Re-evaluation of Chicken CXCR1 Determines the True Gene Structure

CXCL1 (K60) AND CXCL2 (CAF/INTERLEUKIN-8) ARE LIGANDS FOR THIS RECEPTOR∗

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The original report of chicken CXCR1 (Li, Q. J., Lu, S., Ye, R. D., and Martins-Green, M. (2000) Gene (Amst.) 257, 307–317) described it as a single exon gene, with two isoforms (differing in their start codon). In comparison with mammalian CXCR1, the reported chicken CXCR1 was longer at both the NH₂ and COOH termini, and it lacked the conserved (C/S)CXNP motif present in the last transmembrane region of all known chemokine receptors. A re-evaluation of chicken CXCR1, comparing known expressed sequence tags with the chicken genome sequence, suggested that the gene contains two exons. We isolated a CDNA corresponding to our prediction, which was significantly different in sequence to the reported CXCR1. In particular, there were three frameshifts in our sequence, compared with the reported sequence, that restored higher identity in the COOH-terminal half of the protein to mammalian CXCR1 (61% total amino acid identity compared with 52% for the reported CXCR1), restored the (C/S)CXNP motif, and gave a predicted protein of the same length as mammalian CXCR1. In human, CXCR1 is the receptor for CXCL8. In the chicken, there are two syntenic genes, CXCL1i and CXCL2i, which look equally like orthologues of human CXCL8. We demonstrate that both of these chemokines are ligands for chicken CXCR1. We also demonstrate that heterophils express chicken CXCR1 and that the receptor is G0, protein-linked.

CXC chemokines can be classified according to the presence of the tripeptide motif glutamic acid-leucine-arginine (ELR) in the NH₂-terminal region before the first conserved cysteine. In mammals, ELR+ chemokines are specific for polymorphonuclear leukocytes (2), whereas ELR− chemokines attract a variety of leukocytes (3, 4). In mammals, ELR+ CXC chemokines bind to two receptors, CXCR1 and CXCR2, with different affinities. Both receptors are the principal expressed chemokine receptors on neutrophils.

To understand the evolution of the chemokines and their receptors in concert with the evolution of different developmental and responsive modes of the immune system, the recently released chicken genome sequence (5) was used to catalogue a complete list of chicken chemokines and their receptors (6–8). All four classes of chemokines were identified in the chicken genome, although the total number, 24, was fewer than identified in human (42) or mouse (35). For the homeostatic chemokines present, a clear orthologous relationship could be identified, suggesting conservation of function (8). However, for the inflammatory chemokines, the relationships between the chicken chemokines and their mammalian homologues remain unclear (8). All four classes of chemokine receptors were also identified in the chicken genome (8), again with a smaller total repertoire (13 genes) in the chicken as compared with mammals. Similarly to the chemokines, the homeostatic chemokine receptors had obvious mammalian orthologues, whereas the relationships between the inflammatory chemokine receptors remain unclear.

In the absence of clear orthologous relationships with human counterparts, Kaiser et al. (8) proposed a systematic nomenclature for the putative chicken inflammatory chemokines and their chemokine receptors. They were classified according to the classes of chemokine to which they belong (for example CC or CXC), followed by L for ligand and R for receptor. The chemokines were then followed by an “i” for presumed inflammatory function and a number based on the chromosomal location of the gene. A similar nomenclature was given for the receptors, in this case using sequential letters indicating chromosomal location to differentiate between them.

Chicken (ch)² CXCL2i (chCAF (chemotactic and angiogenic factor), described previously as 9E3/CEF4) was the first chicken CX chemokine identified. As its name suggests, it has both chemotactic and angiogenic functions in the chicken (9–14). At low concentrations, chCAF is chemotactic for monocyte/macrophages and lymphocytes. However, at high concentrations chCAF also stimulates sprouting and growth of blood vessels (11). It is most similar in sequence to human (hu) CXCL8 (interleukin-8) (50% amino acid identity) (14). Another CXCL chemokine, chCXCLi1 (K60), was later identified by differential gene expression analysis of lipopolysaccharide-stimulated and unstimulated cells of the chicken macrophage cell line HD11 (15). chCXCLi1 is similar to chCXCLi2 (67% amino acid iden-

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‡The abbreviations used are: ch, chicken; RT, reverse transcription; HA, hemagglutinin; PHA, phytohemagglutinin; ConA, concanavalin A; UTR, untranslated region; hu, human; PMA, phorbol myristate acetate; lono, ionomycin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EST, expressed sequence tag.

