Identifying Bovine Receptors for Interleukin-8: A Key Component of Inflammatory Response

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Abstract

Mastitis most commonly results from bacterial infection that causes mammary gland inflammation. In response to invading bacteria, the cytokine interleukin-8 (IL-8) is released, producing a gradient to the site of infection. Neutrophils follow this gradient and migrate to the infection where they engulf and kill invading bacteria. In most species, neutrophils recognize IL-8 through two receptors, CXCR1 (IL8-R α) and CXCR2 (IL8-R β). CXCR1 and CXCR2 are receptors for CXC chemokines, characterized by cysteines separated by an amino acid (X). The receptors bind chemokines to activate leukocyte adhesion and chemotaxis. Thus far, only the CXCR2 mRNA has been identified in the bovine model. The objective of this study was to identify the presence of both IL-8 receptors by first determining the 5' ends of mRNA using the rapid amplification of cDNA ends (RACE) procedure. The mRNA was reverse transcribed to make a DNA copy (cDNA), amplified using a primer theoretically common to both receptors, and the products sequenced. We identified two distinct 5' ends of the mRNA that matched two separate regions of bovine chromosome 2 with 100% homology using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat). The cDNA sequences were translated into protein sequences and the similarity between the two clones was evaluated using BLAST (http://www.ca.expasy.org/cgi-bin/blast). The translated amino acid sequence of clone 3 and clone 4 matched each other with an

identity score of 80%. Each clone then was compared to the published bovine CXCR2 sequence and IL-8 receptor sequences of different species. Clone 3 shared 98% identity with the published bovine CXCR2 sequence and 76-80% identity with CXCR1 of other species. This indicates that clone 3 is specific for the published bovine sequence and these sequences are closely related to CXCR1 in other species, suggesting the published sequence may be misannotated. In contrast, clone 4 shared only about 85% identity with the published bovine CXCR2 sequence and 76-77% identity with CXCR2 in other species. This suggests that Clone 4 is related but not identical to the published bovine CXCR2 sequence. However, it shares greater homology with CXCR2 in other species, indicating this may be the "true" bovine CXCR2. Future research includes cloning the full-length receptors and evaluating subsequent protein function following introduction of natural mutations that occur in the bovine population. By understanding the functional relationship of both IL-8 receptors in dairy cattle, preventive treatments can be used to target pathways that enhance mastitis resistance.

Introduction

Mastitis is the most devastating disease to the dairy industry, costing the United States industry $2 billion dollars annually and compromising safety of the milk supply (National Mastitis Council, 1996). The disease is most commonly caused by bacteria frequently found in the environment of dairy cows. Bacteria enter the mammary gland through the teat, potentially resulting in an inflamed mammary gland. If a bacterial infection develops, milk is discarded due to antibiotic therapy and an increase in the number of white blood cells in the milk (known in the industry as somatic cell count).

In response to bacterial invasion, endothelial and epithelial cells release the chemokine interleukin-8 (IL-8). IL-8 mediates neutrophil function, allowing neutrophils to resolve bacterial infections by migrating through blood vessel walls and to the site of infection (Kehrli and Harp, 2001). IL-8 also impacts neutrophil killing and survival ability during the inflammatory response (Chertov et al., 2000 and Glynn et al., 2002). Many species are known to have two neutrophil receptors for IL-8, CXCR1 and CXCR2, otherwise known as IL8-Ra and IL8-Rb (Holmes et al., 2001). Both receptors are important for controlling neutrophil activation by chemokines; however, they differ at the 5' end, which corresponds with the N' terminus (Catusse et al., 2002). CXCR1 has been shown to bind IL-8 with high affinity, while CXCR2 binds not only IL-8 both other related chemokines such as epithelial-cell-derived neutrophil attractant-78 and growth-related oncogene α (Wuyts et al., 1998). Because the receptors have the ability to bind the same chemokines, they act as backups for each other during the inflammatory response.

Cummings and co-workers (1999) studied IL-8 receptor function in human septic patients. They found CXCR1 and CXCR2 were expressed equally in unstimulated neutrophils. However, upon neutrophil stimulation, CXCR1 was re-expressed within minutes, while CXCR2 was re-expressed much slower. By blocking CXCR1, migration of neutrophils to the infection site was suppressed and susceptibility to bacterial infection increased. Furthermore, neutrophils maintained CXCR1 expression while down-regulating the expression of CXCR2 by 50%. Although IL-8 receptors are closely related and jointly activate neutrophils, they play different roles in fighting off infection. This human model of CXCR1 and CXCR2 function during the inflammatory response can also be applied to the regulation of disease in different species.

In the bovine model, only one IL-8 receptor (CXCR2) has thus far been identified at the gene level. Identifying whether both receptors are expressed is critical because they act together to help direct the inflammatory response. We hypothesized both CXCR1 and CXCR2 genes are present in bovine. The objective of this study was to identify the presence of both IL-8 receptors in dairy cows by determining the 5' ends of messenger RNA (mRNA) using the rapid amplification of cDNA ends (RACE) procedure.

