Sequence based prediction of putative transcription factors binding sites in the 5'-upstream region of Toll-like receptor 8 gene in Indian goats

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ABSTRACT

Toll-like receptor 8 (TLR8) recognizes single stranded RNA (ssRNA) and is localized in endosomes. Stimulation of TLR8 by ligands activates the production of pro-inflammatory cytokines such as different tumour necrosis factors, interleukins etc. In this study approximately 1.1Kb of 5'-upstream region of goat TLR8 gene was amplified and characterized for the presence of different putative transcription binding factor sites. The sequence analysis revealed the goat TLR8 to be closer to cattle sharing 88% homology followed by other species in the 5-upstream region. The sequence analysis for the search of putative binding sites for transcription factors, showed the presence of 70 potential sites in goat as compared to cattle, where it was found to be having 76 sites in the same region scanned. Further analysis showed the insertion of five nucleotides in goat at two different positions as compared to cattle. The binding factor sites analysis revealed the presence of some important factors binding sites such as PU-1, an important regulatory elements in goat, found to be absent in cattle. Many of the factors were found to be similar in both the species sharing the same position in the sequence. Overall our results showed the presence of important regulatory elements in goat TLR8 5'upstream, which may be important for understanding the regulation of its transcription.

Keywords: Toll-like receptor 8, 5' Upstream, Sequencing, Transcription factors bindings

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INTRODUCTION

Innate immune response develops after the initial sensing of invading microbes, leading to the production of effector molecules that contribute to contain initial infection and to mount the subsequent adaptive immune response (Liese et al., 2008). Toll-like receptors (TLRs) are hallmarks of cellular receptors that recognize pathogen-associated molecules and participate in innate responses to infections (Kaye et al., 2011). There are currently 13 mammalian TLRs described, and can be divided into extracellular: TLR1-2, TLR4-6, and TLR11 (Pifer et al., 2011) or intracellular: TLR3, TLR7-9 and TLR13 (Blasius et al., 2010), and those receptors recognize specific groups of ligands either at the cell surface or in the endosomal compartment, respectively (Kawai et al., 2011). Structurally, TLRs are composed of extracellular /ectodomains leucine-rich repeats (LRR), which recognize the pathogen, and a cytoplasmic signalling domain also known as the Toll-interleukin receptor (TIR) domain. Each TLR detects distinct sets of molecules from viruses, bacteria, fungi and parasites, and upon binding, they recruit different adaptor proteins such as MyD88 or TRIF (Kawai et al., 2011). TLRs initiate innate responses in a variety of ways, leading to the production of inflammatory cytokines by macrophages and different subtypes of dendritic cells (DCs) and of type I interferons (IFN) by inflammatory monocytes, macrophages, and DCs (Kawai et al., 2011).

The microbial molecules recognized by TLRs are conserved polymers, such as bacterial lipopolysaccharides (LPS), peptidoglycans, unmethylated bacterial DNA, and single-stranded or double-strand viral RNA, among others. TLR8 is one of the important recognition receptor, found in endosomes of monocytes and macrophages, recognizing single-stranded RNA from viruses. Activation of TLR8 induces production of pro-inflammatory cytokines such as TNF-α, Interleukins (IL-12) (Gorden et al., 2005). TLR7 and TLR8 genes lie adjacent to each other in the genome of mammals and both genes are homologous, found to be located on X-chromosome of many livestock species (Astakhova et al., 2009). The polymorphism in TLR genes and their promoter regions has been associated with the disease resistance and susceptibility in different livestock

species and human. The variation in TLR8 gene in human has been linked to the higher susceptibility to asthma, tuberculosis, Systemic Lupus Erythematosus (Davila et al., 2008; Deng et al., 2010). The polymorphism within TLR8 is reported to play a crucial and important role in modulation of TLR8-dependent microbicidal response of infected macrophages (Grantier et al., 2010). A novel role of TLR8 as a suppressor of neurite outgrowth clearing its involvement in diverse physiological processes in mammals has also been demonstrated (Ma et al. 2007). Recently, TLR8 has also been implicated in metastasis of tumours in human beings activated through miRNA (Fabbri et al., 2012).

