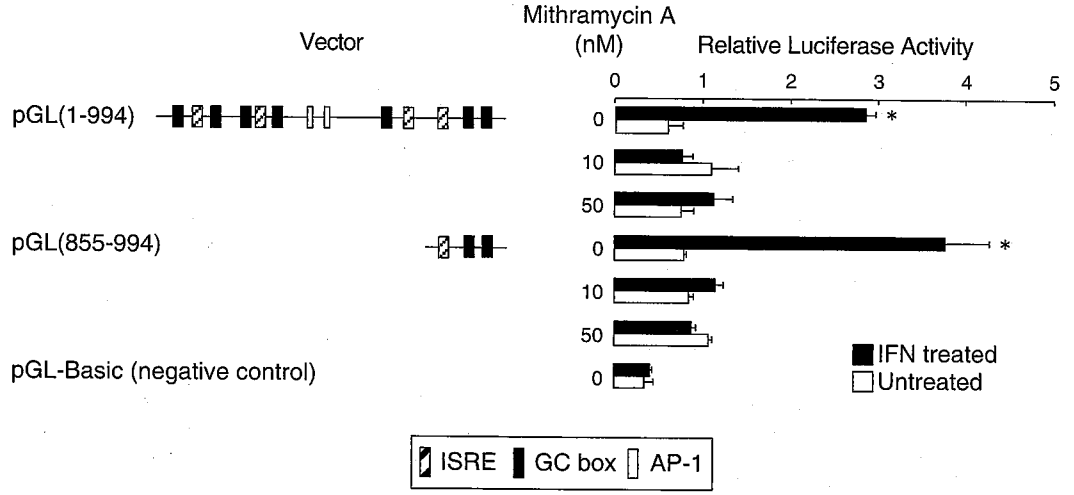

FIG. 6. Promoter activities of bovine *Mx1* treated with 10 and 50 nM mithramycin A. The schema on the left shows a representation of pGL(1–994) and the 5′-deleted pGL(855–994) constructs of bovine *Mx1* promoters, and that on the right shows the promoter activities assayed in MDBK cells transfected with plasmids and treated with 10 or 50 nM mithramycin A for 12 h and then after 1000 IU IFN induction for 6 h. Experiments were carried out independently at least 3 times, and the results are expressed as means ± SD. $^*P < 0.05$ was considered statistically significant when the IFN-treated cells were compared with corresponding untreated cells.

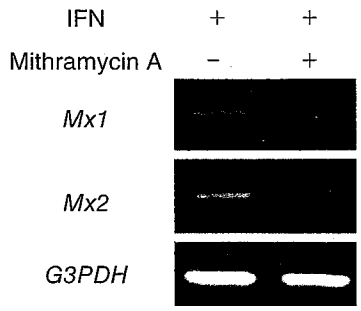

FIG. 7. Bovine *Mx1* and *Mx2* mRNA expressions determined by semi-quantitative RT-PCR in MDBK cells treated with (+) or without (–) 50 nM mithramycin A for 12 h, and then after 1000 IU IFN induction for 6 h (+). Primers synthesized for cDNAs by semi-quantitative RT-PCR were bMx1_421F and bMx1_930R for *Mx1*, bMx2_806F, and bMx2_1400R for *Mx2* and control primers F and R (G3PDH) for *G3PDH*.

box because of many nucleotide variations (Fig. 1). The transcription start point of exon1 of the bovine *Mx1* was located in ISRE1, similar to the results of a previous study showing that transcription start points were located in the ISRE in mouse *PKR* (Tanaka and others 2000).

Then we carried out functional analysis of the *Mx1* promoter using genomic DNA from a Holstein cow (AB443582). The bovine *Mx1* gene clearly showed promoter activity in the response to IFN, although the activity by IFN induction was a relatively small magnification in comparison to that without IFN. Furthermore, several deletion mutants showed that the ISRE has a key function in IFN response in the bovine *Mx1* gene (Figs. 3 and 4). Our results indicating an important role of ISRE in the bovine *Mx1* promoter are consistent with results of other studies (Hug and others 1988; Schumacher and others 1994; Nakade and others 1997; Ronni and others 1998; Asano and others 2003) showing that ISREs in many kinds of *Mx* genes are necessary for IFN induction.

