Provided for non-commercial research and educational use only.
Not for reproduction or distribution or commercial use.

This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator.

All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

http://www.elsevier.com/locate/permissionusematerial

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Developmental and Comparative Immunology 31 (2007) 847–857

Developmental & Comparative Immunology

www.elsevier.com/locate/devcompimm

Cloning and characterizing of the ovine MX1 gene promoter/enhancer region

A.M. Assiri[a,c], T.L. Ott[b,c,*]

[a]Department of Animal Science, Washington State University, Pullman, Washington, USA
[b]Department of Animal and Veterinary Science, University of Idaho, Moscow, Idaho, USA
[c]Center for Reproductive Biology, USA

Received 9 October 2006; received in revised form 2 December 2006; accepted 5 December 2006
Available online 16 January 2007

Author's personal copy

Abstract

Ovine MX1 (MX1) is expressed in the uterus during the estrous cycle and is strongly up-regulated during early pregnancy in the uterus and peripheral blood leukocytes. In this study we cloned the MX1 gene promoter/enhancer, and tested its response to interferon tau (IFN-tau). To address the role of IFN tau in regulating MX1 expression, serial deletion mutants were prepared along with a clone that contained a full-length promoter including the two proximal ISREs but lacking an intronic ISRE site. Promoter deletions showed the two proximal ISRE sites, but not the intronic ISRE site, were required for maximal response to IFN tau. Interestingly, MX1 promoter deletion mutants revealed the presence of distal positive (−920 to −715) and negative (−715 to −437) regulatory regions. Identifying positive and negative regulatory regions in MX1 promoter will help define the complex regulation of MX1 during early pregnancy in ruminants.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Ovine; Pregnancy; GTPase; MX1; Antiviral; Interferon; Endometrium; ISRE; Positive and negative regulatory region; Promoter

1. Introduction

In ruminants, female cyclicity is a uterine-dependent event and rescue of the corpus luteum (CL) requires the presence of the conceptus (embryo and associated membranes) to block the uterine luteolytic signal [1,2]. This process, termed maternal recognition of pregnancy is achieved by conceptus-produced interferon tau (IFN-tau) [3]. IFN-tau is a novel Type I IFN that is secreted by the conceptus mononuclear trophectoderm between days 10 and 21 of pregnancy in sheep [3]. The IFN-tau blocks pulsatile release of the uterine luteolysin, prostaglandin $F_2\alpha$ (PGF) [4]. The antiluteolytic action of IFN-tau is achieved by suppression of endometrial

Abbreviations: IFN, interferon; oGE, ovine glandular epithelial cells; CL, corpus luteum; ER, estrogen receptor; PR, progesterone receptor; OTR, oxytocin receptor; PGF, prostaglandin F2Alpha; ISGF-3, interferon stimulated gene factor-3; ISRE, interferon stimulated response element; ISG, interferon stimulated gene; JAK, the *Janus* family of tyrosine kinase; STAT, the signal transducer and activator of transcription; IRF, IFN regulatory factor; GAS, IFN-γ activated sites; TF, transcription factor; TSS, transcription start site; 5′RACE, 5′Rapid amplification of cDNA ends; 5′UTR, 5′ untranslated region; SRF, serum response factor element; AP-1, activator protein-1; Sp1, stimulating protein-1; NF-κB, NF-kappaB; IL6-RE, interleukin 6 response element.

*Corresponding author. Current address: Dairy and Animal Science Department, Pennsylvania State University, 324 Henning Bldg., University Park, PA 16802, USA. Tel.: +1 814 441 2657; fax: +1 814 863 6042.

E-mail address: tlo12@psu.edu (T.L. Ott).

0145-305X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2006.12.004

estrogen (ER) and oxytocin receptors (OTR) expression [5,6]. In the absence of elevated OTR, oxytocin is unable to elicit pulses of PGF in pregnant ewes. In addition to its antiluteolytic properties, IFN-tau has antiviral, antiproliferative and immunomodulatory activities similar to other Type I IFN [7].

Like other Type I IFN, IFN-tau activates expression of its target genes using the JAK/STAT signaling pathway [8,9]. Binding of Type I IFN to the Type I receptor causes phosphorylation of the receptor-associated Jak-1 and Tyk-2 kinases which leads to the subsequent phosphorylation of Stat-1 and Stat-2 proteins. The phosphorylated Stat-1 (p91) and Stat-2 (p113) form a heterotrimer with p48 (also known as IRF9) and the complex translocates to the nucleus to form interferon stimulated gene factor-3 (ISGF-3). The ISGF-3 complex binds to interferon stimulated response elements (ISRE) in the promoter regions of interferon stimulated genes (ISGs) [9–15]. The conceptus-derived IFN-tau acts locally on the endometrium to regulate a large number of ISGS [16] including the signal transducers and activators of transcription (STAT) 1 and 2 [17], IFN regulatory factor-1 (IRF-1) and IRF-9 [17], interferon stimulated gene-15 (ISG15) [18–20], and the large GTPase, MX1 [18,21–23].

The MX1 proteins are members of the dynamin superfamily of large GTPases and are important components of innate immunity against wide range of viruses [24]. The mouse MX1 protein was the first to be discovered because it conferred resistance to influenza A viral challenge in this species [25]. In most species including fish, birds, and mammals, two to three MX proteins were identified and their sequences are characterized by conserved tripartite GTP binding, dynamin signature and leucine zipper motifs [24,26].

MX1 proteins are expressed in the endometrium of sheep, cattle, pigs, horses, mice and humans [21–23,27,28]. Ovine MX1 mRNA and protein are expressed in the endometrium during estrous cycle; being low but detectable on day 1, substantially increased on day 13 and low on day 15 of the estrous cycle [23]. In response to pregnancy, MX1 expression was increased 10-fold compared to cyclic animals and was expressed in endometrium starting from day 13 through day 19 in luminal epithelium (LE), glandular epithelium (GE), and stroma and myometrium [23]. Temporal MX1 expression correlates with peak levels of progesterone (P4) during the estrous cycle, suggesting that progesterone may also regulate MX1 expression in the uterus [23]. This is particularly interesting because it may link antiviral response in the endometrium to changing steroid levels during the estrous cycle. Several MX1 gene promoters from different species were cloned and the effects of IFN on promoter activity were studied [15,29–36]. Although the chicken MX1 promoter contains one ISRE that is sufficient for stimulation by IFN [35], two proximal ISRE sites act synergistically and are required for MX1 promoter responsiveness to IFN in the human, mouse, zebra fish, and Fugu fish [14,15,31,37].

