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Research paper

Three promoters with different tissue specificity and pathogen inducibility express the toll-like-receptor 2 (TLR2)-encoding gene in cattle

Guangjun Chang[a,b], Tianle Xu[a,b], Bodo Brand[b], Wolfram Petzl[c], Xiangzhen Shen[a], Hans-Martin Seyfert[b,*]

[a] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, PR China
[b] Leibniz Institute for Farm Animal Biology, Institute for Genome Biology, Dummerstorf, Germany
[c] Clinic for Ruminants with Ambulance and Herd Management, Centre of Clinical Veterinary Medicine, LMU Munich, Germany

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ABSTRACT

Toll-like-receptor 2 (TLR2) is a dominant receptor for perceiving presence of bacterial pathogens. The promoter controlling its tissue specific and infection induced expression in cattle was unknown. We structurally defined with 5′-RACE experiments three promoters (P1–3) controlling TLR2 expression in udder, liver and other tissues of cows suffering from *E. coli* mastitis. P1 is 5′-adjacent to exon 1 as defined by the prototypical TLR2 cDNA sequence. Exon 1 is spliced to the protein-encoding exon 2. P2 and P3 reside in intron 1, express exon 1A and exon 1B, respectively which are each spliced to exon 2. Infection induced massively (>30-fold) activity of P1 and P2, but not of P3 in udders and also somewhat in liver. However, the GC-rich housekeeping promoter P3 expressed exon1B in many tissues providing the wealth of TLR2-encoding transcripts. Similar induction data were obtained after challenging primary cultures of mammary epithelial cells (pbMEC) with *E. coli*. Reporter gene analyses in pbMEC and the liver cell line HepG2 collectively validated that P1 and constructs containing segments from P2/P3 are in principle capable to drive gene expression. Our structural data provide the basis for more detailed molecular analyses of the infection and tissue specific regulation of TLR2 expression.

1. Introduction

Mammalian Toll-like-receptors (TLRs) are a family of thirteen receptors (Roach et al., 2005). Their ligands are components from microbial pathogens known as Pathogen Associated Molecular Patterns (PAMPs). Ligand binding activates the TLR and this signal of pathogen presence is transduced into the host cell *via* a multifactorial cascade to ultimately activate the NF-κB factor complex (Akira et al., 2006; Kumar et al., 2013). These factors are known to switching on and orchestrating a multitude of immune genes contributing to the first line immune defence of the innate immune response (Karin and Lin, 2002; Kumar et al., 2009; Vallabhapurapu and Karin, 2009).

TLR2 is a key factor for the recognition of Gram positive bacteria. Its ligands are PAMPSs from Gram positive bacteria, such as lipopeptides (Jin and Lee, 2008; Uematsu and Akira, 2008) and genetic studies unequivocally proved that TLR2 is indispensable for the eradication of *M. tuberculosis* (Drennan et al., 2004). The genes encoding TLR2 in mouse and human are known to be segmented into three exons. Two short 5′ located exons encode the 5′-UTR and are followed by the large exon III encoding the entire reading frame of the protein. Their promoters reside in front of exon I and have been characterized in some detail (Musikacharoen et al., 2001; Wang et al., 2001; Haehnel et al., 2002). The homologous gene from cattle has not been characterized in such depth. The prototypical cDNA sequence from cattle was deposited as early as 2001 from the group of T. Jungi (University of Bern; *AF368419*) and comparison with the meanwhile known genome sequence of cattle suggests that this gene would be segmented into two exons only, with the large exon 2 encoding the entire protein and representing a homolog of exon III from man and mouse.

Abbreviations: CFU, colony forming unit; Nt, nucleotide; RACE, rapid amplification of cDNA ends; RT-qPCR, reverse transcription quantitative PCR; tsp, transcriptional start point.

* Corresponding author at: Leibniz Institute for Farm Animal Biology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany. Tel.: +49 38208 68 713; fax: +49 38208 68 702.
E-mail address: seyfert@fbn-dummerstorf.de (H.-M. Seyfert).