Materials and Methods

CXCR1 and CXCR2 5' mRNA ends were identified using the manufacturer's protocol for the GeneRacer kit (Invitrogen, Carlsbad, California). Briefly, total bovine lung RNA (5 μg) was dephosphorylated using calf intestinal phosphatase to eliminate non-mRNA and mRNA that was not full length. The dephosphorylated RNA was treated with tobacco acid pyrophosphatase to remove the 5' cap from full-length mRNA, and the remaining 5' phosphate was then ligated to the GeneRacer RNA Oligo using RNA ligase. Ligated mRNA was reverse transcribed using the GeneRacer Oligo dT Primer, creating a single strand of complementary DNA. This cDNA strand was amplified by polymerase chain reaction (PCR) using a reverse primer theoretically common to both receptors (5'-CAGGAAGACGAGCACGACAGCAA-3') and the GeneRacer 5' Primer. The PCR products were analyzed on an agarose gel and bands were cut from the gel. The bands were purified and the PCR products cloned into a pCR4-TOPO vector (Invitrogen, Carlsbad, California). Transformed vectors were grown on Lutria Broth plates containing ampicillin (50 μg/ml), colonies were selected, and plasmid DNA isolated and

purified. The DNA was sequenced using M13 forward and reverse primers (Invitrogen, Carlsbad, California) by the University of Tennessee Molecular Biology Core Facility. Sequences were analyzed using publicly available programs BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat), ClustalW (http://www.ebi.ac.uk/clustalw/), BLAST (http://www.ca.expasy.org/cgi-bin/blast) and the DNA to protein translate tool featured at ExPASy (http://ca.expasy.org/). The cDNA sequences were translated into protein sequences, and clones were compared to each other and published sequences of bovine, orangutan, human, and rabbit IL-8 receptors. Percent homology and identity scores were used to evaluate homology of RACE products to published sequences.

Results and Discussion

From 5 colonies, we identified two unique clones of mRNA (clone 3 and clone 4) on opposite strands of bovine chromosome 2. Using BLAT, the nucleotide sequences for clone 3 and 4 were aligned with bovine chromosome 2 (Figure 1). The clones matched two distinct regions of bovine chromosome 2 with 100% homology. The insert for clone 3 was 888 base pairs (bp), and spanned the chromosome in a negative direction over 3,007 nucleotides. The clone sequence covers a large chromosome length because of an intron in the middle of exon 1 and exon 2. This intron, or non-coding segment, is spliced from the RNA before it is transported to the cytoplasm during transcription. This splicing unites exon regions that are later translated into protein. Clone 4 was oriented in a positive direction with an insert length of 836 bp, matching a 787 nucleotide region on chromosome 2. We were unable to compare the clone to the entire chromosome because there is a sequence gap in the assembly of bovine genome at about 64,520,000.

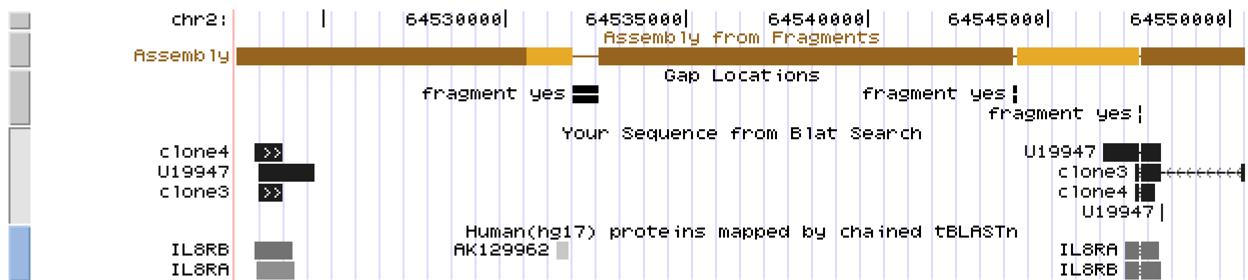

Figure 1. Alignment of cloned sequences to bovine chromosome 2. Clones 3 and 4 are closely positioned to regions of published human IL-8 receptors, as well as the published bovine receptor (accession number U19947).

Because amino acids are more conserved than the nucleotide sequence across species, we translated the nucleotide sequences to amino acid sequences using the translate tool at ExPASy. ClustalW was used to align the translated amino acid clone sequences to each other and the published bovine IL-8 receptor (Genbank accession number U19947). Figure 2 indicates clone 3 and the published bovine IL-8 receptor matched each other with 98% homology, whereas clone 4 was more varied from the bovine sequence with a homology of just 85%. Clone 3 and 4 were 81% homologous, verifying similar sequence structure of the receptors.