Due to the unique production of IFN-tau by ruminant conceptuses and the temporal and spatial expression of MX1 during the estrous cycle and early pregnancy in sheep, this study was conducted to clone the ovine MX1 gene 5′ promoter/enhancer region and to identify regulatory regions which direct its unique temporal and spatial expression in the ruminant uterus.

2. Materials and methods

2.1. Animal procedures, peripheral blood cell collection, genomic DNA isolation

All animal procedures were approved by the University of Idaho's IACUC. Jugular vein blood was collected from commercial Western white-faced ewes in EDTA-containing Vacutainer tubes (BD Biosciences, San Jose, CA) and kept on ice until further manipulations.

Genomic DNA was isolated from whole blood using QIAGEN anion-exchange resin-based kit (QIAGEN Blood & Cell Culture DNA mini kit; QIAGEN, Valencia, CA). The quality and purity of the isolated genomic DNA was assessed by spectrophotometer and by *Dra* I enzyme digestion and gel electrophoresis.

2.2. 5′ Rapid amplification of cDNA ends (5′RACE) and MX1 gene exon–exon structure analysis

Sequence analysis of the two available ovine MX1 cDNA sequences [GI: X66093; GI: AF399856] revealed some discrepancies in their 5′ UTR (Fig. 1). To obtain an accurate 5′UTR sequence and to determine MX1 transcription start site (TSS), we performed 5′ RACE for the ovine MX1 cDNA. The SMART^{TM} RACE cDNA Amplification kit from BD Biosciences was used to synthesize SMART^{TM} first-strand cDNA from endometrial

Author's personal copy

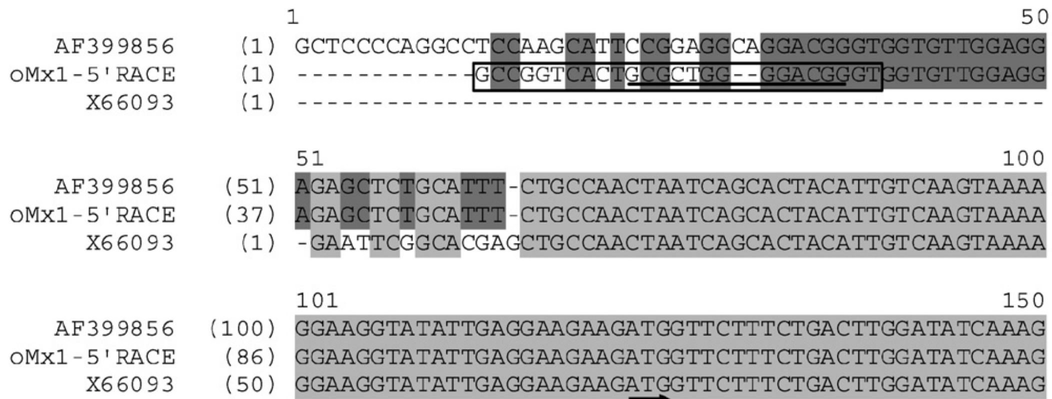

Fig. 1. Multiple sequence alignment (MSA) of the MX1 endometrial cDNA (GeneBank # X66093), oligodendrocyte GTP-binding protein cDNA (GeneBank # AF399856), and this study MX1 5′RACE (GeneBank accession # EF028200). The MSA analysis show sequence discrepancy in the 5′UTR region between the published MX1 sequences. The translation start site (ORF) is indicated by the arrow, MX1 exon 1 sequence is boxed and the 13 bp conserved sequence of MX1 the bovine MX1-a exon 1 (AY251193) is underlined. The white and gray background colors indicate sequence discrepancy and identical sequence, respectively.

total RNA isolated from a pregnant ewe 15 days after mating. The SMART™ cDNA was used to amplify the MX1 5′UTR by using BD Advantage™ 2 PCR kit from BD Biosciences. The polymerase chain reactions (PCR) was carried out using a universal primer A mix (UPM) which is provided with SMART™ RACE kit and the MX1 cDNA specific primer (MX1 5′RACE; GGA TGT CCT TGC CAT ACT TC). Thermocycling parameters were: 94 °C for 5 s; 72 °C for 3 min for 5 cycles; 5 cycles at 94 °C for 5 s, 70 °C for 10 s and at 72 °C for 3 min; followed by 35 cycles at 94 °C for 5 s, 63 °C for 10 s and at 72 °C for 3 min. A single PCR product (~1.1 kb) was gel purified by using GenElute™ Spin Columns (Sigma-Aldrich, St. Louis, MO) and subsequently cloned into PCR® II-TOPO® TA vector (Invitrogen, Carlsbad, CA). Following restriction enzyme screening, clones with the expected size insert were sequenced using M13F and M13R universal sequencing primers. Our MX1 5′UTR sequence (GeneBank accession Nos. EF028200) and the published MX1 sequences (GenBank accession Nos. X66093 and AF399856) were used to generate an MX1 cDNA consensus sequence. The oMX1 cDNA consensus sequence was aligned against the bovine MX1-a genomic sequence (GenBank accession No. 33111869) to map oMX1 gene exon–exon boundaries using the NCBI Spidy program.

2.3. Construction of sheep genomic libraries

The Universal GenomeWalker™ kit (BD Biosciences) was used according to the manufacturer's recommendations. Briefly, four genomic libraries were constructed by digesting sheep genomic DNA with four different restriction enzymes (*Dra I*, *EcoR V*, *Pvu II*, and *Stu I*). The blunt-ends of the digested genomic DNA were ligated to GenomeWalker adapters and used for the subsequent genomic PCR.