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Human CXCL8 binds to two receptors, huCXCR1 (17) and huCXCR2 (18), with high affinity (19). A chicken cDNA encoding a seven-transmembrane domain protein resembling a chemokine receptor, which had 53 and 52% amino acid sequence identity to huCXCR1 and huCXCR2, respectively, was described by Li et al. (1). Using 5’-rapid amplification of cDNA ends, Li et al. (1) claimed that chCXCR1 was expressed from an intronless gene, as they identified different isoforms differing in the transcriptional start point and consequently utilizing different start codons. The isoform 1 transcript was 438 bp longer than that of isoform 2. Isoform 1 was expressed in bursa, brain, liver, pancreas, small intestine, large intestine, stomach (sic), and embryonic fibroblasts (1). The chCXCR1 isoform 1 receptor was expressed in murine NIH3T3 cells, on which it conferred the ability to respond to recombinant chCXCLi2, with increased intracellular calcium and ERK (extracellular signal-regulated kinase) 1/2 phosphorylation (20). Closest analysis of the published chCXCR1 sequence revealed several anomalies in the context of knowledge of mammalian CXCR. Most CXCR genes (including human CXCR1) have two exons, with a single intron in the 5’-UTR sequence. The published chCXCR1 was described as a single exon gene. The published chCXCR1 isoform 1 sequence is longer at both the NH2 and COOH termini as compared with huCXCR1. The published chCXCR1 COOH terminus is proline-rich, which would be unique among chemokine receptors. Finally, a conserved (C/S)CXNP motif present in the last transmembrane region of all known chemokine receptors was absent in the published chCXCR1. We therefore decided to re-examine chicken CXCR1.

**EXPERIMENTAL PROCEDURES**

**Isolation of Chicken Peripheral Blood Monocytes and Heterophils**—Isolation of monocytes was carried out according to Wigley et al. (21). Isolation of heterophils was carried out according to Kogut et al. (22).

**Stimulation of Splenocytes, Thymocytes, and Bursal Cells with Mitogen**—Single-cell suspensions were obtained from 8-week-old line C.B12 chicken spleens, bursae, and thymuses by passing through a nylon filter. Lymphocytes were then extracted using Histopaque 1119, washed three times with Dulbecco’s modified Eagle’s medium, and adjusted to 5 x 10⁶ cells/ml (10 ml/flask). Mitogen was then added to each flask to a final concentration of concanavalin A (ConA) (1 μg/ml), phytohemagglutinin (PHA) (12 μg/ml), or phorbol myristate acetate + ionomycin (PMA + Iono) (800 and 100 ng/ml, respectively). The cultures were incubated at 41 °C, 5% CO₂ for 6 h. The cells were then scraped off the flask, washed three times in PBS, and RNA-extracted using a Qiagen RNeasy kit.

**Determination of the Structure of the chCXCR1 Gene**—Inserts of a cDNA library prepared from purified splenocytes from a juvenile line 0 chicken and cloned into the mammalian cell expression vector pchN were excised with XhoI and ligated with mismatch adapters as described (23). The sequences of a chCXCR1 EST (CK607506) and of the chCXCR1 genomic DNA were used to design internal PCR primers corresponding to the 5’-UTR and 3’-coding regions (5’-GGAATTCGAGCTGGAGAAGCT-3’ and 5’-TCAGAGGTGTTGAGGTGTT-5’). These were used to amplify chCXCR1 from the cDNA library in combination with an adapter primer (5’-GCAGTAATTCGATATCAAGC-3’). Nested PCR was then carried out with nested primers (5’-AGATTGGATTGCGGGCAG-3’ and 5’-CCGATGAGCCGTAGATGAT-5’), respectively) (Fig. 1). Cycling conditions were as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 15 s, 56 °C for 15 s and 72 °C for 2 min, with a final extension of 72 °C for 6 min. PCR products were TA-cloned (Invitrogen TOPO TA cloning kit) into the vector pCR2.1 and sequenced.

