Polymorphisms in the 5' Upstream Region of the *CXCR1* Chemokine Receptor Gene, and Their Association with Somatic Cell Score in Holstein Cattle in Canada

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ABSTRACT

Identification of regulatory elements in 5' regions of chemokine genes is fundamental for understanding chemokine gene expression in response to infection diseases. The *CXCR1* receptor is expressed on the surface of neutrophils and interacts primarily with CXCL8 (IL-8), the most potent chemoattractant for neutrophils. The aim of this study was to characterize the 5' upstream region (2.1 kb) of the bovine *CXCR1* chemokine receptor gene for polymorphism content and to identify in silico potential transcription-factor binding sites. The 5' flanking region was found by mining the NCBI GenBank (www.ncbi.nlm.nih.gov/). A DNA sequence from the whole genome shotgun sequence project with reference number AC150887.4 contained the *CXCR1* 5' flanking sequence. Computer analysis revealed potential binding sites for the transcription factors nuclear factor κ B (NF- κ B), binding factor GATA-1, barbiturate inducible element (Barbie), nuclear factor of activated T-cells, and activator protein 1. Polymorphism discovery in this region was conducted by constructing an inclusive DNA pool including 2 phenotypic extreme groups, 20 bulls with high estimated breeding values (EBV) for somatic cell score (SCS), and 20 bulls with low EBV for SCS. Independent amplicons along the 5' flanking region of bovine *CXCR1* were generated for polymorphism discovery by sequencing. Three novel single nucleotide polymorphisms (SNP), *CXCR1c.*-344T>C, *CXCR1c.*-1768T>A, and *CXCR1c.*-1830A>G, and a previously identified SNP in the coding region, *CXCR1c.*777G>C, were identified. The 4 SNP were genotyped in Canadian Holstein bulls (n = 338) using tetra-primer amplification refractory mutation system (ARMS)-PCR. Average allele substitution effects were estimated to investigate associations between the 4 SNP and EBV for SCS in first, second, and third and

later lactations. Multiple trait analysis revealed that the SNP *CXCR1c.*-1768T>A was associated with EBV for SCS in the first and second lactations and over all 3 lactations. Haplotype analysis substantiated this association with EBV for SCS in the first lactation. Given the location of SNP *CXCR1c.*-1768T>A and the surrounding potential binding recognition sequences for NF- κ B, GATA-1, and Barbie transcription-factors, this SNP may be implicated in gene regulation and warrants further research.

Key words: chemokine receptor, haplotype, single nucleotide polymorphism, somatic cell score

INTRODUCTION

Mastitis continues to be one of the most frequently occurring bovine diseases, causing significant monetary losses to the dairy industry worldwide (Nash et al., 2003). Many approaches to control this complex disease have been proposed, including improving management strategies, using dry-cow antibiotic therapy and vaccines, monitoring clinical mastitis, and, recently, the creation of transgenic cattle resistant to *Staphylococcus aureus* infections through the expression of lysostaphin in the mammary gland (Wall et al., 2005). Another widely used strategy has been to record SCS, obtain EBV, and select for decreased SCS. This strategy is effective because of the positive genetic correlation between clinical mastitis and SCS ($r = 0.7$; Rupp and Boichard, 1999). A study by Rupp et al. (2006), for example, determined that intramammary infections occurred less frequently in sheep with low SCS after one generation of breeding for this phenotype. The Canadian dairy industry currently selects against bulls with EBV for increased SCS (Mark et al., 2002).

The somatic cells in milk are primarily macrophages, neutrophils, and lymphocytes. These leukocytes play an important role in host resistance to IMI. Macrophages are the dominant cell type in cisternal milk from a healthy lactating mammary gland (Prgomet et al., 2005). Neutrophils, on the other hand, are the dominant cell type in alveolar milk, and during clinical mastitis

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they may represent more than 90% of the total mammary leukocyte population (Sordillo and Streicher, 2002). Although neutrophils are important host effector cells during the initial onset of IMI, their rapid elimination by macrophages is essential because prolonged exposure of the mammary epithelium to the oxygen radicals and proteases released by these cells can lead to tissue damage, permanently affecting milk production and quality (Bannerman et al., 2003).

The CXCR1 receptor is expressed on the surface of neutrophils (Proudfoot, 2002) and interacts primarily with CXCL8 (IL-8), the most potent chemoattractant for neutrophils. The *CXCR1* gene has been mapped to *Bos taurus* autosome 2 at 90.3 cM and is part of the gene family encoding the serpentine seven transmembrane-domain G-protein coupled receptors (Murdoch and Finn, 2000). The annotation of *CXCR1* was recently corrected by Pighetti and Rambeaud (2006). These authors reported that the sequence for *CXCR2* with GenBank reference number NM_174360.2 actually corresponds to *CXCR1*. Although this correction had no impact on the current study, it indicates that previous association and functional studies performed by Youngerman et al. (2004) and Rambeaud and Pighetti (2005) investigated polymorphisms in *CXCR1* rather than *CXCR2*.

The CXCR1 is a promiscuous chemokine receptor that interacts with additional chemokines including CXCL6 (granulocyte activating protein-2), and CXCL7 (neutrophil activating peptide-2) (Mantovani et al., 2006).