2.4. Cloning of the ovine MX1 gene intron A, exon 1 and partial promoter

Following sequence analysis of the MX1 5′ UTR and exon–exon mapping of the MX1 cDNA, MX1 exon 2-specific primers were designed using the Primer Designer program. Sheep genomic libraries were used as template and the primary PCR was carried out using Adapter primer1 (AP1; GTA ATA CGA CTC ACT ATA GGG C) which recognized the GenomeWalker adapter and an MX1 exon 2-specific primer (MX1-ex2; TAG TGC TGA TTA GTT GGC AGA AAT GCA GAG). The BD Advantage™ 2 PCR kit was used for amplification with following thermocycler settings; 5 cycles at 94 °C for 25 s, 72 °C for 5 min with progressive decrease of 1 °C/cycle, followed by 32 cycles at 94 °C for 25 s, 67 °C for 5 min and then final extension at 67 °C for 10 min. The secondary PCR was performed on 1:50 dilution of the primary PCR reaction using Adapter primer 2 (AP2; ACT ATA GGG CAC GCG TGG T) and an MX1 exon 2 nested primer (oMx2-exnst; CAG AAA TGC AGA GCT CTC CTC CAA C). The secondary PCR used the following thermocycler settings; 5 cycles at 94 °C for 25 s, then at 72 °C for 3 min with a decrease of 1 °C/cycle, 20 cycles at 94 °C for 25 s, then 67 °C for

3 min and followed by final extension at 67 °C for 7 min. The secondary PCR products were separated on a 1% agarose gel and major bands (~0.25, 0.35, and 3 kb) were gel-purified and cloned into PCR® II-TOPO® TA vector. A 3 kb clone was isolated that contained partial exon 2, intron A, exon 1 and a partial promoter sequence (~0.2 kb). Our cloning and sequencing analysis revealed the size of MX1 exon 1 (25 bp) and intron A (~2.8 kb). Nucleotide sequence and size of exon1 confirmed our MX1 5′RACE results and the Spidy program exon–exon junction mapping.

2.5. Cloning of MX1 gene promoter/enhancer region

The MX1 intron A 5′ nucleotide sequence was utilized to design primers to walk upstream of intron A. The following primer sets (AP1; GTA ATA CGA CTC ACT ATA GGG C, and MX1INT121; TCC TAG TCT GCG CAA TAC CGG CAT C) and (AP2; ACT ATA GGG CAC GCG TGG T, and MX1DG2; NNN CCG TCC CCA GCG CNN N, where N = A, C, G or T) were used for primary and secondary PCR, respectively. The nested degenerative primer MX1DG2 was designed to contain the complimentary sequence for the conserved 13 nucleotides between MX1 and the exon 1 of bovine MX1-a gene. The primary and secondary PCR conditions were similar to those used for amplification of the MX1 intron A region, with the exception that the annealing/final extension temperatures were increased from 72 to 74 °C and from 67 to 69 °C. The secondary PCR reaction was separated on a 1% agarose gel and two major bands (~0.7 and ~1.7 kb) were gel purified and cloned into PCR® II-TOPO® TA vector.

2.6. Generating oMX1 serial deletion constructs

The MX1 promoter specific primers linked with *Mlu* I and *Xho* I restriction sites (Table 1) were designed to clone a full-length, ~2.1 kb, (−1666 bp; Luc1) genomic fragment containing ~1.7 kb promoter/enhancer, exon 1 (25 bp), and ~0.4 kb partial intron A. The serial deletion mutants which ranged from −1425 to −99 bp (Luc2–9), relative to the TSS, and MX1 promoter/enhancer lacking putative intronic-ISRE site (Luc10) were similarly cloned (Fig. 3). The cycling parameters were similar to those previously described for intron A amplification. The PCR reactions generated the expected size products which were cloned into PCR® II-TOPO® TA vector and sequenced. The linked restriction sites were used to release MX1 promoter mutants

Authors personal copy

Table 1
List of primers that were used to clone MX1 promoter deletion constructs Luc1–10

oMx1 p-Luciferase	Regions: TSS	Forward primer-*Mlu* I (located upstream of TSS)	Reverse primer-*Xho*I (located downstream of TSS)
Luc1	−1666 bp	(oMx1PF-39) *ATACGCGT*ATTGCTGGAGAAGCCTGTGT	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc2	−1425 bp	(oMx1PF-280) *ATACGCGT*TGTACGTCCTTCAGGGGTTC	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc3	−1198 bp	(oMx1PF-507) *ATACGCGT*TGGCTCCTCTCTGACCTGTT	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc4	−920 bp	(oMx1PF-785) *ATACGCGT*GTGGGCTTTCCAAAATGAAA	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc5	−715 bp	(oMx1PF-990) *ATACGCGT*CCCTGGTCAAGAAACCAAGA	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc6	−437 bp	(oMx1PF-1268) *ATACGCGT*TACCTTCGGCTTTGGATGAC	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc7	−354 bp	(oMx1PF-1351) *ATACGCGT*GGGTAGCTGGGTCCTACTCC	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc8	−132 bp	(oMx1PF-1573) *ATACGCGTG*CAGCCCTGAAAACTCTACG	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc9	−99 bp	(oMx1PF-1606) *ATACGCGT*AGAGGCTGGGTGGGAGAT	(oMx1PR-2080) *ATCTCGAG*CAGTCGTGTCCGACTCTGTG
Luc10	−1666 bp*	(oMx1PF-39) *ATACGCGT*ATTGCTGGAGAAGCCTGTGT	(oMx1PR-1872) *ATCTCGAG*TTTCCAGGCAGGAGTACTGG

The *Mlu* I and *Xho* I restriction sites are in italic font.

from the TOPO plasmid by double digestion with *Mlu* I and *Xho* I enzymes (Promega, Madison, WI). Subsequently, inserts were gel-purified using NucleoTrap® Gel Extract Kit (BD Biosciences) and cloned into *Mlu* I and *Xho* I digested Promega pGL3Basic luciferase plasmid to generate MX1p-Luciferase (Luc1–10) constructs. To determine the orientation of inserts, the pGL3Basic flanking sequence and the 5′ and 3′ ends of inserts were sequenced. The MX1p-Luciferase (Luc1-10) constructs were purified using QIAGEN HiSpeed Midi plasmid DNA purification kit, and the purity and concentration of DNA preparation were determined by spectrophotometery and gel electrophoresis.