One-step RT-PCR was carried out using Ready-to-Go RT-PCR beads (Amersham Biosciences). Primers were designed spanning the stop (5’-ATGGGGAATTTTACGCTGA-3’) and stop (5’-TCAAGGGGTGTTGAGGTG-3’) codons of chCXCR1. RNA was extracted using a Qiagen RNeasy kit from spleens of line C.B12 and N chickens. Genomic DNA from line N chickens was used as a positive control. One-step RT-PCR was carried out according to the manufacturer’s instructions except that betaine (Sigma) was added to the PCR mixture to a final concentration of 1 M. The resulting PCR products were then TA-cloned and sequenced. Reverse transcriptase-negative controls were carried out according to the manufacturer's protocol. The sequences of chCXCR1 for line 72, N, and C.B12 chickens were then deposited into ENSEMBL (accession numbers AJ973196, AJ973197 and AJ973198, respectively).

**Expression of chCXCR1 in Tissues, Mitogen-stimulated Lymphoid Tissues, Monocytes, and Heterophils by One-step RT-PCR**—A panel of tissues (bursa, thymus, spleen, bone marrow, brain, cecum, cecal tonsil, liver, kidney, upper ileum, lower ileum, large intestine, muscle, skin, trachea, pancreas, proventriculus, heart, lung, duodenum, esophagus, and crop) were collected from 2-week-old Rhode Island Red chickens. RNA was extracted from these tissues using a Qiagen RNeasy kit as well as from heterophils, monocytes, and mitogen-stimulated lymphoid tissues, isolated as described above. One-step RT-PCR was carried out using Ready-to-Go RT-PCR beads (Amersham Biosciences) according to the manufacturer’s instructions, using the following primers: 5’-TGCAATGAGAATACCCCA-3’ and 5’-CCGATGAGCCGTAGATGAT-3’. RNA concentrations were standardized using β-actin primers spanning an intron as follows: 5’-TGCTGGTGTTCACATCTACGC-3’ and 5’-TGTTGACAAATACCCATGTT-5’. Generation of a Hemagglutinin-tagged chCXCR1 Clone—The chicken CXCR1 gene was amplified from line N genomic DNA by PCR using the sense primer 5’-ATTTGGAATTTCCGGACATTTTTCGAC-3’ and the antisense primer 5’-TACGCTCTAGATGATGTCGACG-5’ and TA-cloned (Topo.TA cloning kit, Invitrogen). The chCXCR1
ChCXCR1 was then excised from the TOPO vector with EcoRI (New England Biolabs) and XbaI (New England Biolabs) and then ligated into the same sites of a modified pcDNA3 vector containing a hemagglutinin epitope 5′ to the multiple cloning site (24), to generate a recombinant chCXCR1 with an HA epitope at the 5′ end.

Expression of Recombinant chCXCL1 and chCXCL2—ChCXCL1 and chCXCL2 were transiently expressed in COS-7 cells as described previously (25, 26).

Transient Expression of chCXCR1 in Murine L1.2 Cell Line—The murine pre-B cell line L1.2 was maintained in HEPES-modified RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2 as described previously (27). pcDNA3 either unmodified or containing HA-tagged chCXCR1, HA-tagged CCR1 (24), and FLAG-tagged huCXCR1 constructs was introduced into L1.2 cells by electroporation (28). To enhance cell surface receptor expression, transient transfectants were cultured for 24 h in medium supplemented with 20,000 cells (20,000)


to enhance cell surface receptor expression, transient transfectants were cultured for 24 h in medium supplemented with 10 μm sodium butyrate (Sigma) prior to use. Cells were analyzed for chemokine receptor expression by flow cytometry.