The activity of CXCR1 receptor is strongly associated with the inflammatory response to gram-negative bacteria infections, and consequently CXCR1 is a key player activating the innate immune response (Oviedo-Boyso et al., 2006; Rainard and Riollot, 2006). Bacterial membrane components such as LPS induce the expression of CXCR1 via interaction with the toll-like receptor 4 (TLR-4) receptor complex. This subsequently leads to the induction of the transcription factor NF- κ B that translocates into the nucleus, binding to its DNA response element in the 5' regulatory region of the *CXCR1* gene. Receptor-ligand interaction between CXCR1 and IL-8 induces conformational changes in neutrophils that permit their chemotaxis to the site of infection where they release their antimicrobial components (Olson and Ley, 2002).

Variation in the inflammatory and immune response to pathogens is mediated, in part, by variation in the DNA sequence of immune-related genes (Bidwell et al., 1999; Shelley and Hill, 2003). Inflammatory and immune responses are polygenic traits by nature, and numerous studies have demonstrated statistical and functional associations between inflammatory diseases

Table 1. Descriptive statistics of the EBV of the Holstein bulls sampled for the association study for SCS at first (SCS1), second (SCS2), and third or later (SCS3) lactations

Statistic	EBV (in units of score)			Accuracy ¹
	SCS1	SCS2	SCS3	
n	309	309	309	309
Mean	3.01	3.00	3.02	0.94
SD	0.30	0.36	0.40	0.04
Maximum	4.40	4.15	4.10	0.99
Minimum	3.39	3.23	3.09	0.73

¹Average accuracy of EBV across lactations.

such as mastitis and polymorphisms in various immune-related genes (Youngerman et al., 2004; Sharma et al., 2006; Leyva et al., 2007). A single nucleotide polymorphism (SNP) located in the *CXCR1* gene at position +777, for example, is reported to be associated with subclinical mastitis, SCS, milk yield, and neutrophil function (Youngerman et al., 2004; Rambeaud and Pighetti, 2005). This SNP is within a region that encodes the third intracellular loop of the CXCR1 receptor and is important for G-protein coupling neutrophil activation that leads to cell conformational changes that favor chemotaxis.

Little is known about the regulatory regions of chemokine genes. Thus, in the present study we investigated the presence of SNP in the 5' upstream region of the bovine *CXCR1* chemokine receptor gene and identified potential transcription-factor responsive elements in silico. In addition to this, associations between these SNP and EBV for SCS for 3 different lactations were investigated. We hypothesized that polymorphisms in this gene may contribute to variation in EBV for SCS.

MATERIALS AND METHODS

Animals and Traits

Semen samples donated by The Semex Alliance (Guelph, Ontario, Canada) were used as the source of DNA to conduct this experiment. Animal selection was based on extreme EBV for protein yield and SCS. Three hundred thirty eight bulls were selected from a collection of 2,166 semen samples from Canadian Holstein bulls. Most of the selected bulls came from half-sib families, whose sizes ranged from 2 to 30 offspring. The EBV for SCS for the first (SCS1), second (SCS2), and third or later (SCS3) lactations were used as trait phenotypes for each of the bulls. The EBV were obtained from the Canadian Dairy Network (Guelph, Canada) database relative to the national genetic evaluation published in May 2006. Bull EBV for SCS are calculated using the Canadian test-day model and each bull receives a separate proof for first, second, and third (including later) lactations. Table 1 presents simple de-

Table 2. Sequencing primers for the 5' upstream region of the bovine chemokine receptor gene (*CXCR1*)

Gene scanned	Primer sequences (5'-3')	Annealing temperature, °C	Amplicon size, bp
<i>CXCR1c.777G>C</i>	Forward: CATCAGCATGGACCGCTAC Reverse: TGGATGCCACCGAGATCCTG	60	490
<i>CXCR1-1</i>	Forward: TGCAGAAAGTCAAAGTCAGCA Reverse: ATAAGATGACCAGCATCACCAG	60	549
<i>CXCR1-2</i>	Forward: CCAGTGGTTAGGATTTCAGCA Reverse: AGGCTGCTTTAAGGATCAGC	59	578
<i>CXCR1-3</i>	Forward: CCACCCCTCCTCAGCAGAA Reverse: GCTCTCAGATGCCCTTTC	60	574
<i>CXCR1-4</i>	Forward: AAAAGGTGTGGGTTTGAATGA Reverse: CCCACAGAATTGTTTCAGCAC	60	524
<i>CXCR1-5</i>	Forward: TCACAGGCTCTAGGCACGTA Reverse: TCATTCAAACCCACACCTTTT	60	337

scriptive statistics of the EBV of the 338 bulls used in this study.

DNA Extraction

Frozen semen samples from 338 bulls were used to obtain high-quality DNA, using the classical phenol/chloroform extraction protocol described by Winfrey et al. (1997). The quantity and quality of DNA were measured with spectrophotometry at 260/280 nm using an Eppendorf BioPhotometer (Berlin, Germany).