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We had previously reported that the expression of TLR2 is upregulated in the udder of cows suffering from acute mastitis (Goldammer et al., 2004). This observation has meanwhile been validated in several studies. Hence, we recently included TLR2 into a collection of candidate genes to examine the contribution of epigenetic mechanisms to regulate immune gene expression in liver during the systemic reaction elicited by acute mastitis (Chang et al., 2015). Such analyses mandate that the relevant promoter expressing the candidate gene is known. We learned in RT-qPCR analyses that the DNA sequence immediately upstream of that exon 1 from cattle cannot be the sole and strongest promoter expressing TLR2 in liver. Rather, initial 5′-RACE experiments revealed very quickly that a promoter residing in intron 1 is most strongly driving the expression of this gene. We subsequently found that the chromatin in this area of the gene is remodeled during infection related up-regulation of gene expression (Chang et al., 2015).

We now report a more comprehensive analysis of the promoters expressing TLR2 in cattle and show that at least three promoters express this gene. They contribute differentially to the tissue specific base line and the pathogen related up-regulated expression revealing that they have distinct functions.

2. Materials and methods

Animals, tissue and cell collection: All tissues have been collected from Holstein Frisian (HF) cows having been culled in the abattoir of the Leibniz Institute for Farm Animal Biology. This facility is operated under the supervision of the regional authorities surveying compliance with the pertinent ethical rules. Samples from udder tissues and liver were from experimental and control animals (referred to below as "gold standard" control cows) from a trail in which a single quarters of primiparous mid-lactating cows had been infected for 24 h with an inoculum of 500 CFU of *E. coli*$_{1303}$, as previously described in detail (Mitterhuemer et al., 2010). All the infected quarters suffered from acute mastitis. All other samples were from healthy HF heifers having served as foster mothers for embryos during establishing a F2 reference family (Weikard et al., 2012). Small cubes (0.5 cm^3) from the tissues were excised and immediately snap frozen under liquid nitrogen. RNA was extracted using Trizol (Life Technologies), as described (Petzl et al., 2008). RNA samples from primary bovine mammary epithelial cells (pbMEC) were taken from an experiment challenging these cells (two biological replica with cultures from two different cows) for different times with heat inactivated *E. coli*$_{1303}$ particles, as described (Günther et al., 2011).

5′-RACE: We used the GeneRacer kit (Life Technologies, # 45-0168) essentially as prescribed by the manufacturer. Amplificates were cloned into pGEM-T Easy (Promega) and sequenced.

RT-qPCR: One μg of total RNA was used to generate cDNA with the SuperScript II Reverse Transcriptase (Life Technologies). The mRNA abundance was assessed with the Sybr Green Fast Start I kit and the LightCycler II device (both from Roche), basically as described (Goldammer et al., 2004). The cDNA was primed for these assays with the gene specific reverse primer b_T2r4 (5′-CCTCAGAGTCTTCAGGTTCAC) binding to exon 2 and oligo(d)T to allow for measuring reference genes. Aliquots of the purified cDNA equivalent to 75 ng of input RNA were distributed to different vials and mixed with 25 pM of the common reverse amplification primer (T2_902 r, 5′-AGACCAGAGGGGATGGAGTT, also binding to exon 2). Next, 25 pM of either of the exon-specific forward primers were added; for exon 1 the primer T2_902 f (5′-TGCGTTGGTTTGGATAGTGA), for exon 1A the primer T2_ex1Af (5′-GGCTGTGGAAGGCGCTG-CAA) and for exon 1B the primer T2_ex1Bf (5′-TCACTCGAAGCGGCTGTGAC). Relative copy numbers were titrated against external standards consisting of dilution series (10^6 to 10 copies) of the linearized subclones harboring the respective amplificates. The concentration of β-actin (ACTB) was also monitored using the primers 5′-AACTGGGACGACATGGAGAAGAT (forward) and 5′ GCCAAGTCCAGACGCAGGAT (reverse). Relative copy numbers from liver and mammary gland samples collected from the infection experiment were referenced against the cDNA of the splicing factor, serine/arginine-rich, 4 (SFRS4;) using 5′- ATGGCAGTTACGGTTCTGGAC and 5′-CCTGCCTGACGCATATAATCC as forward and reverse primers, respectively.