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Clone3          MTIILKDLSNSSILWEGFEDE-FGNYSG--TPPTEDYDYSPCEISTETLNKYAVVVIYAL 57
BoIL8R|U19947   MTIILKDLSNSSILWEGFEDE-FGNYSG--TPPTEDYDYSPCEISTETLNKYAVVVIDAL 57
CLONE4          MAETKFTSNIEGFNWENYSDEDFGNYSYNTDLPSILTDSAPCRPEILDINKHAVVVIYAL 60
                *:      . ..: **.:.** *****     *:   * :**. .   :**:***** **

Clone3          VFLLSLLGNSLVMLVILYSRIGRSVTDVYLLNLAMADLLFAMTLPIWAASKAKGWIFGTP 117
BoIL8R|U19947   VFLLSLLGNSLVMLVILYSRIGRSVTDVYLLNLAMADLLFAMTLPIWTASKAKGWVFGTP 117
CLONE4          VFLLSLLGNSLVMLVILYSRIGRSVTDVYLLNLAMADLLFAMTLPIWAASKAKGWVFGTP 120
                ***********************************************:*******:****

Clone3          LCKVVSLLKEVNFYSGILLLACISMDRYLAIVHATRTLTQKRHWVKFICLGIWALSVILA 177
BoIL8R|U19947   LCKVVSLLKEVNFYSGILLLACISMDRYLAIVHATRTLTQKWHWVKFICLGIWALSVILA 177
CLONE4          LCKVVSLLKEVNFYSGILLLACISMDRYLAIVHATRTLTQKRHWVKFICLGIWALSVILA 180
                ***************************************** ******************

Clone3          LPIFIFREAYQPPYSDLVCYEDLGANTTKWRMIMRVLPQTFGFLPPLLVMLFCYGFTLRT 237
BoIL8R|U19947   LPIFIFREAYQPPYSDLVCYEDLGANTTKWRMIMRVLPQTFGFLLPLLVMLFCYGFTLRT 237
CLONE4          LPVFIFRRAIHPPYSSAVCYEDMGANTTKWRMVMRVLPQTFGFLLPLLVMLFCYGFTLRT 240
                **:****.* :****. *****:*********:*********** ***************

Clone3          LFSAQMGHKHRAMRVIFAVVLVF------------------------------------- 260
BoIL8R|U19947   LFSAQMGHKHRAMRVIFAVVLVFLLCWLPYNLVLIADTLMRAHVIAETCQRRNDIGRALD 297
CLONE4          LFSAQMGQKHRAMRVIFAVVLVF------------------------------------- 263
                *******:***************
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Figure 2. ClustalW multiple sequence alignment of clones to bovine CXCR2 (U19947). Clone 4 differs from U19947, as seen from consensus symbols. "*" means nucleotides in the column are identical, ":" means substitutions are conserved, and "." means that semi-conserved substitutions are observed.

In order to evaluate nucleotide similarity between bovine receptors and human IL-8 receptors, we compared the protein sequences of the clones to published human sequences using BLAST. Clone 3 was more closely related to human CXCR1 (identity score of 76%), while clone 4 matched human CXCR1 and CXCR2 both with 75% homology (Table 1).

	Clone 3	**Clone 4**	**Bo IL8-R**	**Hu CXCR1**	**Hu CXCR2**
Clone 3	100	81	98	76	73
Clone 4		100	85	75	75
Bo IL8-R			100	74	75
Hu CXCR1				100	78
Hu CXCR2					100

Table 1. Comparison of clones to published bovine and human sequences using BLAST. Clone 3 is 98% homologous with the bovine IL8-R and most similar to human IL8-R CXCR1.

The clones also were compared to IL-8 receptor protein sequences of gorilla (Gorilla gorilla), orangutan (Pongo pygmaeus), rabbit (Oryctolagus cuniculus), and house mouse (Mus musculus) Clone 3 was more similar to CXCR1 receptors of all inquired species with an identity score range of 67-73%. Clone 4 showed more likeness to

CXCR2 receptors (identity range of 72-74%). For both clones, comparison with the house mouse CXCR1 and CXCR2 generated lower identity scores. Clone 3 matched mouse CXCR2 with an identity score of 65%, while clone 4 showed similarity to mouse CXCR1 at 61% identity. The low amino acid sequences identity scores between bovine and mouse comparison are likely due to a large variation in genome due to excess inbreeding; this difference should be considered when interchanging mouse studies with human, as the bovine genome would be a more closely related model. These findings are additional evidence that clones 3 and 4 are homologous to CXCR1 and CXCR2, respectively, regardless of the species origin.

Because of the position of these clones on chromosome 2 and their identity scores with published human and bovine IL-8 receptors, we found clone 4 to be more like CXCR2 and clone more closely related to CXCR1 and the published bovine receptor. Interestingly, although clone 3 is homologous with CXCR1, the published bovine receptor is labeled as CXCR2, indicating that the receptor most likely has been misannotated.

In conclusion, the identification of the 5’ sequences of clones 3 and 4 suggest that both IL-8 receptors are present in bovine. Future work will identify full-length sequences of these genes so more in depth functional studies can be conducted. By understanding the functional relationship of both IL-8 receptors in dairy cattle, preventative treatments can be developed to target pathways that enhance mastitis resistance and therapy. Controlling the severity and frequency of mastitis is socially significant, resulting in healthier cows and higher quality dairy products that increase revenue and food safety.

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