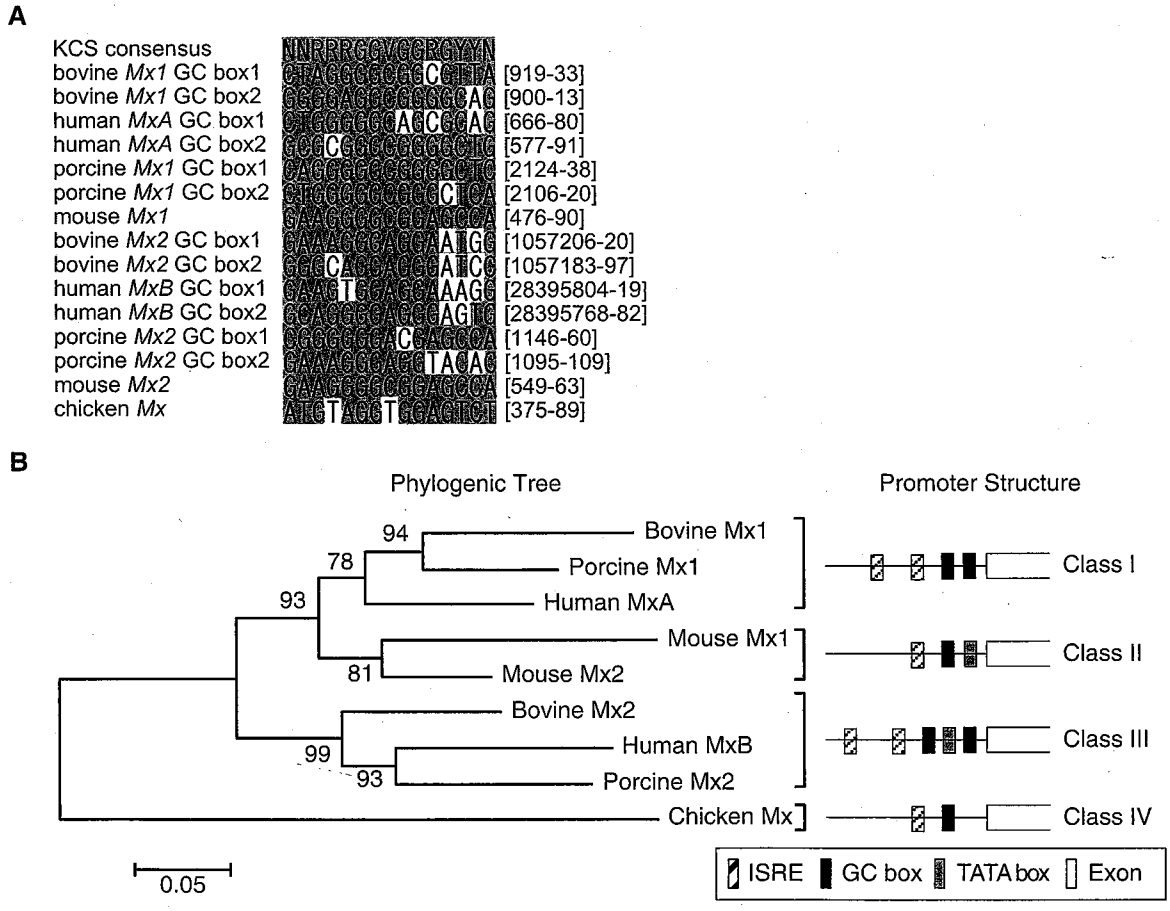

FIG. 8. (**A**) Alignment of KCS-like sequences in several kinds of *Mx* promoters. The nucleotides corresponding to the KCS consensus sequence (upper) are shaded. The positions of the nucleotides are shown on the right. Nucleotide sequences of the promoter regions were based on GenBank accession No. AB443582 for bovine *Mx1*; X55639 for human *MxA*; DQ144503 for porcine *Mx1*; M21104 for mouse *Mx1*; NW_001493784 for bovine *Mx2*; NT_0115.10 for human *MxB*; DQ444963 for porcine *Mx2*; AB086958 for mouse *Mx2*; and Z23167 for chicken *Mx*. (**B**) Evolutionary relationship of several kinds of *Mx* promoters. The left side shows a phylogenic tree of N-terminal regions of Mx proteins. The tree was drawn using clustalX1.83 for multiple alignment, Mega3.1 for phylogenic tree construction and the bootstrap test. The right side shows a classification of the promoter structure that includes the position and number of potential regulatory elements in *Mx* genes. Amino acid sequences were based on accession No. P79135 for bovine Mx1, P27594 for porcine Mx1, AAO31807 for human MxA, CAJ18612 for mouse Mx1, AAH07127 for mouse Mx2, AAK25824 for bovine Mx2, CAB90555 for human MxB, BAF76735 for porcine Mx2, and CAA80686 for chicken Mx.

In addition, the proximal ISRE1, rather than the distal ISRE2, showed a potential induction by IFN in the bovine *Mx1* promoter, as well as in mouse *Mx1* (Hug and others 1988) and *Mx2* (Asano and others 2003) and human *MxA* (Ronni and others 1998; Asano and others 2003).

In this study, not only the ISRE but also the proximal GC boxes in the bovine *Mx1* gene were demonstrated to be essential for the IFN response with the deletion mutants (Figs. 5 and 6). In this case, the 2 GC boxes in the *Mx1* promoter exhibited a synergistic activation in the IFN response. GC boxes located near the proximal ISRE have been reported to be important for the IFN response in the human and mouse *Pkr* promoters and have been termed KCS (Kuhen and others 1998; Tanaka and others 2000). For the *Mx* genes, we showed first that GC boxes in the bovine *Mx1* promoter are important for IFN induction and that these GC boxes would be