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Genomic structure, promoter analysis and expression of the porcine (*Sus scrofa*) *TLR4* gene

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Abstract

Toll-like receptor 4 (TLR4) is essential for initiating the innate response to lipopolysaccharide (LPS) from Gram-negative bacteria by acting as a signal-transducing receptor. As the pig industry faces a unique array of related pathogens, it is anticipated that the genotype of swine *TLR4* could be of crucial importance in future strategies aimed at improving genetic resistance to infectious diseases. In order to help in investigating *TLR4* as a candidate disease-resistance gene in pigs, we established its genomic structure and produced sufficient flanking intronic sequences to enable simple PCR amplification of the coding portions of the gene. Expression in different porcine tissues was studied and showed splicing variations in mRNA sequences. The cDNA sequence for *poTLR4* contains an open reading frame of 2526 bp that codes for 841 aa, 98 and 568 bp in the 5′- and 3′-UTRs, respectively. Overall, the general organization of porcine, human, murine, and avian TLR4 genes is quite similar: three exons with the third one very long. A high level of conservation of the size and the sequence, especially for the two last exons and particularly in the sequence corresponding to the LRRs and TIR domain, is observed between species. The important antimicrobial properties of these proteins may account for a conservative selection pressure on these TLR4 coding sequences. Several putative binding sites described in the human and murine promoter of *TLR4* genes have been identified in the 5′-flanking region of *poTLR4*. Conversely, this region lacks a TATA box, consensus initiator sequences, or GC-rich regions. The basic sequence data gathered will allow the establishment of an inventory of naturally occurring variation in porcine *TLR4*, so that alleles can be tested for disease association studies.

Keywords: Toll-like receptor; Lipopolysaccharide; LRR; TIR domain

1. Introduction

Multicellular organisms have developed strongly sensitive innate mechanisms of immunological defense that allow rapid recognition of invading pathogens and provide early signaling to initiate nonspecific as well as adaptive immune responses. In this context, the mammalian Toll-like receptors (TLRs), which are members of the type-1 transmembrane receptors family, play a crucial role. Whereas the signaling domains of the 10 known TLRs are highly conserved, the leucine-rich repeat ligand-recognition domains are more diverse to accommodate recognition of different pathogen-associated molecular patterns. Toll-like receptor 4 (TLR4) specifically recognizes lipopolysaccharide and the structurally similar lipoteichoic acid components of Gram-negative and Gram-positive bacterial cell walls, respectively (Chow et al., 1999; Takeuchi et al., 1999; Lien et al., 2000; Kopp and Medzhitov, 2003). Recently, data have accumulated showing that lack of, or mutations in *TLR4* can cripple immune responses to pathogens that produce these ligands, thus implying that polymorphisms in the coding sequence or in the promoter of *TLR4* can underlie different resistance/susceptibility patterns to infectious diseases. For instance, objective data are already available for *Salmonella typhimurium* (O'Brien et al., 1980), *Pasteurella pneumotropica* (Chapes et al., 2001), *Streptococcus pneumoniae* (Malley et al., 2003), *Brucella abortus* (Campos et al., 2004), *Haemophilus influenzae* (Wang et al., 2002), *Klebsiella pneumoniae* (Branger et al., 2004) and *Mycobacterium*

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bovis (Heldwein et al., 2003). As the pig industry faces a unique array of related pathogens, i.e. *Salmonella* spp., *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Bordetella bronchiseptica*, or *Haemophilus parasuis* (Jubb et al., 1993), it is anticipated that the genotype of swine *TLR4* could be of crucial importance in future strategies aimed at improving genetic resistance to infectious diseases. A partial cDNA sequence for porcine *TLR4* has been reported with 72 and 63% amino acid similarities to human and mouse *TLR4*, respectively (Smirnova et al., 2001; Strausberg et al., 2002). However, several issues need to be resolved to further investigate *TLR4* as a candidate disease-resistance gene in pigs. First, the genomic structure needs to be established and sufficient flanking intronic sequences gathered to enable simple PCR amplification of the coding portions of the gene. Then, a basic knowledge of the promoter region needs to be obtained as an allelic variation there can significantly alter absolute levels and/or tissue-specificity of TLR4 expression. The objectives of the present study were to report such basic sequence data along with exon-specific PCR protocols, a comparison of the genomic organization of the pig *TLR4* locus with its known mammalian counterparts and expression of *TLR4* in porcine tissues.