2.7. Cell culture and transient transfection

All in vitro experiments were repeated 2–3 times, with 3 wells/treatment. All transfections included the β-galactosidase plasmid to monitor transfection efficiency and cells were harvested using Promega 1X passive lysis buffer (PLB) according to the manufacturer's protocol.

Ovine glandular epithelial (oGE) cells [38] were cultured in 12-well plates (Corning Inc, Corning, NY), 5% CO_2 at 38.5 °C for 24 h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Lois, MO) supplied with 10% FBS (GIBCO; Invitrogen, Carlsbad, CA). At approximately 90–95% confluency, cells were either mock transfected or transfected with 500 ng/mL of pGL3Basic (negative control), Promega pGL3Control (positive control) or MX1p-Luciferase constructs and 50 ng/mL β-galactosidase expression vector (pEF1/Myc-His/LacZ, Invitrogen) using LipoFectamine2000™ reagent (Invitrogen) according to the manufacturer's protocol. Briefly, culture medium was aspirated and cells were washed twice with Opti-MEM Reduced Serum media (Opti-MEM RS; Invitrogen) and after the final wash, 800 μL of Opti-MEM RS media was added to each well. Lipofectamine2000 reagent was mixed with Opti-MEM RS (1.5 μL/100 μL) and incubated at room temperature for 5 min and then combined with DNA (500 ng/100 μL of Opti-MEM RS) and incubated for 20–40 min at room temperature. A total of 200 μL of DNA-Lipofectamine2000 reagent mixture was added to each well and cells were incubated for 4–6 h, after which 500 μL of DMEM-10% FBS without antibiotic was added to each well. Cells were incubated for 24 h and observed for evidence of cytotoxicity by microscope.

2.8. Effect of time and dose of IFN-tau on full-length MX1 promoter activity

To determine effect of time on MX1 promoter activity, oGE cells were transfected with the full-length MX1 promoter (Luc1), treated with 10,000 antiviral units (AVU)/mL of IFN-tau and then harvested at 0 (control), 3, 6, 12, 24, 36, and 48 h after IFN-tau treatment. To establish the effect of various IFN-tau doses, oGE cells were transfected with the full-length MX1 promoter (Luc1) and then treated with different doses of IFN-tau (0–10,000 AVU/mL) for 12 h and then harvested.

2.9. Effect of IFN-tau on MX1 full-length promoter and MX1 promoter deletion mutants' activity in oGE cell line

Ovine GE cells were transfected with MX1 promoter constructs (6 wells/construct) and 24 h after transfection; cells were incubated (3 wells/treatment) with medium only or medium containing 10,000 AVU/mL of IFN-tau. Cells were harvested after 12 h of IFN-tau treatment.

2.10. Luciferase and β-galactosidase assays

Cell lysates from each well were assayed for luciferase and β-galactosidase activity using recommended protocols of the Luciferase Assay System (Promega, Madison, WI) and Galactolight Plus (Applied Biosystems, Foster City, CA), respectively. Luciferase and β-galactosidase assays were run using a VICTOR2 Wallac1420 luminometer (Perkin Elmer, Boston, MA).

2.11. Sequence analysis and bioinformatics

The DNA sequencing was performed at the DNA sequencing core, Washington State University. Sequence alignment was generated using Vector NTI Suite 9 (Invitrogen). The MX1 cDNA consensus sequence was generated using a contig alignment program (CAP, Infobiogen, France). The Spidy program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) was used to map the exon–exon junctions of the MX1 cDNA consensus sequence. The MX1 promoter/enhancer sequence was searched for putative transcription factor (TF) binding sites using the default settings of the web-based program, the Transcription Elements Search System (TESS) (http://www.cbil.upenn.edu/tess).

Author's personal copy

2.12. Statistical analysis

Luciferase units from untreated and IFN-tau-treated cells were analyzed using general linear models (GLM) procedures of the Statistical Analysis System (version 9.1, SAS Inc., Gary, NC). The model included main effect of treatment and construct and their interaction. Results are reported as fold increase or decrease compared to un-induced controls.

3. Results

3.1. 5′ Rapid amplification of cDNA ends (5′RACE) analysis

Sequence analysis revealed that the MX1 5′UTR (GenBank accession No. EF028200) had about 99% sequence homology (Fig. 1) with known sheep MX1 sequences. Our RACE clone had an additional 50 and 18 nucleotides compared with the published MX1 sequences X66093 and AF399856, respectively. Interestingly, our sequence had 13 nucleotides which were identical to the published full-length bovine MX1a exon 1, (GenBank accession No. AY251193). The obtained nucleotide sequence was used to generate MX1 mRNA consensus sequence and establish MX1 gene exon structure (data not shown) to facilitate cloning of the promoter region.

3.2. Analysis of MX1 gene promoter/enhancer sequence

An approximately 2.1 kb genomic fragment (GenBank accession No. EF028199) containing the MX1 promoter/enhancer region was cloned. Computer assisted analysis of the MX1 gene promoter/enhancer region revealed the presence of the following putative TF binding sites (Figs. 2 and 3); two promoter-ISRE sites (−101 and −145) and one intronic-ISRE site at +244 that had high