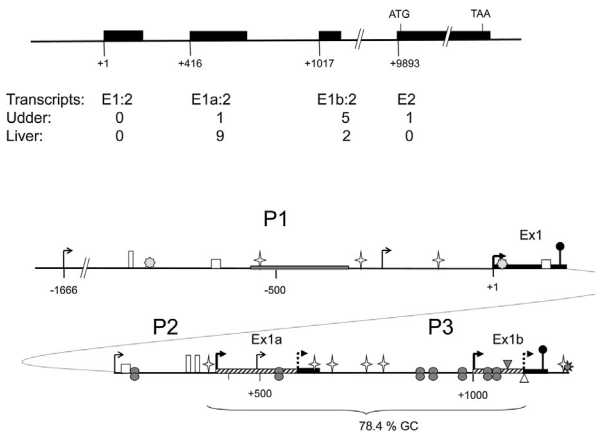

Fig. 1. Segmentation and promoter features of the TLR2-encoding gene. (A) Genomic arrangement and sizes of exons and introns. Positions and exons (black boxes) as identified by 5′-RACE are indicated. Exon 2 is homolog to exon III of the human and mouse TLR2 gene. Position +1 is the 5′-end of the original data base entry of the TLR2 mRNA from cattle (AF368419). Transcript indicates the structure of the 5′-UTRs of the TLR2 mRNA. (B) Positions of attachment sites for relevant transcription factors. Grey box denotes a repeat element. Exons retrieved from liver or mammary gland RNA as templates are indicated by black or hatched boxes, respectively. Arrows with filled heads indicate the longest RACE clones retrieved from liver or mammary RNA (stippled). Promoter segments between open arrows and knobbed vertical lines have been used for reporter gene construction of P1 (upper line) or P2 combined with P3 (lower line). Attachment sites for various transcription factors are indicated (see main text for more information).

Expression clones and reporter genes assays: Reporter gene clones were established by amplifying the respective segments with primers attaching 5′-K*pn*I and 3′-X*ho*I restriction sites to the end of the fragments. After subcloning in pGEM®-Teasy (Promega) the segments were excised with those restriction enzymes, purified and recloned into the multiple cloning site of the Firefly luciferase-encoding, but promoter-less vector pGL 3-basic (Promega). The long segment from P1 was amplified with the primers (forward) 5′-AGCTGGTACCATGTT-ATTGACATTGTTCAAAGTAG and (reverse) 5′-AGCTCTCGAGCACTATCCAAACCAACGCAGCA, amplifying 1793 bp (restriction sites are underlined). Establishment of the shorter segment (389 bp) used the same reverse primer, but as forward primer 5′-AGCTGGTACCATGAATGCTTCTTCAGTCTCTGA. Intron 1 sequence elements harboring P2 fused to P3 were amplified as long version (973 bp) with the primers (forward) 5′-GATCGGTACC-GTGGGCCGTGTAGATATGCTG and reverse 5′-AGCTCTCGAGCGTCACAGCCGCTTCGAGTGA. The short version (627 bp) used the same reverse primer, but as forward primer 5′-AGCTGGTACCGCCACAGGGGTTACGCGCA. Primer positions are indicated in Fig. 1B and Supplementary file 1A.

Lipofectamin 2000 (Life Technologies) was used to transfect target cells grown in six well plates with 400 μg of endotoxin free preparations of these plasmids together with 20 ng of the ELAM-promoter driven and Renilla-luciferase expressing NF-κB measuring plasmid, as previously described in detail (Yang et al., 2008). After recovery, cells were distributed into six wells of a 24 well plate. In the case of pbMEC, three of these wells were kept as controls while the other three were stimulated with heat killed