Determination and Analysis of the 5'-Upstream Sequence from the *CXCR1* Coding Region

Initial in silico determination of the 5'-upstream region from the *CXCR1* coding gene was implemented by using the public database from the *Bos taurus* (ORGN) nucleotide-nucleotide BLAST (BLASTn) search tool at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/BLAST/). The bovine *CXCR1* mRNA (GenBank accession number U19947) was used as an input sequence to perform the BLASTn search. A 157,543-bp whole shotgun sequence (GenBank accession number AC150887.4) was found harboring the bovine *CXCR1* gene and its flanking sequences. Subsequent alignment of the bovine *CXCR1* mRNA sequence with the retrieved genomic sequence AC150887.4 was pursued to identify the boundary between the 5' flanking sequence and the coding region of the *CXCR1* gene. Further verification of the 5' flanking sequence from the bovine *CXCR1* gene was performed by PCR and sequencing. Primer design was conducted using Primer3 software to generate overlapping PCR amplicons for the 2.1 kb of the retrieved 5' upstream region of the bovine *CXCR1*. The primers are shown in Table 2. The PCR reactions were carried out in a T-Gradient thermocycler (Biometra, Montral Biotech Inc., Kirkland, Canada) in a final volume of 50 µL con-

taining 50 ng of pooled template, 10 pmol of each primer, 10 mM of each dNTP, 1.5 mM MgCl₂, 1× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), and 1 U of *Taq* polymerase (Invitrogen, Life Technologies, Carlsbad, CA) with the following conditions: 94°C for 3 min, followed by 35 cycles of denaturation 94°C for 30 s, annealing temperature for 30 s (see Table 2 for specific annealing temperatures), extension 72°C for 30 s, and final extension of 5 min at 72°C. The amplicon sizes were confirmed using 1.5% agarose gels stained with ethidium bromide.

The region of the bovine *CXCR1* 5' shown in Figure 1 was confirmed by direct sequencing of the PCR amplicons in forward and reverse directions using an ABI Prism System Version 3.4.1. (Applied Biosystems, Foster City, CA). The 2.1 kb of the 5' upstream region of the bovine *CXCR1* gene was then analyzed in silico for potential DNA binding sites with the public software MOTIF Search (<http://motif.genome.jp/>). A threshold of 90% similarity was implemented for the query.

DNA Pooling and SNP Detection

Estimated breeding values for SCS were used to select 20 high and 20 low Canadian Holstein bulls to maximize the variance for this trait for the construction of an inclusive DNA pool for SNP detection. A 2-step DNA quantification approach was implemented to generate equal concentrations of 40 DNA samples. In the first step, quantification was performed by spectrophotometry at 260 nm using an Eppendorf BioPhotometer. Samples were then diluted to a concentration of 25 ng/µL. In the second step, double-stranded DNA was quantified using the PicoGreen protocol (Molecular Probes, Invitrogen) and a Victor 3 fluorescent plate reader (Perkin Elmer, Wellesley, MA). The DNA samples were adjusted to a final concentration of 5 ± 0.5 ng/µL. Equal amounts of the 40 DNA samples were then pooled into a single tube. The pooled samples were

Figure 1. Nucleotide sequence of the 5' upstream region (hg18_refGene_NM_001557.2) of the bovine chemokine receptor gene *CXCR1*. The 5' flanking region is indicated in uppercase letters, and position +1 (start codon) is indicated in lowercase letters. Putative binding sites are shown in gray: NF-κB = nuclear factor kappa B; GATA-1 = binding factor GATA-1; Barbie = barbiturate inducible element; NF-AT = nuclear factor of activated T-cells; GATA-1 = nuclear factor GATA-1; and AP-1 = activator protein 1. Single nucleotide polymorphisms *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* are underlined and in bold; CG sites are underlined.

used as a template for PCR, sequencing, and SNP detection.

Single nucleotide polymorphism discovery was performed using primers from Table 2 and PCR conditions previously used to annotate the 5' upstream region of the bovine *CXCR1*. Sequencing reactions were performed in forward and reverse directions for each amplicon and polymorphism detection was performed by scrutinizing the forward and reverse chromatograms generated from the sequencer. Three SNPs were detected in the 5' upstream region of the *CXCR1* gene and a previously identified SNP was observed in the coding region. Figure 1 shows chromatograms with the SNPs *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C*.

SNP Genotyping

The 4 SNPs were genotyped in the 338 bulls using tetra-primer amplification refractory mutation system

(ARMS)-PCR protocol as described previously by Ye et al. (2001) and Rincon and Medrano (2003). The primer design was carried out with Cedar Genetics Software (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). These genotyping primers are shown in Table 3. The tetra-primer ARMS-PCR reactions were carried out in a final volume of 25 µL containing 50 ng of template, 0.2 pmol of each outer primer, 2.0 pmol of each inner primer, 0.2 mM of each dNTP, 1.5 mM of MgCl₂, 1× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), and 1 U of *Taq* polymerase (Invitrogen). Touch-down thermocycling conditions were used for all of the SNPs as follows: 94°C for 3 min, followed by 35 cycles of denaturation 94°C 30 s, annealing temperatures were reduced 1°C per cycle from 72°C × 30 s until the appropriate annealing temperatures were reached (see Table 3 for appropriate annealing temperatures), extension at 72°C for 30 s, and final extension at 72°C for 5 min. These reactions were carried out in a T-

Table 3. Primer information for single nucleotide polymorphism (SNP) genotyping by tetra-primer amplification refractory mutation system-PCR in the bovine chemokine receptor gene (*CXCR1*)