Fluorescence-activated Cell Sorter Analysis of Chemokine Receptor Expression—Approximately 5 × 10^5 cells were harvested, washed once with fluorescence-activated cell sorting buffer (0.25% bovine serum albumin and 0.01% NaN3 in PBS), and then incubated with either 10 μg/ml of murine monoclonal anti-human CXCR1 clone 42705.111 (Sigma) or murine monoclonal anti-HA clone 16B12 (Covance) for 20 min in a final volume of 100 μl. Cells were then washed with fluorescence-activated cell sorting buffer and incubated with the fluorescein isothiocyanate-coupled secondary antibody (1:20 diluted goat polyclonal anti-mouse IgG from Dako) for 20 min in a final volume of 100 μl. Subsequently, cells were washed with fluorescence-activated cell sorting buffer and resuspended in a final volume of 500 μl before being analyzed by flow cytometry as described previously (29). All incubations were carried out on ice.

Assays of Chemotactic Responsiveness—Assays of chemotactic responsiveness were carried out as described previously (30) using 96-well chemotaxis plates with 5-μm pores (Receptor Technologies, Adderbury, UK). Cells were harvested and washed twice with PBS and then resuspended in serum-free HEPES modified RPMI 1640 medium (Sigma). Cells (20,000) were placed onto the chemotactants. RPMI 1640 medium containing 0.1% BSA and 2.5 × 10^6 cells in 100-μl total volume was loaded into the upper wells and the plate incubated at 37 °C in 5% CO2 for 4 h.

Treatment of Heterophils with Pertussis Toxin—Briefly, cells were treated with 1 μg of pertussis toxin (Sigma)/10^6 cells/ml in RPMI 1640 medium containing 0.1% BSA for 1 h at 37 °C. Cells were washed with a 5-fold excess of RPMI 1640 medium containing 0.1% BSA and centrifuged at 250 × g for 10 min at 4 °C. Cells were then resuspended in RPMI 1640 medium containing 0.1% BSA and used in chemotaxis assays.

RESULTS

Determination of the Gene Structure of chCXCR1—As described earlier, most CXCR genes (including human CXCR1) have two exons, with a single intron in the 5′-UTR sequence (Fig. 1A). The published chCXCR1 (1) was described as a single exon gene, with two isoforms. The isoform 1 sequence was longer at both the NH2 and COOH termini as compared with the EST sequence (1). All three ESTs were different from the published sequence (1) identified three EST sequences (BX258468, CB018048, and CK607506). When aligned with the published sequence (1), the isoform 1 and 2 mRNA structures. The translational start-point in isoform 1 lies within the putative intron in our gene, whereas that for isoform 2 agrees with our prediction. Primers were designed spanning across the putative intron to amplify a chCXCR1 cDNA by nested RT-PCR. F1 and R1, first-round PCR primers, F2 and R2, nested PCR primers, B, gene and mRNA structures for human CXCR1. CDS, coding sequence, shown shaded.

A, published chCXCR1 mRNA (1) was expressed from an "intronless" gene in two isoforms. Our predicted gene structure and mRNA are shown below the isoform 1 and 2 mRNA structures. The translational start-point in isoform 1 lies within the putative intron in our gene, whereas that for isoform 2 agrees with our prediction. Primers were designed spanning across the putative intron to amplify a chCXCR1 cDNA by nested RT-PCR. F1 and R1, first-round PCR primers, F2 and R2, nested PCR primers, B, gene and mRNA structures for human CXCR1. CDS, coding sequence, shown shaded.

B, fluorescence-activated cell sorting analysis of chemokine receptor expression was performed as described previously. Approximately 5 × 10^5 cells were harvested, washed once with fluorescence-activated cell sorting buffer (0.25% bovine serum albumin and 0.01% NaN3 in PBS), and then incubated with either 10 μg/ml of murine monoclonal anti-human CXCR1 clone 42705.111 (Sigma) or murine monoclonal anti-HA clone 16B12 (Covance) for 20 min in a final volume of 100 μl. Cells were then washed with fluorescence-activated cell sorting buffer and resuspended in a final volume of 500 μl before being analyzed by flow cytometry as described previously (29). All incubations were carried out on ice.