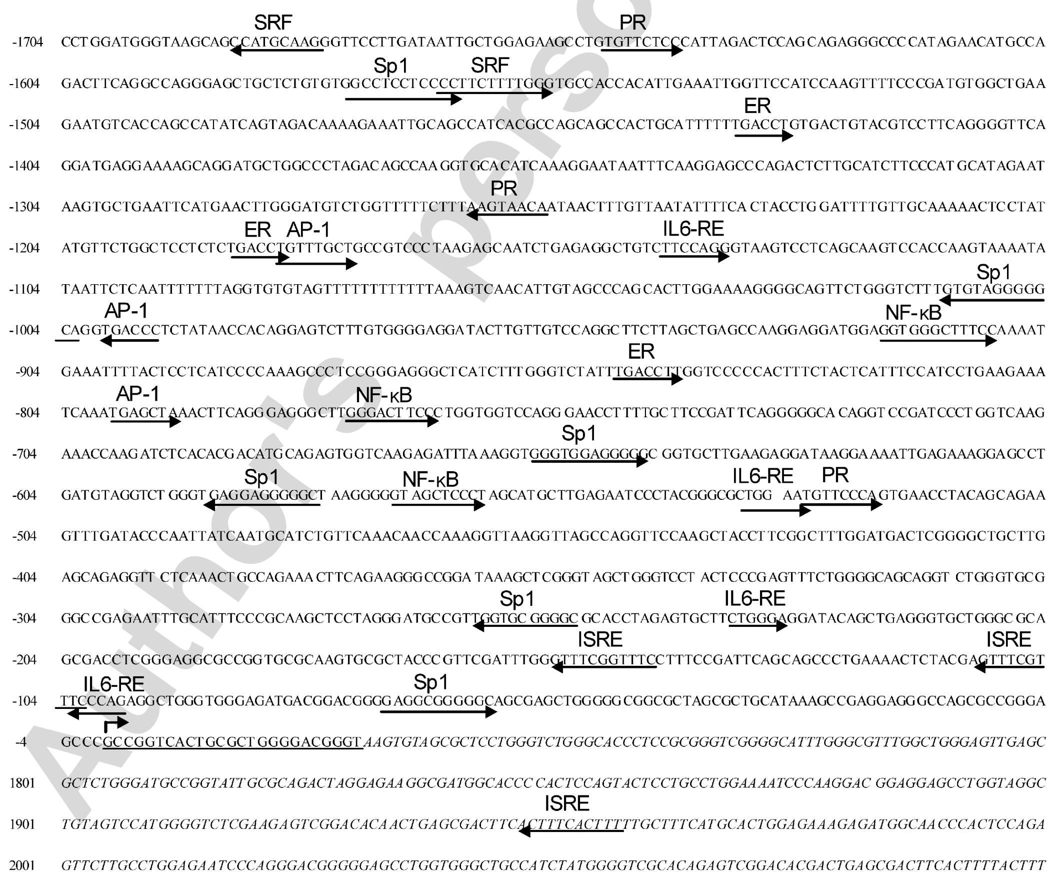

Fig. 2. The 5′ nucleotide sequence of the MX1 gene including 1.7 kb of promoter/enhancer, 25 bp exon 1 (underlined), and partial intron A (in italics). The transcription start site is indicated with bent arrow. The putative transcription factor binding sites (TFBS) are marked with arrows pointed left to right (sense strand) and right to left (reverse strand) to indicate orientation of TFBS. AP-1, activator protein-1; ER, estrogen receptor; ISRE, interferon stimulatory response element; IL-6RE, interleukin-6 response element; NF-κB, nuclear factor-kappa B; PR, progesterone receptor; Sp1, stimulating protein-1; SRF, serum response factor.

Author's personal copy

Fig. 3. Schematic diagram showing 5′ region of MX1 gene deletion mutants and putative transcription factor binding sites. Luc1 construct represents the full-length promoter/enhancer region including all three predicted ISRE sites while Luc2–7 constructs represent truncated forms of the full-length maintaining the two proximal ISRE sites. Luc8 and 9 represent constructs that lack one and two proximal ISRE sites, respectively. Luc10 construct represent the full-length (Luc1) but missing the intronic ISRE site.

Author's personal copy

sequence similarity to the ISRE consensus sequence; [(G/A/T)GAAAN(1-2)GAAA(G/C)(A/T/C)] [33]. In addition several ISRE core sequences GAAAN and GC-rich boxes were identified in the MX1 promoter/enhancer region. The MX1 promoter lacked a TATA box and IFN-γ activated sites (GAS). Putative half-sites for progesterone receptor (PR; −1650, −1263, −530), and estrogen receptor (ER; −1437, −1188, −849) were also predicted. The MX1 promoter contained two putative serum response factor elements (SRF) (−1688 and −1567), three putative activator protein-1 (AP-1; −1183, −1001, −800) and several stimulating protein-1 (Sp1; −1576, −1016, −657, −590, −262, −72) binding sites. There were three predicted NF-kappaB (NF-κB) binding sites at −920, −776, and −573, and four IL6 response elements (IL6-RE) at −1144, −536, −237, and −104.

3.3. Effect of IFN-treatment on MX1 promoter/enhancer constructs

To determine the effect of different doses of IFN-tau on the full-length MX1 promoter/enhancer (Luc1) luciferase activity, transfected oGE cells were treated with different doses of IFN-tau and luciferase activity was measured. There was an approximate 2-fold and 14-fold increase in luciferase counts in response to 1 and 1000 AVU/mL of IFN-tau, respectively (Fig. 4A). The effect of different treatment times with 10,000 AVU/mL of IFN-tau was also tested (Fig. 4B). The luciferase activity driven by the MX1 promoter/enhancer increased (5-fold) after 3 h, peaked (9-fold) at 12 h and remained elevated (4-fold) after 48 h of treatment with IFN-tau.

To identify transcriptional elements regulating the MX1 promoter/enhancer and the minimal sequence that was required for IFN-tau responsiveness, serial deletion mutants were generated by PCR and transfected into oGE and then treated with IFN-tau. In oGE cells (Fig. 5), IFN-tau increased the luciferase activity ($P<0.01$) of the MX1 full-length and truncated promoter constructs (Luc1-8 and 10). There were approximately 8- to 10-fold increases in the transcriptional activity of Luc1–4, 6, 7, and 10, only 3-fold in Luc5, and 2-fold in Luc8. Treatment with IFN-tau decreased ($P<0.01$) luciferase levels by 1.5-fold in the Luc9 promoter construct.