Polymorphism analyzed	Primer sequence ¹ (5' to 3')	Annealing temperature, °C	Amplicon size, bp
<i>CXCR1c.-1830A>G</i>	Forward inner primer: CCACATCTCCCCTTTT ^T TAGGGACGA Reverse inner primer: ATAAACGTCAAATCAGAAAAAGTCATACC Forward outer primer: GAGAGTGGGGGCTACTCTCTAGTTGAG Reverse outer primer: ATCCAAATTGAGGGAGGCAGAGTAAATA	62	292 (G allele) 195 (A allele) 432 (from 2 outer primers)
<i>CXCR1c.-1768T>A</i>	Forward inner primer GCCCTGCGGCTTAGTCTTTTGTGAAGAT Reverse inner primer: CACCTTTTAATATCTCTGCCAGGACTACT Forward outer primer: CTTAGTAGCTGCAGCTTGCAGGCTCTAG Reverse outer primer: AGCTATGTGACCTTGAGTGGGTCCTTTT	62	280 (A allele) 187 (T allele) 410 (from 2 outer primers)
<i>CXCR1c.-344T>C</i>	Forward inner primer: CTCGGGGTTCTGGAAGCTGACAAAAT Reverse inner primer: CAGGCTGCTTTAAGGATCAGCTTCAT ^A AG Reverse outer primer: TGGCCCTTCCCTGAATGTATAGAACAAC Reverse outer primer: TCAGATCCAAATCACTCTCATCAAGGATG	62	240 (C allele) 160 (T allele) 344 (from 2 outer primers)
<i>CXCR1c.777G>C</i>	Forward inner primer: CGCACGCTATTTT ^T CAGCCCAAATGGGGGAC Reverse inner primer: AAAGATGACCCGCATGGCCCGGTGCAT ^C Forward outer primer: AGGCATCTGGGCCCTGTCCGTGATCCTG Reverse outer primer: GCCAATGTGCGGCGCTGACAGGTC	69	262 (G allele) 175 (C allele) 379 (from 2 outer primers)

¹The nucleotide underlined in each primer sequence represents the unmatched nucleotide to increase annealing specificity to the corresponding SNP.

Gradient thermocycler (Biometra). Genotypes were determined by resolving PCR amplicons on 1.5% agarose gels stained with ethidium bromide as shown in Figure 2.

Statistical Analysis

Average Allele Substitution Effects. Single nucleotide polymorphism average allele substitution effects were analyzed using the software ASREML (Gilmour et al., 2000) implementing the following model:

$$Y_{jk} = \mu + \sum_{i=1}^4 \beta_i G_i + S_j + e_{jk},$$

where y_{jk} = trait EBV for the j th bull from the k th sire, μ = overall mean, β_i = fixed regression coefficient for

the i th SNP, G_i = the genotype of the i th SNP recoded as in Zeng et al. (2005):

$$G_i = \begin{cases} -1 & \text{for Homozygous genotype 1} \\ 0 & \text{for Heterozygous genotype} \\ +1 & \text{for Homozygous genotype 2} \end{cases},$$

S_j = random polygenic effect of the j th sire ($j = 1$ to 28), and e_{jk} = random error.

The covariance among sire polygenic effects was modeled through the sires' numerator relationship matrix, which included 127 animals from a pedigree going back 2 generations.

Bulls' EBV for SCS in the first (SCS1), second (SCS2), and third (and later; SCS3) lactations were used in this association study as trait phenotypes.

Bulls' EBV for SCS were also analyzed in a multiple trait model, assuming an unstructured sire polygenic and residual covariance matrix among the 3 SCS traits (first, second, and third lactation SCS EBV).

Comparison-wise probability levels are shown for all comparisons carried out. In addition, an adjustment for multiple tests proposed by Cheverud (2001) was performed and comparisons significant at an experimental-wise level are also shown.

Cheverud (2001) proposed the following procedure to adjust for multiple correlated tests:

Step 1: Calculate the correlation matrix for the variables (e.g., SNP).

Figure 2. Agarose gel (1.5%) stained with ethidium bromide showing the tetra-primer amplification refractory mutation system (ARMS)-PCR amplicons for resolution of the 3 genotypes for single nucleotide polymorphism *CXCR1c.-1768T>A*. M represents the 50-bp DNA ladder.

Step 2: Estimate the effective number (**Me**) of independent tests from the eigen values of the correlation matrix as:

$$Me = 1 + (M - 1) \left(1 - \frac{V_\lambda}{M} \right)$$

$$V_\lambda = \sum_{i=1}^M (\lambda_i - 1)^2 / (M - 1)$$

where M is the number of tests (e.g., the number of SNP in the analysis for 1 trait phenotype) and λ_i ($i = 1, \dots, M$) are the eigen values.

Step 3: Adjust the test criteria as though there were Me independent tests with the Sidak (1967) correction:

$$\alpha_c = 1 - (1 - \alpha_e)^{1/Me}$$

where α_e is the experimental-wise significance level desired (5% in this investigation), and α_c is the adjusted comparison-wise significance level to be used.