Assays of Chemotactic Responsiveness—Assays of chemotactic responsiveness were carried out as described previously (30) using 96-well chemotaxis plates with 5-μm pores (Receptor Technologies, Adderbury, UK). Cells were harvested and washed twice with PBS and then resuspended in serum-free HEPES modified RPMI 1640 medium (Sigma). Cells (20,000) were loaded into a total volume of 20 μl into the upper compartment of a microchemotaxis chamber. Chemottractants were loaded in a final volume of 31 μl at increasing concentrations in the lower compartment. Two compartments were separated by a polyvinylpyrrolidone-free polycarbonate filter with 5-μm pores. The chemotaxis chamber was incubated in a humidified chamber at 37 °C in 5% CO2 for 6 h. The filter was then removed, and the number of cells migrating into each compartment was counted using a hemocytometer. All conditions were tested in duplicate. For chemotaxis assays in 24-well transwell plates (Corning Inc.), wells were first blocked with 600 μl of RPMI 1640 medium containing 1% BSA for 20 min. Chemotactants were then diluted 1:10 in RPMI 1640 medium containing 0.1% BSA and 600 μl loaded into the lower wells. Wells containing filter bases with 5-μm pores were placed onto the chemoattractants. RPMI 1640 medium containing 0.1% BSA and 2.5 × 10^6 cells in 100-μl total volume was loaded into the upper wells and the plate incubated at 37 °C in 5% CO2 for 4 h.
Primers were then designed spanning the predicted coding sequence, and the full coding sequence of chCXCR1 was determined by two methods: single-step RT-PCR from splenic mRNA from two lines (72 and C.B12) of chickens and PCR from line N chicken genomic DNA. All three templates generated a PCR product that was then sequenced. The predicted amino acid sequences are shown aligned with the previously published chCXCR1 sequence in Fig. 3. The differences to the published sequence give changes in four amino acid codons in the first half of the protein and three frameshifts in the remainder, leading to changes in transmembrane regions and a shorter coding region overall, with the COOH terminus of our chCXCR1 sequence aligning with those of human CXCR1 and CXCR2 (Fig. 3). The four amino acids changes to the published chCXCR1 sequence aligning with those of human CXCR1 and CXCR2 (Fig. 3). The alignment also reveals a conserved motif, (C/S)CXXNP, in the last transmembrane region, which is typical of chemokine receptors and is present in our chCXCR1 sequence and the human CXCR1 and CXCR2 sequences, but not in the published chCXCR1 isoform sequence (Fig. 3).

Expression Profiles of chCXCR1—One-step RT-PCR was carried out to determine the expression profile of chCXCR1 in a panel of tissues, cells, and mitogen-stimulated lymphoid cells. chCXCR1 mRNA was expressed in several lymphoid tissues (bursa, spleen, bone marrow, and cecal tonsil). It was also expressed in the cecum, ileum, and large intestine (Fig. 4). It was expressed in monocytes and heterophils (Fig. 4, lanes 24 and 25). Upon stimulation with ConA or PHA, chCXCR1 mRNA expression was slightly up-regulated in bursal cells. In splenocytes, chCXCR1 expression was down-regulated in all mitogen-stimulated samples, especially after stimulation with PMA + ionomycin (Fig. 4). chCXCR1 was not expressed in thymocytes (Fig. 4, lane 34). However, after stimulation with mitogen, chCXCR1 expression was detected at low levels (Fig. 4, lanes 35–37).