Many studies have demonstrated that the mRNA levels of different TLRs change after activation of immune response (Severa et al., 2007). These changes in TLR expression could have positive or negative consequences in regulation of immune response. The mechanisms responsible for the changes in TLR expression are poorly defined, and only few studies focused on the characterization of TLR transcriptional regulation have been carried out (Rehli et al., 2000; Takeshita et al., 2004). Moreover, very little information is available on the 5'-upstream sequence of TLR8 and its transcription factor binding sites in livestock species. We report here the sequence characterization and prediction of transcription factor binding sites in the 5'-upstream region of TLR8 gene of goat, a species not well studied for immune response genes.

MATERIALS AND METHODS

Amplification of 5'-upstream of goat TLR8: Genomic DNA was extracted from the whole blood of Sirohi goat, collected from breeding tract, by using standard SDS-ProteinaseK method (Sambrook and Russel, 2001). Quality and quantity of the DNA extracted was assessed by Nanodrop 1000 (Thermo Scientific, USA) as well as 0.8% agarose gel electrophoresis before further use. For the characterization of the 5'-upstream region of goat TLR8 gene, three sets of overlapping primers were designed (Table-1) from cattle sequence already available in Ensemble genome browser under the ID ENSBTAT00000029896, by using PrimerSelect programme of Lasergene software (DNASTAR Inc., USA).

Polymerase chain reaction (PCR) was performed in a total volume of 20 µl containing 50-100 ng of genomic DNA, 0.5 µl of 10 pmol each forward and reverse primers, 0.5 µl of 10 mM dNTPs, 10X PCR buffer with 15mM MgCl2 and 1U of Taq DNA polymerase (Bangalore Genei, India). The products were amplified using cycling conditions with initial denaturation at 95°C for 2.5 min followed by 32 cycles of 94°C for 30 sec, primers with respective annealing temperature (Table 1) for 30 sec and 72°C for 1 min and final extension at 72°C for 10 min and specific bands were visualised by running in ethidium bromide stained 1.5% agarose gel. The PCR products generated were sequenced from both ends after purification. At least two independent PCR products were sequenced for confirmation.

Table 1. List of the primers designed and used for the 5'UTR amplification of goat TLR8 gene

Primer	Sequence	Annealing Temp. (°C)	Size (bp)
TLR8 Prom P1	F: 5'-GCCAGCGTTTCCTTGAGTTATG -3'		
	R: 5'-AGAGATGAGCAGGAAAAGGGATGT -3'	54.6	442
TLR8 Prom P2	F: 5'-TGGTCCTCAGGCAAGATACATCC -3'		
	R: 5'-AACATGGAAAGCTTTGGCAGAA -3'	55.2	803
TLR8 Prom P3	F:5'- TGTTAAGGAAGAATCGCAGAAAG -3'		
	R:5'- TCAAGGAGGAAGAAAGGACAAAT -3'	52.7	896

Sequence analysis : Nucleotide sequence of PCR products, representing 5'-upstream region of goat TLR8 gene in overlapping fragments assembled into 1.01 kb contig and has been submitted to NCBI GenBank (Acc. No. JQ911705). The nucleotide sequence was further analyzed by alignment with TLR8 of other mammalian species, available in Ensemble, using MegAlign programme of Lasergene to draw a phylogenetic tree. The transcription factor binding sites in the 5' upstream region of goat and cattle were defined by Alibaba2.1

(http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and Patch (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) program and were compared to confirm the location of the putative transcription factors binding sites.

RESULTS AND DISCUSSION

Goat rearing is the backbone of the economy of small and landless farmers in India. India being a country having the second-largest goat population in the world, it is an insurance against crop failure and provides alternate source of livelihood to the farmers all year round. Also the goat farming is being considered as the best choice for the rural people in developing countries because of the low investment, wide adaptability, high fertility and fecundity, low feed and management needs, high feed conversion efficiency. The productivity of Indian goat is low, yet considering the nutritional and physical environmental conditions under which they are reared, it cannot be considered inefficient. Peste des petits ruminants (PPR), goatpox, FMD and enterotoxaemia are the major viral and bacterial diseases, which are the main causes for low productivity in Indian goats.

Expression profiling and full gene characterization of TLRs has been studied in goat by earlier workers (Raja et al., 2011), but still analysis of upstream sequence of TLRs and the transcription factors binding sites have not been carried out in goat. So to characterize immediate upstream region of the TLR8 gene in goat comprising a total stretch of 1016 nucleotide sequence was amplified and analyzed. When compared with other livestock species, the nucleotide sequence of goat TLR8, showed a maximum homology of 88% with cattle and with rest of the species relatively low levels of homology was seen, as expected (Results not shown). When the sequence based phylogenetic analysis was carried out, goat shared the closest place with cattle, as compared to other species (Fig. 1). The same results of phylogeny has been reported in the full gene characterization of TLR8 in buffalo by Dubey et al. (2012), there also buffalo TLR8 shared the closest place to goat then with cattle and other species.