This procedure was used to adjust the comparison-wise significance level for both the number of independent SNP and independent SCS EBV analyzed. An effective number of tests was obtained, using the steps 1 and 2 above, considering both the correlation matrix among the SNP (**Mem**) and the correlation matrix among SCS EBV (**Mes**). Then, the total number of independent tests (**Met**) was obtained multiplying Mem by Mes . Finally, Met was used into the Sidak (1967) correction to obtain the adjusted comparison-wise significance level. This procedure resulted in Met equal to 6 and an adjusted comparison-wise significance level equal to 0.008.

Haplotype Reconstruction. The HAPROB algorithm and software was implemented to reconstruct haplotype probabilities (Boettcher et al., 2004). This algorithm is designed for a half-sib population structure, for which no parental genotypes are required in the analysis. The software runs a first step using a Monte Carlo approach reconstructing conditional parental haplotype probabilities based on offspring genotypes and allelic frequencies. In the second step, conditional offspring haplotype probabilities were assigned based on parental probabilities. A minimum of 2 offspring per sire is required to reconstruct haplotype probabilities with the HAPROB software. Thirteen haplotypes were reconstructed in the Holstein bulls genotyped. Eight haplotypes that had low probabilities were pooled as a single haplotype designated Haplo 6. Haplotype effects were estimated by regressing EBV on haplotype probabilities using the software ASREML (Gilmour et al. 2000), implementing the following model:

$$Y_{jk} = \mu + \sum_{i=1}^6 \beta_i \text{Hap}_{ik} + S_j + e_{ijk}$$

where: Y_{jk} = trait EBV for the k th animal from the j th sire, μ = overall mean, β_i = fixed linear regression coefficient for the i th haplotype, Hap_{ik} = probability of the i th haplotype of the k th bull, S_j = random polygenic effect of the j th sire ($j = 1$ to 28), and e_{ijk} = random error.

Trait EBV and covariance among sire polygenic effects were as previously described for the average allele substitution effect analysis.

RESULTS

Determination and Analysis of the 5'-Upstream Sequence

A sequence of 157,543 bp (GenBank reference number AC150887) was retrieved by BLAST search using the mRNA from the bovine *CXCR1* gene as a query. Sequencing chromatograms of the 2.1-kb DNA fragment of the 5' upstream region from the bovine *CXCR1* were used to confirm the sequence and size of the correct fragment. Further alignment of the 2.1-kb fragment from the 5' upstream region of the bovine *CXCR1* was carried out using the corresponding mouse and human *CXCR1* sequences with GenBank numbers NM_009909 and NM_001557. This analysis revealed that the 5' upstream region of the bovine *CXCR1* was approximately 45% homologous with the corresponding genes in these species.

The 2.1-kb 5' upstream region of the bovine *CXCR1* was analyzed in silico for potential DNA binding sites with the open software MOTIF search. Potential nuclear factor binding sites were identified using 90% similarity as a threshold. The transcription-factor response elements that were identified included nuclear factor κ B (**NF- κ B**), binding factor GATA-1 (**GATA-1**), barbiturate inducible element (**Barbie**), nuclear factor of activated T-cells (**NF-AT**), and activator protein 1 (**AP-1**). Figure 1 depicts these transcription-factor binding sites.

SNP Assessment

Sequencing PCR amplicons from pooled DNA from animals carrying extreme EBV for SCS proved to be an effective detection system for SNP discovery. Three novel SNP were detected in the 5' upstream region of bovine *CXCR1*. A transition SNP (T/C) was identified at position -344n, a transversion SNP (T/A) at position -1768n, and a second transition SNP (A/G) at position -1830n. The 3 SNP were submitted to NCBI, and are

Table 4. Observed genotypic frequencies of the single nucleotide polymorphisms (SNP) *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C*

SNP	Genotype	Coded genotype	Frequency	Percentage
<i>CXCR1c.-1830A>G</i>	AA	-1	239	73.3
	GA	0	79	24.2
	GG	+1	8	2.5
	Total		326	
<i>CXCR1c.-1768T>A</i>	TT	-1	209	62.8
	AT	0	106	31.8
	AA	+1	18	5.4
	Total		333	
<i>CXCR1c.-344T>C</i>	TT	-1	20	6.1
	CT	0	142	43.6
	CC	+1	164	50.3
	Total		326	
<i>CXCR1c.777G>C</i>	GG	-1	62	18.6
	GC	0	183	54.8
	CC	+1	89	26.6
	Total		334	

located in the 126 build of dbSNP with the reference numbers dbSNP rs41255712 (*CXCR1c.-344T>C*), dbSNP rs41255711 (*CXCR1c.-1768T>A*), and dbSNP rs41255709 (*CXCR1c.-1830A>G*). The SNP *CXCR1c.-344T>C* is located 125 bp downstream from an AP-1 transcription-factor binding site and 68 bp upstream from a GATA-1 transcription-factor binding site. The SNP *CXCR1c.-1830A>G* is located 20 and 15 bp downstream from the NF- κ B and GATA-1 transcription-factor binding sites, respectively. The SNP *CXCR1c.-1768* is located 17 bp upstream of the Barbie transcription-factor binding site. Figure 1 depicts the SNP positions from the 5' *CXCR1* upstream region and *CXCR1* coding region.