chCXCR1 Is the Orthologue of huCXCR1 and Transfected L.1.2 Cells Expressing chCXCR1 Chemotaxed toward huCXCL8, chCXCL1, and chCXCL2—To determine whether chCXCL1 and chCXCL2 are ligands for chCXCR1, and if chCXCR1 is the orthologue of huCXCR1, pcDNA3 containing either recombinant HA-tagged chCXCR1 or FLAG-tagged huCXCR1 was transfected into murine L.1.2 cells (which do not respond to huCXCL8 (31)) for transient expression of the receptors. pcDNA3 containing HA-tagged huCXCR1 and pcDNA3 alone were included as controls. Expression of the receptors was measured by flow cytometry (Fig. 5). chCXCR1 was transiently expressed in the cells at high levels, with ~50% of the cells being transfected (Fig. 5A). As positive controls, L.1.2 cells were transfected with HA-tagged huCCR1 (Fig. 5B) and FLAG-tagged huCXCR1 (Fig. 5D). pcDNA3 was transfected into L.1.2 murine cells as a mock control and stained negative throughout the experiment (Fig. 5C). Once demonstrated as positively transfected, the cells were then used in chemotaxis assays.

To determine the ligand-receptor relationships, the responsiveness of HA-chCXCR1 or FLAG-huCXCR1 L.1.2 transfectants to purified recombinant huCXCL8, chCXCL1, chCXCL2, or a mock control was determined by transwell chemotaxis assays. Fig. 6A shows that both huCXCR1 and chCXCR1 transfectants respond to huCXCL8 in a dose-dependent manner. The huCXCR1 transfectants had a typical prozone response, with lowered migration at higher concentrations of huCXCL8. The responsiveness of FLAG-huCXCR1 and HA-chCXCR1 expressing transfectants to chCXCL1 and chCXCL2, the predicted ligands for chCXCR1, was also tested. Both chCXCL1 and chCXCL2 induced the chemotaxis of chCXCR1 transfectants, but not huCXCR1 transfectants, in a dose-dependent manner (Fig. 6B), confirming the functional relationship between chCXCR1 and the ligands chCXCL1 and chCXCL2.

Chemotaxis of Heterophils to chCXCL1 and chCXCL2—Pertussis toxin catalyzes the ADP-ribosylation of the Goi regulatory component of adenylyl cyclase (32, 33), inhibiting various metabolic responses of mammalian neutrophils to chemotactic factors (17, 31, 34–37). Heterophils, the avian equivalent to neutrophils, express chCXCR1 on their surface (Fig. 4, lane 25). To determine whether chCXCL1 and chCXCL2 chemoattract heterophils and whether the receptor is Goi protein-linked, a chemotaxis assay was carried out using untreated heterophils and pertussis toxin-treated heterophils.

Fig. 7 shows the percentage of cells migrating in response to chCXCL1 and chCXCL2. Heterophils responded to both chCXCL1 and chCXCL2, with a higher proportion of cells migrating in response to chCXCL1 (~50%) as compared with chCXCL2 (~28%). Pertussis toxin treatment abolished the chemotactic response of heterophils to chCXCL1 and chCXCL2, reducing it to background levels seen with the pCI-neo transfected and untransfected controls, suggesting that the receptor is Goi protein-linked.
**ChCXCR1 and Its Ligands**

**A**

| Gene   | Sequence                                                                 |
|--------|-------------------------------------------------------------------------|
| huCXCR1 | GSSSGHTSTTL                                                           |
| huCXCR2 | -SSSVNVSSNL                                                         |
| chCXCR1iso2 | QVLGFSTVASTPSSTASSGRSFATASSRSWHSVASSARMLWHATAAPPTL           |
| chCXCR1new | EILGFLHSCLNPIIYAFIGQNFRHGFLKILAMHGLVSKEFLARHRVTSYT       |
| huCXCR2 | MRVIFAVVLIFLLCWLPYNLVLLADTLMRTQVIQESCERRNNIGRALDAT         |
| huCXCR1 | VIIAYALVFLLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALT          |

**B**

| Gene   | Sequence                                                                 |
|--------|-------------------------------------------------------------------------|
| chCXCR1iso2 | MGTFYADELLDILYNYTSDYCN--YSLVLPDIDVSSSPCRNEGSVANKYL       |
| huCXCR1 | -----MSNITDPQMWDFDDLN----FTGMPPADEDYSPCMLETETLNKYV         |