4. Discussion

This study reports the cloning and characterization of the 5′ flanking region of the ovine MX1 gene. Previously, only two sheep MX1 cDNA sequences were available (GenBank accession Nos. X66093 and AF399856). The X66093 sequence was derived

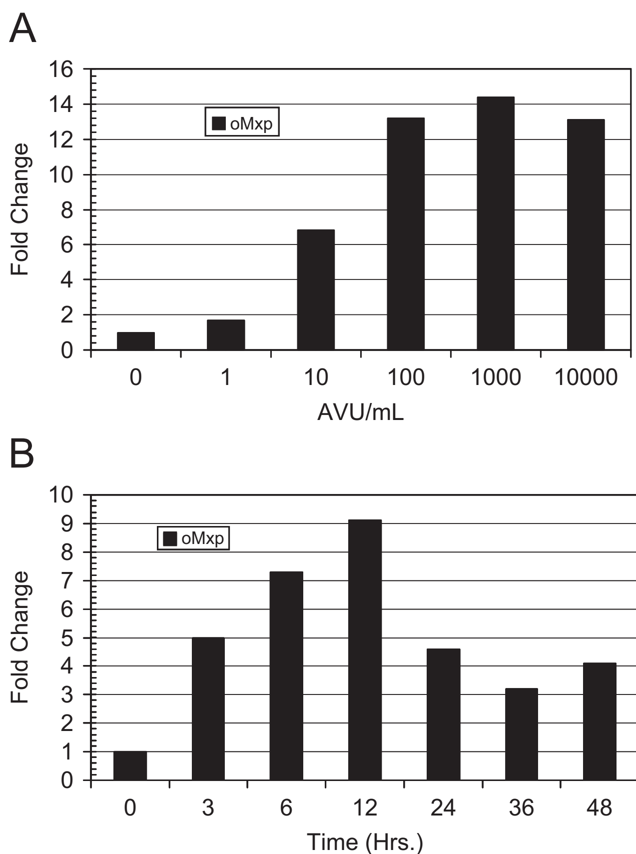

Fig. 4. Full-length ovine MX1 promoter/enhancer luciferase activity increased with increasing concentrations of IFN-tau: (A) and treatment with 10,000 AVU/mL IFN-tau increased luciferase activity 5- and 9-fold at 3 and 12 h, respectively (B).

from pregnant ewe endometrium [22] while the AF399856 sequence was cloned from sheep brain [39]. Although they share approximately 99% sequence homology, the reported 5′ UTRs had some sequence discrepancies shown in Fig. 1. To determine the MX1 gene TSS, we cloned the 5′ UTR of the MX1 cDNA. Utilizing day 15 pregnant ewe endometrial cDNA, MX1 5′ RACE generated a single product (~1.1 kb) which suggested that MX1 mRNA transcript originated from a single TSS. Sequencing analysis of the 5′ RACE product revealed a unique nucleotide sequence which is not found in the published sheep MX1 sequences (Fig. 1). The MX1 5′ RACE contained an extra 50 and 18 nucleotides compared to published ovine MX1 sequences X66093 and AF399856, respectively. Exon 1 of MX1 is 25 bp in length and contains 13 bp which represents the entire exon 1 sequence in bovine MX1-a gene (GenBank accession No. AY251193) [27,29]. In addition, several rounds of PCR amplifications of the 5′ region of the MX1 gene confirmed exon 1 sequence and size. Therefore, the sequence disagreement between this report and the other reports is probably due to unknown splice variants in the sheep MX1 gene downstream of exon 1.

Unlike the mouse MX1 promoter, the ovine MX1 promoter does not contain a TATA box. This is similar to the promoter region in human MX1 (formerly MxA), bovine MX1, chicken MX1, zebra fish MX1, and Fugu fish MX1 [14,29,33–37]. The ovine MX1 promoter possesses two ISRE sites which are located upstream of the TSS and one site was located in intron A. In other species, MX1 promoters contained at least two functional ISRE, with exception of chicken MX1 promoter that has a single ISRE [14,15,31,35,37]. In addition, the MX1 promoter has several GAAAN type sequences that represent the ISRE core sequence [(G/A/T) GAAAN(1-2)GAAA(G/C)(A/T/C)] [34], which are found in most MX1 promoters and can act as binding sites for IFN stimulated gene factor-3, ISGF-3 [12,14,29]. Analysis of the 5′ flanking region of the MX1 gene revealed the presence of several putative binding sites for cytokine and steroid TFs such as IL6-RE, NF-κB, PR and ER half sites, AP-1, SRF and Sp1. These TF binding sites are found in human MX1, mouse MX1, and bovine MX1-a promoter sequences as well (Ott, TL and Assiri AM, unpublished observations) which may suggest common regulation of these genes in different species. The presence of putative steroid receptor (PR and ER) binding sites in the ovine MX1 gene promoter/enhancer was not entirely unexpected because previous studies showed that MX1 expression was regulated by steroids in the ovine uterus [23,40]. In ovariectomized ewes, intrauterine administration of IFN-tau failed to induce MX1 expression. However, estrogen, progesterone, and estrogen plus progesterone treatment increased MX1 expression in response to IFN-tau [40]. The effect of both steroids on MX1 stimulation by IFN-tau was abolished by a progesterone receptor antagonist [40]. However, further studies are needed to verify if these steroid receptor binding sites are functional.