When the goat TLR8, 5'-upstream region was compared with that of cattle it showed the maximum variability in the region start codon to 808 nucleotides upstream. Transcription factor binding sites analysis in goat TLR8 revealed presence of total 70 potential sites, located at different sites whereas, in cattle a total of 76 putative binding sites were found (Fig. 2). Important factors like PU-1, which is an essential binding factor that regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors (DeKoter et al.,1998), was found to be present in 5'UTR sequence of goat TLR8 as compared to cattle where it was found to be absent. The PU-1 binding factor has been associated with many important functions in human, such as PU-1-dependent transcriptional program determining the pulmonary response to LPS (Berclaz et al., 2007); the induction of PU-1/PIP binding to the TLR4 promoter is found to be involved in endotoxin response in-vivo and may mediate transcriptional changes in TLR4 gene expression (Tetyana et al., 2005). Also PU-1 alongwith interferon consensus sequence-binding protein has been reported to be involved in regulation of the

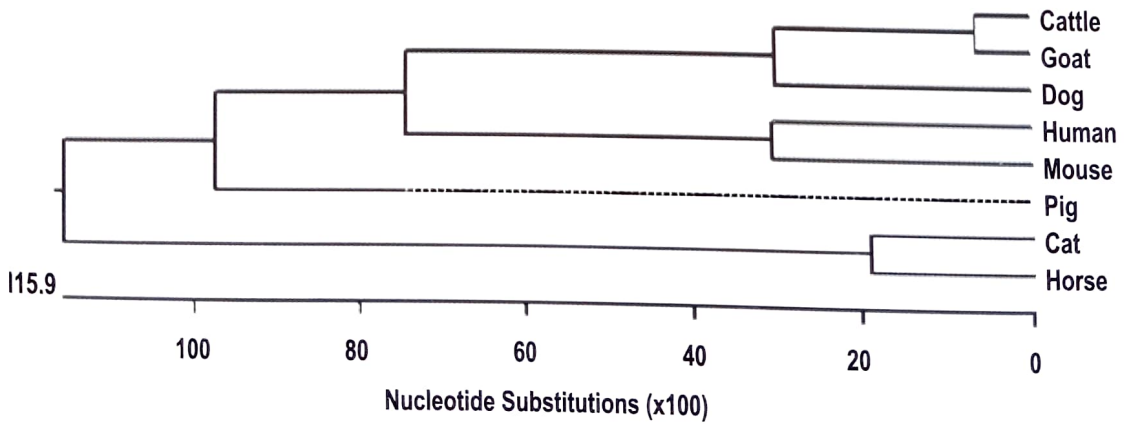

Figure 1. Nucleotide sequence based phylogenetic analysis of 5'-upstream of TLR8 gene comparing goat with other species.