2. Materials and methods

2.1. Identification of a BAC clone containing the porcine TLR4 gene

To obtain the *TLR4* gene, gridded array nylon filters containing a porcine genomic bacterial artificial chromosome (BAC) library (C.H.O.R.I., Oakland, CA, USA) was screened, under conditions of high stringency hybridization, using one 164-nt α-32P-tagged probe, spanning from nt 41 to nt 204 of TLR4 sequence (GenBank No. AJ583830). Four BAC clones were positive and then cultured in 100 ml LB in the presence of 20 μg/ml of chloramphenicol. Plasmid DNA was extracted using standard alkaline lysis procedure, precipitated with isopropanol and resuspended in 10 mM Tris–HCl (pH 8). A two-step confirmatory procedure was used: (i) a PCR aimed at amplifying fragments of exon 3 (sense, 5′-tcgctgctaacatcatccag-3′ and antisense, 5′-ctgcaggacgatgaagatga-3′) and 3′-untranslated sequence (UTR) (sense, 5′-gaagttggagaagtccctgct-3′ and antisense, 5′-tgtaccatgatgcagtttcc-3′) and (ii) sequencing of the produced amplicons and confirmation by sequence comparison with porcine *TLR4* cDNA sequence (AY289532).

2.2. Sequencing of the insert from the BAC clone 363F10

After DNA extraction from the BAC clone, DNA was broken by Hydroshear® (GeneMachines, San Carlos, CA, USA) in 2.5–5 kb fragments. After end repair, fragments with the size from 2.5 to 5 kb were selected on 0.7% agar gel. Purified fragments were then cloned in *Sma*I-digested and dephosphorylated PUC18 vector. Ligation product was transformed in ElectroTenBlues *Escherichia coli* cells (Stratagene, La Jolla, CA, USA) by electroporation and cultured in 2TY broth with ampicillin (100 μg/ml), IPTG (1 mM; Sigma Aldrich, St Louis, MO, USA) and XGal (120 μg/ml; Euromedex, Mundolsheim, France). Primers M13 forward and reverse were used for sequencing and sequences were joined with the Phrap (0.960731) software (Washington University, USA).

2.3. Amplification of poTLR4 cDNA 5′- and 3′-ends

Total RNA from a porcine spleen was extracted with TRIzol (Invitrogen) as described by the manufacturer. The SMART RACE technology (Clontech Laboratories) was used to clone *poTLR4* 3′-end. For first-strand cDNA synthesis, and according to the *poTLR4* gene sequence obtained before, gene-specific primer was designed for the 5′- and 3′-RACE PCR 5′-CTGTTTCAAACTGGAATGCTGGAAATC-3′ (corresponding to nt 1282 to nt 1308; AB188301.1) and 5′-CCCAGCACTTCATACAGAGCCGATG-3′ (corresponding to nt 101 to nt 125; AY289532), respectively. Reverse transcriptase and polymerase chain reaction (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification kit. The 5′- and 3′-RACE products were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen), TA-cloned into pCRII-TOPO (Invitrogen) and seeded on kanamycin plates. Minipreps were obtained from colonies grown in 5 ml LB-Kan broth.

2.4. RT-PCR from different porcine tissues

Total RNA (0.5 μg) from spleen, lymphatic node, liver, stomach, lung, small intestine, and colon from one healthy pig was reverse transcribed using Improm II (Promega). RNA (0.5 μg) from spleen of another healthy pig and of two wild boars was also used. The full-length cDNA was then generated by long distance PCR using Taq polymerase (Promega) with primers designed from the distal ends of both 5′- and 3′- RACE products: 5′-tgaccagcagatacagaggg-3′ (sense) and 5′-agggatgtttctgaactgac-3′ (antisense). The procedures recommended by the manufacturer were followed, with the following cycling parameters: 10 min at 94 °C, then 34 cycles including (i) 1 min at 94 °C, (ii) 1 min at 56 °C and (iii) 3 min 30 s at 68 °C, followed by a final extension at 68 °C for 10 min (elongase, Invitrogen). The RT-PCR products (around 3.5 and 1.4 kb) were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen), TA-cloned into pCRII-TOPO (Invitrogen) and seeded on kanamycin plates. Minipreps were obtained from colonies grown in 5 ml LB-Kan broth.

2.5. Validation of exon-specific PCR protocols

PCR fragments corresponding to the predicted exons were then produced from genomic DNA extracted from the BAC