The full-length MX1 promoter/enhancer responded to the lowest dose (1 Unit) of IFN-tau that was used and to higher dosages as well which suggests that the promoter is very sensitive to IFN-tau induction. In addition, the full-length MX1 promoter was induced within 3 h, and showed maximal response at 12 h of incubation with IFN-tau. Although still higher than the control, the decline in transcriptional activity 12 h after IFN-tau

Author's personal copy

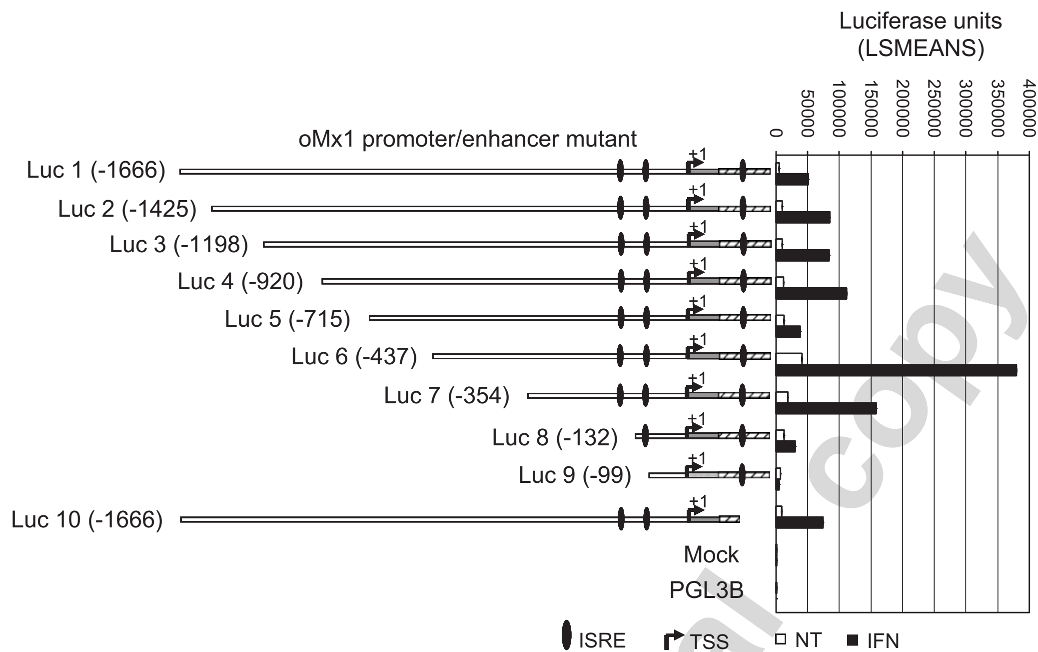

Fig. 5. Transcriptional activity of various MX1 promoter/enhancer deletion constructs in oGE cells in the absence and presence of IFN-tau. Treatment with IFN-tau increased the luciferase activity ($P < 0.01$) of the MX1 full-length and truncated promoter constructs (Luc1–8 and 10). There was an approximate 8- to 10-fold increase in the transcriptional activity of Luc1–4, 6, 7, and 10, only 3-fold in Luc5, and 2-fold in Luc8. Treatment with IFN-tau decreased ($P < 0.01$) luciferase levels in MX1 promoter constructs; Luc9 by 1.5-fold.

Author's personal copy

induction could be attributed to either binding of negative regulatory factors such as interferon regulatory factor 2 (IRF2), a known suppressor of ISGs [17], or post-transcriptional/translational modifications which may affect the luciferase mRNA/protein stability.

Transfection of oGE of serial deletions showed the transcriptional behavior of truncated MX1 promoter/enhancer in response to IFN-tau treatment. Both the ISRE positioned at −101 and −145 were required for maximal MX1 promoter response to IFN-tau. Deletion of the distal ISRE site (−145) decreased luciferase activity from approximately 8-fold (Luc7) to 2-fold (Luc8). Interestingly, the presence of intronic ISRE at +244 (Luc8) did not compensate for the loss of the promoter ISRE site (−145). In addition, deleting both proximal ISRE sites (−101 and −145) and maintaining the intronic ISRE site (Luc9) resulted in a decrease in promoter activity in response to IFN-tau treatment by 1.5-fold compared to non-treated constructs. However, deleting the intronic ISRE site while maintaining the two proximal ISRE sites in Luc10, did not affect the luciferase activity; with this deletion construct showing the maximal response to IFN-tau. Deletion of a distal region (−920 to −715) resulted in a 6-fold reduction in luciferase activity in response to IFN-tau treatment. However, subsequent deletion of the adjacent −715 to −437 region restored maximal promoter response (~9-fold) to IFN-tau. The simplest interpretation of these results is that the regions −920 to −715 and −715 to −437 have positive and negative regulatory element binding sites, respectively. However, experiments are underway to confirm this speculation. Although MX1 and ISG-15 are both stimulated by IFN-tau, their spatial expression is different in the ewe endometrium [18]. Both proteins are expressed in GE and stroma during early pregnancy, however, MX1 is only expressed in LE [18]. These findings are rather intriguing, because both proteins are ISGs and presumably, like other ISGs, activated through the JAK–STAT signaling pathway. Expression of MX1 in LE would suggest that in addition to JAK-STAT, other signaling mechanisms may be involved in MX1 regulation. There is support for STAT-1-independent gene activation by IFN-tau in the LE [41]; however, to this phenomenon remains an area of active investigation.

In this study, the MX1 ‘5 UTR sequence, TSS were determined. In addition, the 5′ regulatory region of the MX1 gene was cloned and its ability to drive reporter plasmid expression was studied. Results from this study suggest that IFN-tau activates the MX1 promoter/enhancer through the proximal ISRE sites. The importance of the two

proximal ISRE sites for MX1 promoter activation by IFN is consistent with results for the human MX1 and mouse MX1 promoters. Although, a predicted intronic-ISRE is present in the human MX1 promoter, this is the first report to test its activity in any species. Results presented here show that this site may not be required for MX1 promoter activation by IFN-tau in sheep. In addition, MX1 promoter/enhancer transcriptional activity was affected by the presence of upstream positive and negative regulatory regions in the MX1 enhancer region. To the best of our knowledge, this is the first report to show the presence of upstream activator and suppressor regions in MX1 enhancer. Overall, these results contribute to understanding of the complex regulation of the MX1 gene in the uterus and suggest the possibility that factors other than IFN-tau may regulate transcription of the MX1 gene.

Acknowledgments

This work was supported in part by USDA NRICGP grant 2002-02398 and NIH NCRR grant P20-RR15587-01 to T.L.O and by the King Faisal Specialist Hospital and Research Center graduate fellowship, Riyadh, Saudi Arabia to A.M.A.