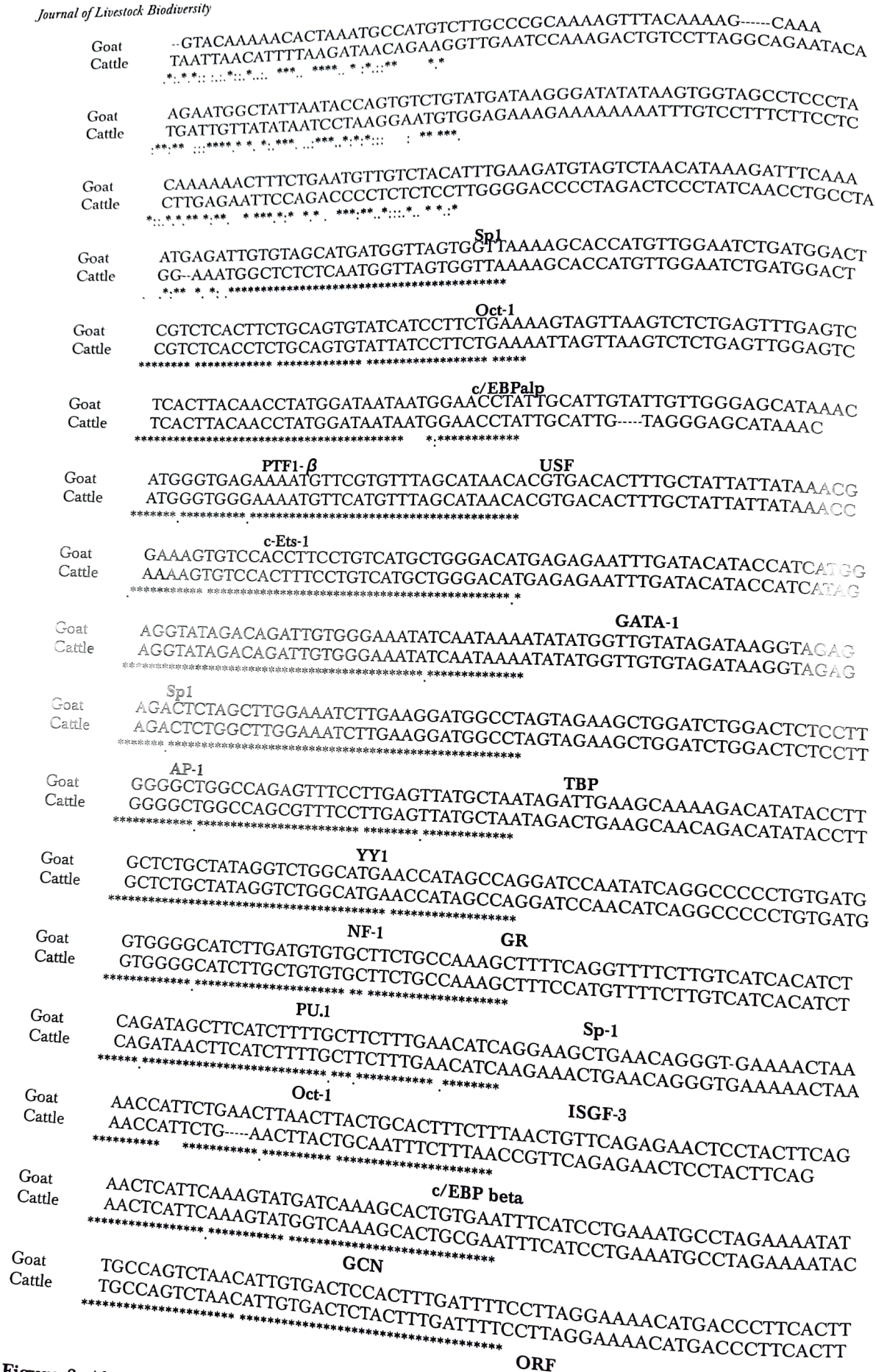

Figure 2. Alignment of 5-upstream sequence showing variable putative transcription factor binding sites in goat TLR8 as compared to cattle.

myeloid expression of the human toll-like receptor 4 gene (Rehli et al., 2000). Presence of this important factor binding site in goat TLR8 5' upstream sequence may also have implication in gene regulation due to the transcriptional changes in it.

Another important factor C/EBP which has been classified in six different classes and is said to be involved in different cellular responses, such as control of cellular proliferation, growth and differentiation, metabolism, and in immunity was found to present in both goat and cattle in different forms in TLR8 sequence. The role of these C/EBPs in the regulation of transcription in human has been studied by different workers (Zannetti et al., 2010). Another major factor GATA-1, which is important in erythroid development (Xavier et al., 2011) was found to be present in goat and cattle both. Its function and critical role in the regulation of transcription has been studied in mouse by Roger et al., (2005). Another important binding factor ETS, implicated in the development of different tissues as well as cancer progression was found to be present in goat but not in cattle. Activator Protein-1 (AP-1) was found to be present in both goat and cattle TLR8 sequence. The role of these two important binding factors has been previously studied in regulation of mouse TLR4 gene expression by Roger et al. (2005).

Further comparison of TLR8 5'-upstream sequence of goat with cattle, showed the insertion of 5 nucleotides at two different positions. The insertion at position 149 upstream to open reading frame was found to have an extra important binding factor Oct-1 site which was found to be missing in cattle. Oct-1 is said to be critically involved in self-renewal of undifferentiated embryonic stem cells and its regulation of expression will cause differentiation of the cells (Niwa et al., 2000). Also its role in TLR2 gene regulation has been previously studied by Frantz et al. (2001). Another insertion which was found at nucleotide 657 upstream to coding region in goat did not show any extra binding factor site.

These results thus indicate wide variation in the TLR8 5'-upstream region of goat and these variations could affect the different transcription factors binding sites, influencing the expression of this important gene.

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