KCS. This correlation between IFN induction and KCS-like GC boxes was also shown by using mithramycin A, an agent coating GC boxes (Figs. 6 and 7). Mithramycin A inhibited the expression of not only bovine *Mx1* mRNA but also *Mx2* mRNA. We have found that the bovine *Mx2* promoter also contains KCS-like GC boxes (unpublished data). Therefore, we searched for nucleotide sequences corresponding to KCS-like GC boxes in databases from other animals and compared the sequences as shown in Figure 8A. It was found that KCS-like GC boxes are conserved in many animals, and they are expected to have an important role in the expression of *Mx* genes.

We also compared the numbers and positions of ISRE, KCS-like GC box and TATA box, which are thought to have an important role in the expression of *Mx* genes. As shown in Figure 8B, two ISREs and two KCS-like GC boxes exist in bovine *Mx1*, porcine *Mx1*, and human *MxA* (Class I), one ISRE, one KCS-like GC box, and one TATA box exist in mouse *Mx1* and *Mx2* (Class II), two ISREs, two KCS-like GC boxes, and one TATA box sandwiched by two GC boxes exist in bovine *Mx2*, porcine *Mx2* and human *MxB* (Class III), and one ISRE and one KCS-like GC box exist in chicken *Mx* (Class IV). Curiously, these characteristics among the four classes in number and position of important elements in the *Mx* promoters are consistent with groups classified by a phylogenic tree based on amino acid sequence homology (Fig. 8A).

The nucleotide sequence, positions of ISRE and GC box and absence of a TATA box in the bovine *Mx1* promoter resembled those of the porcine *Mx1* promoter (Tungtrakoolsub and others 2008). It was reported that there were several polymorphic variations in both porcine *Mx1* and *Mx2* promoters and that some nucleotide substitutions were correlated with the difference in promoter activities after IFN induction. Since we found at least 5 nucleotide variations in the bovine *Mx1* promoter among Holstein, Charolai, and Brahman breeds (Fig. 1 and Table 1), further study should be carried out to determine whether the variations cause different promoter activities after IFN induction.

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