References

[1] Ott TL. Pregnancy, in farm animals. Encyclopedia of reproduction, vol. 3. 1999; p. 980–85.
[2] Spencer TE, et al. Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 2004;82–83: 537–50.
[3] Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. Am J Reprod Immunol 1997; 37(6):412–20.
[4] Bazer FW, Ott TL, Spencer TE. Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology 1994;41:79–94.
[5] Fleming JA, et al. Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon-tau. Endocrinology 2001;142(7):2879–87.
[6] Spencer TE, et al. Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. J Mol Endocrinol 1995;15(2):203–20.
[7] Bazer FW, Spencer TE, Ott TL. Placental interferons. Am J Reprod Immunol 1996;35(4):297–308.
[8] Stewart MD, et al. Interferon-tau (IFNtau) regulation of IFN-stimulated gene expression in cell lines lacking specific IFN-signaling components. Endocrinology 2001; 142(5):1786–94.
[9] Stewart DM, et al. Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. Endocrinology 2001; 142(1):98–107.
[10] Stewart MD, et al. Roles of Stat1, Stat2, and interferon regulatory factor-9 (IRF-9) in interferon tau regulation of IRF-1. Biol Reprod 2002;66(2):393–400.
[11] Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. Virology 2006;344(1):119–30.
[12] Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev 2001;14(4):778–809 table of contents.
[13] Harada H, et al. Regulation of IFN-alpha/beta genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN-alpha/beta. Genes Cells 1996;1(11):995–1005.
[14] Hug H, et al. Organization of the murine Mx gene and characterization of its interferon- and virus-inducible promoter. Mol Cell Biol 1988;8(8):3065–79.
[15] Ronni T, et al. The proximal interferon-stimulated response elements are essential for interferon responsiveness: a promoter analysis of the antiviral MxA gene. J Interferon Cytokine Res 1998;18(9):773–81.
[16] Gray CA, et al. Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol Reprod 2006;74:383–94.
[17] Choi Y, et al. Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. Biol Reprod 2001;65(4):1038–49.
[18] Johnson GA, et al. The Interferon Stimulated Genes (ISG) 17 and Mx have different temporal and spatial expression in the ovine uterus suggesting more complex regulation of the Mx gene. J Endocrinol 2002;174(2):R7–R11.
[19] Johnson GA, et al. Interferon-tau and progesterone regulate ubiquitin cross-reactive protein expression in the ovine uterus. Biol Reprod 2000;62(3):622–7.
[20] Johnson GA, et al. Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. Biol Reprod 1999;61(1):312–8.
[21] Hicks BA, et al. Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J Anim Sci 2003;81(6):1552–61.
[22] Charleston B, Stewart HJ. An interferon-induced Mx protein: cDNA sequence and high-level expression in the endometrium of pregnant sheep. Gene 1993;137(2): 327–31.
[23] Ott TL, et al. Effects of the estrous cycle and early pregnancy on uterine expression of Mx protein in sheep (Ovis aries). Biol Reprod 1998;59(4):784–94.
[24] Haller O, Kochs G. Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic 2002; 3(10):710–7.
[25] Lindenmann J. Resistance of mice to mouse-adapted influenza A virus. Virology 1962;16:203–4.
[26] Lee SH, Vidal SM. Functional diversity of Mx proteins: variations on a theme of host resistance to infection. Genome Res 2002;12(4):527–30.
[27] Ellinwood NM, et al. Cloning and characterization of cDNAs for a bovine (Bos taurus) Mx protein. J Interferon Cytokine Res 1998;18(9):745–55.

Author's personal copy

[28] Chang KC, Goldspink G, Lida J. Studies in the in vivo expression of the influenza resistance gene Mx by in-situ hybridisation. Arch Virol 1990;110(3–4):151–64.
[29] Gerardin JA, et al. Genomic structure, organisation, and promoter analysis of the bovine (Bos taurus) MX1 gene. Gene 2004;326:67–75.
[30] Altmann SM, et al. Cloning and characterization of an Mx gene and its corresponding promoter from the zebrafish, Danio rerio. Dev Comp Immunol 2004;28(4): 295–306.
[31] Yap WH, et al. Molecular cloning of the pufferfish (Takifugu rubripes) Mx gene and functional characterization of its promoter. Immunogenetics 2003;54(10):705–13.
[32] Kojima T, et al. The bovine MX1 gene: characterization of the gene structure, alternative splicing, and promoter region. Biochem Genet 2003;41(11–12):375–90.
[33] Asano A, Jin HK, Watanabe T. Mouse Mx2 gene: organization, mRNA expression and the role of the interferon-response promoter in its regulation. Gene 2003;306:105–13.
[34] Collet B, Secombes CJ. The rainbow trout (Oncorhynchus mykiss) MX1 promoter. Structural and functional characterization. Eur J Biochem 2001;268(6):1577–84.
[35] Schumacher B, et al. The chicken Mx promoter contains an ISRE motif and confers interferon inducibility to a reporter gene in chick and monkey cells. Virology 1994;203(1):144–8.
[36] Chang KC, et al. Molecular and functional analysis of the virus- and interferon-inducible human MxA promoter. Arch Virol 1991;117(1–2):1–15.
[37] Altmann SM, et al. Molecular and functional analysis of an interferon gene from the zebrafish, Danio rerio. J Virol 2003;77(3):1992–2002.
[38] Johnson GA, et al. Development and characterization of immortalized ovine endometrial cell lines. Biol Reprod 1999;61(5):1324–30.
[39] Szuchet S, Plachetzki DC, Karialukas R. Oligodendrocytes express an alpha/beta-interferon-susceptible Mx gene: molecular characterization of the encoded protein. Glia 2002;37(2):183–9.
[40] Ott TL, et al. Expression of the antiviral protein Mx in the ovine uterus is regulated by estrogen (E), progesterone (P), and interferon-tau (IFN-τ). Biol Reprod 1999;60(Suppl. 1):211.
[41] Kim S, et al. Identification of genes in the ovine endometrium regulated by interferon tau independent of signal transducer and activator of transcription 1. Endocrinology 2003;144(12):5203–14.

Author's personal copy