Genotypic and Allelic Frequencies

Table 4 shows the genotype frequencies observed for 3 novel SNP detected in the 5' upstream region of the *CXCR1* gene and for the previously identified SNP *CXCR1c.777G>C* in the coding region. Genotype TT for *CXCR1c.-344T>C* was present at a low frequency

(6.1%) in the sampled bulls. The estimated frequency of the T allele was 28%. For the SNP *CXCR1c.-1768T>A*, genotype AA was at a low frequency (5.4%) and the frequency of allele A was 21%. Genotype GG for *CXCR1c.-1830A>G* had the lowest frequency in the sampled bulls (2.5%) with an estimated frequency of allele G of 15%. For *CXCR1c.777G>C*, the genotype frequencies of the homozygous genotypes were at a more intermediate value with an estimated frequency of the C allele of 54%. Previously reported genotypic frequencies in a US Holstein population by Youngerman et al. (2004) were also intermediate; however, the frequency of allele C was 43%.

The individual frequencies of the genotypes were in Hardy-Weinberg equilibrium for all the SNP by χ^2 test ($P > 0.05$). In addition, a measure of linkage disequilibrium (r^2 ; Hill and Robertson, 1968) was calculated and tested for all possible pairwise combinations among the 4 SNP. In all cases, the SNP were in joint equilibrium, with r^2 values ranging from 0.008 to 0.01 ($P > 0.05$).

SNP Associations

The association of *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* with EBV for SCS in the first, second, and third lactations are shown in Table 5. The SNP *CXCR1c.-1830A>G*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* were not associated with SCS EBV in any of the lactations. The SNP *CXCR1c.-1768T>A*, however, was associated at a comparison-wise level with EBV for SCS in the first ($P = 0.025$) and second ($P = 0.047$) lactations, but not with third lactation ($P = 0.333$). The corresponding allele substitution effects ($\pm$ SE) were 0.070 ± 0.031 , 0.071 ± 0.035 , and 0.039 ± 0.040 SCS, respectively.

The estimated allele substitution effects from the multiple trait model analysis are presented in Table 6. The SNP *CXCR1c.-1830A>G*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* were again not associated with SCS in any of the lactations. The SNP *CXCR1c.-1768T>A* was, however, consistently associated at a comparison-

Table 5. Association of the single nucleotide polymorphisms (SNP) *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* with bulls' EBV for SCS at first (SCS1), second (SCS2), and third or later (SCS3) lactations¹

SNP	Substitution	EBV (in units of score)		
		SCS1	SCS2	SCS3
		Average allele substitution effect $\pm$ SE (P -value)		
<i>CXCR1c.-1830A>G</i>	(A/G)	0.041 ± 0.039 (0.287)	0.061 ± 0.045 (0.177)	0.066 ± 0.051 (0.196)
<i>CXCR1c.-1768T>A</i>	(T/A)	0.070 ± 0.031 (0.025)	0.071 ± 0.035 (0.047)	0.039 ± 0.040 (0.333)
<i>CXCR1c.-344T>C</i>	(T/C)	-0.003 ± 0.032 (0.916)	-0.033 ± 0.037 (0.367)	-0.046 ± 0.042 (0.272)
<i>CXCR1c.777G>C</i>	(G/C)	-0.017 ± 0.029 (0.571)	-0.027 ± 0.033 (0.423)	$+0.002 \pm 0.038$ (0.953)

¹Results from univariate analyses.

Table 6. Association of the single nucleotide polymorphisms (SNP) *CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C* with EBV of bulls for SCS at first (SCS1), second (SCS2), and third or later (SCS3) lactations¹

SNP	Substitution	EBV (in units of score)			Multivariate test across lactations
		SCS1	SCS2	SCS3	
		Average allele substitution effect $\pm$ SE (<i>P</i> -value)			<i>P</i> > <i>F</i>
<i>CXCR1c.-1830A>G</i>	(A/G)	0.043 $\pm$ 0.038 (0.259)	0.060 $\pm$ 0.044 (0.178)	0.066 $\pm$ 0.050 (0.188)	0.609
<i>CXCR1c.-1768T>A</i>	(T/A)	0.073 $\pm$ 0.031 (0.019)	0.074 $\pm$ 0.035 (0.035)	0.043 $\pm$ 0.040 (0.290)	0.007*
<i>CXCR1c.-344T>C</i>	(T/C)	-0.011 $\pm$ 0.032 (0.720)	-0.040 $\pm$ 0.036 (0.274)	-0.054 $\pm$ 0.041 (0.192)	0.329
<i>CXCR1c.777G>C</i>	(G/C)	-0.013 $\pm$ 0.029 (0.650)	-0.022 $\pm$ 0.033 (0.501)	+0.005 $\pm$ 0.038 (0.896)	0.152

¹Results from multivariate analysis.

*Hold significance at an experimental-wise 5% significance level.

wise level with EBV for SCS in the first ($P = 0.019$) and second ($P = 0.035$) lactations, but not in the third lactation ($P = 0.290$); the corresponding allele substitution effects were 0.073 ± 0.031 , 0.074 ± 0.035 , and 0.043 ± 0.040 SCS, respectively. The overall multivariate Wald *F*-test for the association of SNP *CXCR1c.-1768T>A* with the 3 EBV traits was highly significant ($P = 0.007$) and retained significance at an experimental-wise level (Table 6).