**DISCUSSION**

The elucidation of the sequences of chCXCL11, chCXCL12, and their receptor chCXCR1 in the chicken provides a potential animal model to study and characterize the function of CXCL8, which is absent in the mouse. However, the definitive characterization of ligand-receptor relationships is a necessary prerequisite for such studies. A putative chicken CXCR1 sequence has been published (1) and described as a single exon gene, but the data reported here raise substantial doubts about the validity of that sequence. First, splice junctions are generally highly conserved across species (38–40). Most chemokine receptor genes have at least 2 exons, including human CXCR1, whose primary transcript is derived from 2 exons. This suggested that chCXCR1 should be a 2-exon gene.

Li et al. (1) reported two isoforms of chCXCR1 mRNA. Although the shorter of these used the same initiation codon as our proposed transcript, the longer form was proposed to use an alternative start codon 135 nucleotides upstream, leading to a 45-amino acid NH2-terminal extension compared with human CXCR1. This extension would be unique among known CXCR1 receptors. The alternative start codon resides in the intron in the gene structure proposed in this study. It seems likely that the longer isoform mRNA cloned by Li et al. (1) may have been either unprocessed pre-mRNA or accidentally cloned genomic DNA.

In the amino-terminal half of the protein, there are four amino acid residue differences between the published chCXCR1 sequence and our predicted sequence. For all four residues, the residues in our predicted sequence are identical to those in human CXCR1 and CXCR2. There were also three frameshifts in the COOH-terminal half of our sequence compared with the COOH-terminal half of the sequence reported by Li et al. (1). A prediction of the transmembrane regions of the published chCXCR1 sequence, using the TMpred program, revealed that the frameshifts disrupted the 7-transmembrane structure of the chemokine receptor, with only 6 transmembrane regions being predicted, as opposed to the 7-transmembrane structure reported by Li et al. (1). Our predicted sequence has significant amino acid identity with human CXCR1 in these regions, and the 7-transmembrane structure is restored.

The changes in the published cytoplasmic COOH terminus of the protein might be expected to have profound effects on the outcome of chemotaxis, as this region of chemokine receptors typically couples to Go proteins (41). The COOH terminus of the published chCXCR1 is proline-rich, unlike any other known chemokine receptors. Although Li et al. (20) reported Ca2+ mobilization in response to recombinant CXCL12 in cells transfected with their long isoform cDNA, they did not report the definitive chemotactic response in these cells. Ca2+ mobilization mediated by huCXCR1 is dependent on different residues (Arg-199, Arg-203, and Asp-265) in the receptor (42) than those responsible for the chemotactic response, and these three residues are outside the cytoplasmic

chCXCR1iso2, in blocks of 60 nucleotides, showing only those blocks where there are nucleotide differences. Those causing an amino acid substitution are indicated by asterisks. The three frameshifts are indicated by an ampersand.

**FIGURE 3.** A, alignment of the amino acid sequences of our predicted chCXCR1 (chCXCR1new), the published chCXCR1 isoform 2 (chCXCR1iso2), huCXCR1, and chCXCR2. The four amino acid differences between chCXCR1new and chCXCR1iso2 in the first half of the protein are indicated by asterisks. The areas affected by frameshifts in the second half of chCXCR1iso2 are boxed. Amino acids important for ligand/receptor activation in huCXCR1 (42) than those responsible for the chemotactic response, and these three residues are outside the cytoplasmic regions of chCXCR1new and chCXCR1iso2. B, alignment of the nucleotide sequences of chCXCR1new and chCXCR1iso2.
Thus it is possible that the Ca\(^{2+}\) mobilization response was present, despite the absence of the conserved COOH-terminal sequences, in the experiments reported by Li et al. (20). Alternatively, it may be that the sequence differences result from sequencing errors, and the clone used by Li et al. (20) does in fact contain the correct sequence. Finally, it is formally possible that the frameshift mutations could be present in the line of chickens studied by Li et al. (1), although they are not present in the three chicken lines studied here, in EST sequences, or in the red jungle fowl genomic sequence. We propose that there is a single functional CXCR1 transcript in the chicken, with the sequence described here.