Haplotype Analysis

All 4 SNP (*CXCR1c.-1830A>G*, *CXCR1c.-1768T>A*, *CXCR1c.-344T>C*, and *CXCR1c.777G>C*) were used in the haplotype reconstruction. Table 7 shows the estimated haplotype frequencies for the 13 haplotypes reconstructed in the sampled Holstein bull population. The most common haplotype was designated as Haplo1 carrying alleles ATCC with frequencies equal to 0.22, followed by 4 haplotypes (from Haplo2 to 5) with frequencies equal to 0.18, 0.17, 0.15, and 0.13, respectively. The haplotype frequencies for Haplo6 to Haplo13 decreased from 0.05 to 0.001, and were therefore pooled

into a single haplotype designed Haplo6. The linear effects of the 6 haplotypes were estimated, constraining the Haplo6 to have an estimated effect equal to zero to account for a linear dependency among haplotype effects. Table 8 shows the estimated haplotype effects and their levels of significance for EBV for SCS in the first, second, and third lactation and for later lactations. Haplo4 (AACG) was associated at a comparison-wise level with the EBV for SCS in the first lactation ($P = 0.022$), but not in the second ($P = 0.230$) or third ($P = 0.368$). The corresponding allele substitution effects ($\pm$ SE) were 0.117 ± 0.051 , 0.071 ± 0.059 , and 0.061 ± 0.068 SCS, respectively.

DISCUSSION

Numerous studies have revealed the importance of chemokines and chemokine receptors in inflammatory diseases. Increased CXCL8 protein and mRNA expression for example, occurs during bovine mastitis (Lee et al., 2006). Murine *CXCR1* knockout studies have also demonstrated the importance of this chemokine in LPS-induced acute lung injury and neutrophil chemotaxis.

Table 7. Estimated haplotype frequencies in the sampled Holstein bulls

Haplotype	Single nucleotide polymorphism				Estimated frequency
	<i>CXCR1c.-1830A>G</i>	<i>CXCR1c.-1768T>A</i>	<i>CXCR1c.-344T>C</i>	<i>CXCR1c.777G>C</i>	
Haplo1	A	T	C	C	0.2262
Haplo2	A	T	T	C	0.1866
Haplo3	A	T	C	G	0.1766
Haplo4	A	A	C	G	0.1560
Haplo5	G	T	C	G	0.1321
Haplo6 ¹	A	T	T	G	0.0522
Haplo7 ¹	A	A	C	C	0.0256
Haplo8 ¹	A	A	T	G	0.0153
Haplo9 ¹	A	A	T	C	0.0131
Haplo10 ¹	G	T	C	C	0.0065
Haplo11 ¹	G	T	T	G	0.0052
Haplo12 ¹	G	T	T	C	0.0033
Haplo13 ¹	G	A	C	G	0.0012

¹The rare haplotypes (from Haplo6 to 13) were pooled into Haplo6 for the analysis.

Table 8. Estimated haplotype effects ($\pm$ SE, with *P*-value in parentheses) on EBV for SCS at first (SCS1), second (SCS2), and third or later (SCS3) lactations

Haplotype ¹		EBV (in units of score)		
		SCS1	SCS2	SCS3
Haplo1	(ATCC)	0.029 $\pm$ 0.048 (0.555)	0.012 $\pm$ 0.056 (0.830)	-0.013 $\pm$ 0.064 (0.837)
Haplo2	(ATTC)	0.082 $\pm$ 0.051 (0.108)	0.084 $\pm$ 0.059 (0.161)	0.067 $\pm$ 0.068 (0.326)
Haplo3	(ATCG)	0.001 $\pm$ 0.061 (0.982)	-0.027 $\pm$ 0.071 (0.704)	-0.039 $\pm$ 0.081 (0.627)
Haplo4	(AACG)	0.117 $\pm$ 0.051 (0.022)	0.071 $\pm$ 0.059 (0.230)	0.061 $\pm$ 0.068 (0.368)
Haplo5	(GTCG)	0.050 $\pm$ 0.063 (0.366)	0.049 $\pm$ 0.063 (0.434)	0.066 $\pm$ 0.072 (0.364)

¹Haplo6 (pool of rare haplotypes) was constrained to have a solution equal to zero to eliminate a linear dependency among haplotype effects.

In one study, *CXCR1*^{+/+} mice under LPS inhalation treatment showed normal polymorphonuclear leukocyte migration into bronchoalveolar lavage fluid, whereas *CXCR1*^{-/-} mice under the same treatment showed impaired polymorphonuclear leukocyte migration (Reutershan et al., 2006).

Given the significance of the expression of this gene during bovine mastitis, the primary objective of this study was to characterize the 5' upstream region of bovine *CXCR1* by scanning this region for the presence of SNP and performing in silico detection of transcription-factor binding factor sites. The mRNA sequence for the bovine *CXCR1* was sufficient to perform a BLAST search, which identified the *Bos taurus* bacterial artificial chromosome clone (AC150887.4) harboring the query sequence. A total length of 15.7 kb was obtained in the query, and alignment with LAGIN software (<http://www.ch.embnet.org/index.html>) was used to identify 5' flanking sequence.

The sequence of the 5' upstream region of *CXCR1* revealed several potential transcription-factor binding sites, including NF- κ B, Barbie, NF-AT, GATA-1, and AP-1. Flanking sequences from mouse and human *CXCR1* were aligned with the bovine 5' upstream region of *CXCR1* and revealed only 45% homology. The human 5' flanking sequence also revealed potential binding sites for the same transcription factors as those revealed in the bovine sequence. Analysis of the mouse sequence, however, revealed binding sites for NF-AT, GATA-1 and AP-1, but none for NF- κ B or Barbie. A potential TATA box was also located -53 bp from exon 1, and several potential CG sites were identified in the 2.1-kb region upstream of bovine *CXCR1*.

Pooling of DNA followed by sequencing proved to be a powerful SNP discovery tool by including DNA from animals with extreme EBV for SCS. Three novel SNP were detected in the 5' upstream region of the *CXCR1* at positions -1830n (*CXCR1c.-1830A>G*), -1768n (*CXCR1c.-1768T>A*), and -344n (*CXCR1c.-344T>C*) from the start codon, plus an SNP, *CXCR1c.777G>C*, that was previously identified by Youngerman et al.

(2004). None of the SNP were positioned within a transcription-factor binding site; however, SNP *CXCR1c.-1830A>G* was positioned +20 and +15 bp from the NF- κ B and GATA-1 sites, respectively, and SNP *CXCR1c.-1768T>A* was located -17 and -40 bp from the transcription-factor binding sites Barbie and NF-AT, respectively. Last, SNP *CXCR1c.-344T>C* was located between possible AP-1 and GATA-1 transcription-factor binding sites located at +125 and -133 bp, respectively.

The SNP *CXCR1c.-1768T>A* was the only SNP found to be associated with SCS. Results from the single trait analyses were consistent with the multiple trait analysis, in that SNP *CXCR1c.-1768T>A* was associated with EBV for SCS during the first 2 lactations. The multiple trait analysis revealed stronger evidence (i.e., lower comparison-wise *P*-values) for these associations and an overall significant multivariate Wald *F*-test for the association of SNP *CXCR1c.-1768T>A* with the 3 EBV traits, which retained significance at an experimental-wise level.

The allele A, for example, increased the EBV for SCS by approximately 0.07 SCS (approximately one-fifth of the EBV SD) in both lactations. Because Rupp and Boichard (1999) and others have reported moderate genetic correlations between clinical mastitis and SCS, it is possible that allele A of SNP *CXCR1c.-1768T>A* may also predispose cows to clinical mastitis via increased neutrophil trafficking to the mammary gland.

Haplotype analysis revealed significant associations with EBV for SCS at a comparison-wise level, supporting the allele substitution effects for the first lactation. Haplo4 (AACG), containing the allele A for the SNP *CXCR1c.-1768T>A*, which is responsible for greater SCS, showed a significant effect on SCS (approximately one-third of the EBV SD) in the first lactation. An association between Haplo4 and EBV for SCS in subsequent lactations was not significant, despite a trend toward increased EBV for SCS in later lactations.

Although the present study did not demonstrate an association between SNP *CXCR1c.777G>C* and SCS, this SNP has been previously associated with subclini-

cal mastitis, SCS, milk yield, and neutrophil function in Holstein dairy cattle by others (Youngerman et al., 2004; Rambeaud and Pighetti, 2005). Youngerman et al. (2004), for example, demonstrated that Holstein cows with the GG genotype for SNP *CXCR1c.777G>C* had decreased SCS and a tendency to have an increased risk of clinical mastitis, whereas cows with the CC genotype had a greater incidence of subclinical mastitis. Rambeaud and Pighetti (2007) focused on the functional characterization of *CXCR1c.777G>C* and demonstrated that cows with the CC genotype had lower neutrophil binding affinity for IL-8 and significantly less Ca^{++} release following IL-8 stimulation in vitro, suggesting that CXCR1 signaling may be different between these genotypes.

Reasons for the discrepancy between the results from Youngerman et al. (2004) and the present study are not obvious, but might be because of different sample sizes. The current investigation used a sample of 338 Holstein bulls, whereas Youngerman et al. (2004) reported an association in 37 Holstein cows, but not in a sample of 42 Jersey cows. It is interesting to note that the haplotype analysis in the present study revealed a significant effect of Haplo4 on SCS EBV in the first lactation, and that this haplotype included the G allele for SNP *CXCR1c.777G>C* and the A allele for SNP *CXCR1c.-1768T>A*, suggesting potential confounding between the effect of these alleles if they are not estimated simultaneously.

With respect to the genomic localization of the bovine *CXCR1* gene, a relevant QTL for SCS has been previously mapped on *Bos taurus* autosome 2, with the most likely position at 91.5 cM (Bennewitz et al., 2003). This QTL is close to the *CXCR1* gene (90.3 cM) and to *CXCR2* gene, which are 20 kb apart and on opposite strands (Pighetti and Rambeaud, 2006).

In summary, evidence of association between SNP *CXCR1c.-1768T>A* and EBV for SCS during the first and second lactations was found. Haplotype analysis supported this association for the first lactation. This result combined with the proximity of potential transcription-factor binding sites and the location of *CXCR1* next to a reported QTL raises the possibility that this SNP may affect expression of *CXCR1*. The hypothesis of such an effect warrants further investigation.

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