Li et al. (1) measured the expression of chCXCR1 in different tissues. However, the primers they used were designed to the region including our predicted intron. As their reported ampli-
Evidence from infection studies has suggested that chCXCL1 and chCXCL2 might have different roles (47, 48). In both studies, chCXCL1 was up-regulated to a higher level following Salmonella infection than chCXCL2. chCXCL2 induces the migration of both blood-derived mononuclear cells and heterophils (49) but at different concentrations. The migration of blood-derived mononuclear cells was induced at low concentrations of chCXCL2, whereas the migration of heterophils required higher concentrations (49). As shown here, chCXCL1 was more efficient in inducing the migration of blood-derived heterophils than chCXCL2. The chicken therefore seems to have evolved two different CXCL8-like chemokines, with chCXCL2 more efficient at inducing the migration of monocytes and chCXCL1 more efficient at inducing the migration of heterophils.

The main role of chemokines is to direct cells along a chemokine gradient. The migration of chCXCR1 transfectants in response to huCXCL8, and the similarity of the response of huCXCR1 transfectants to huCXCL8, suggested that chCXCR1 is an orthologue of huCXCR1. Although both chCXCL1 and chCXCL2 are similar to huCXCL8, they do not induce the migration of huCXCR1-transfected cells. chCXCR1 and huCXCR1 are more highly conserved (61% amino acid similarity), both in sequence and function, compared with the chemokines (~50% amino acid similarity). huCXCL8 is the major chemokine for chemoattracting neutrophils, which highly express chCXCR1 on their surface. Although chCXCL1 and chCXCL2 chemoattract heterophils, the avian equivalent to neutrophils, chCXCL1 does this more efficiently.

In mammals CXCR1 is the principal chemokine receptor expressed on neutrophils (17, 38). Treatment with pertussis toxin abolished the chemotaxis of neutrophils migrating in response to CXCL8 (34, 50). Heterophils, the avian equivalent of neutrophils, also express chCXCR1. Treatment of heterophils with pertussis toxin abolished the chemotaxis of heterophils to chCXCL1 and chCXCL2, suggesting that the receptor for both chemokines, chCXCR1, is also coupled to Goi protein.

chCXCL2 is expressed by and is chemotactic for peripheral blood-derived monocytes (49). chCXCL2 is also chemotactic for heterophils (49). However, the concentration required for chemoattracting heterophils is 100 times more than that required to chemoattract monocytes (49). Based on this and the data in this study, we hypothesize that chCXCL1 is the orthologue of huCXCL8 and that it mainly chemoattracts heterophils, whereas chCXCL2, although similar to huCXCL8, chemoattracts mainly monocytes. Although both chemokines use chCXCR1 as their receptor and both heterophils and monocytes express chCXCR1, how the different cell types regulate their response to both chemokines remains unknown.

Although the ligand CXCL8 is absent in the mouse genome, two groups have recently identified and cloned a CXCR1 orthologue in the mouse (51, 52). Moepps et al. (51) went on to express mouse CXCR1 as a recombinant protein and showed that mouse CXCR1 was not activated by a large panel of known CXC chemokines of human and murine origin. These findings suggest that a homologue or orthologue of huCXCR1 is expressed in the mouse to be activated by a previously unknown
CXCL chemokine or that it is nonfunctional, given that its ligand is absent in the mouse genome. The elucidation of the ligand-receptor relationship for chCXCR1 and its ligands, chCXCL1 and chCXCL2, therefore presents the chicken as an animal model for studying the roles and functions of CXCL8.

The chicken has a different repertoire of inflammatory chemokines and chemokine receptors to mammals (8). The approach described here to establish ligand-receptor relationships for chCXCR1 can be extended to characterize the ligand-receptor relationships for the entire repertoire of chicken chemokines and their receptors.

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