Genomic Organization and Evolution of the CX3CR1/CCR8 Chemokine Receptor Locus*

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The chemokine receptors CCR8 and CX3CR1 are key players in adaptive immunity and are co-receptors for human immunodeficiency virus. We describe here the genomic organization and evolutionary history of both of these genes. CX3CR1 has three promoters that transcribe three separate exons that are spliced with a fourth exon containing the coding region. CCR8 has two promoters. One promoter produces a transcript of two spliced exons, and the other promoter transcribes an exon containing the coding region and lacks introns. We analyzed these promoters in the context of a luciferase reporter and identified several positive and negative regulatory elements. Identification of the genomic organization of these genes in mouse demonstrates a similar organization for CCR8, but mouse CX3CR1 lacks two of the human promoters and has an additional mouse-specific promoter that transcribes only the exon containing the coding region and therefore resembles the organization of the human and mouse CCR8 genes. We also identify two nontranscribed regions that are highly conserved between human and mouse CX3CR1 containing possible regulatory elements. Examination of the CX3CR1 and CCR8 genes and surrounding genomic regions indicates that these genes are the result of the duplication of an ancestral gene prior to the divergence of teleost fish. We characterize single nucleotide polymorphisms in the promoters of human CCR8 and CX3CR1 and establish linkage relationships between CX3CR1 promoter polymorphisms and two previously described CX3CR1 coding polymorphisms associated with human immunodeficiency virus disease progression and arteriosclerosis susceptibility.

Chemokines (chemotactic cytokines) are an extensive family of homologous proteins initially described for their ability to control leukocyte chemotaxis. Their role in immune biology has expanded to include secretagogue, angiostatic, and angiogenic functions as well as modulators of hematopoiesis and possibly organogenesis (1). Chemokines share several functional properties and are identified and classified on a structural basis (1–3). The CC and CXC are the two largest subfamilies, containing four conserved cysteine residues and can be differentiated by the presence of intervening amino acid between the first and second conserved cysteines (1, 2). Lymphotactin-a/-XCL1 and lymphotactin-2/XCL2 are the sole C chemokines and lack the first and third cysteines of the four-cysteine motif (1). Fractalkine/CX3CL1 is the only member of the CX3C subfamily and has three intervening amino acids between the first and second conserved cysteines.

The receptors for chemokines are members of the serpentine, heterotrimeric G-protein coupled, seven-transmembrane-spanning receptor superfamily (1, 2). Each chemokine receptor has a distinct set of chemokine agonists and is expressed on a specific subset of leukocytes. The array of chemokine receptors displayed on a leukocyte surface regulates their chemotactic responsiveness (1, 2, 4). Chemokines and their respective receptors orchestrate the immune response by regulating leukocyte infiltration and subsequent leukocyte effector functions as well as leukocyte emigration and homing to secondary lymphoid organs.

CX3CR1 is the receptor of the chemokine fractalkine (CX3CL1), only one of two chemokines present in both a membrane-bound and a soluble form following proteolytic cleavage (5). CX3CR1/fractalkine have been demonstrated to function in inflammatory responses and in particular appear to be linked to Th1 adaptive immune responses (6). CX3CR1 serves a unique role in microglia chemotaxis and communication with neurons in the brain in addition to its role in immune cell migration (7). CX3CR1 can also be antagonized by vMIP-VMIPII from human herpesvirus 8 (8). Furthermore, like many other chemokine receptors, CX3CR1 is a coreceptor for HIV1 entry (9). In fact, HIV infection leads to dramatic increase in the expression of CX3CR1 in lymphoid tissues (plasma cells and dendritic cells) and a corresponding increase in CX3CR1 expression on CD4 T-cells (10). Recently, a number of coding polymorphisms have been characterized in CX3CR1 and shown to alter susceptibility to both HIV infection and arteriosclerosis (11–15). The two mutations are a valine to isoleucine substitution at position 249 (V249I) and a threonine to methionine substitution at position 280 (T280M). These two polymorphisms are in complete linkage disequilibrium, so that all

1 The abbreviations used are: HIV, human immunodeficiency virus; BAC, bacterial artificial chromosome; RACE, rapid amplification of cDNA ends; AA, amino acid; contig, group of overlapping clones; SNP, single nucleotide polymorphism.
chromosomes possessing the Met260 mutation also have the Ile242 mutation. The Ile242 mutation, however, also exists together with the common Thr260 form of the receptor. The polymorphism leading to a threonine to methionine substitution at position 280 has been shown to increase susceptibility to HIV in French Caucasians and to have a modest delay in AIDS onset and all-cause death in North American Caucasians (11, 13, 14). The Ile242 mutation, on the other hand, is associated with decreased susceptibility to arteriosclerosis (12, 15).

CCR8 is the chemokine receptor for I309 (CCL1) and is also a coreceptor for HIV (16–20). Increased expression of CCR8 correlates with activated Th2 T cells, although the receptor plays a role in the trafficking of other cell types such as monocytes and endothelial cells (21–24). Deletion of CCR8 in mice leads to impaired TH2 responses and decreased eosinophil recruitment (25). As with CX3CR1, VMIP-VMIPII is also an antagonist to CCR8, as is MC148, a chemokine-like molecule from Molluscum contagiosum virus (26, 27). VMIP-1, another chemokine from human herpesvirus 8, on the other hand, can function as a full CCR8 agonist (27). Like many chemokine receptors, both CCR8 and CX3CR1 are located on human chromosome 3.

Whereas chemokine receptors have been identified for a number of years, relatively little work has been done in examining their gene structure and promoter function. CXCR1, -2, and -4 as well as CCR2, -3, and -5 have been examined (28–33), but the more than 15 other members have yet to be analyzed. Furthermore, it is unclear how chemokine receptor gene structure is conserved between species. Mouse models are commonly used to study gene function due to the ease with which knockouts and transgenic animals can be created. However, translating the data from mouse models to human clinical applications can be difficult, particularly in regard to gene regulation.

In this study, we have determined the gene structure and identified the functional promoter elements in both human genes for CX3CR1 and CCR8. In addition, we have identified several noncoding polymorphisms and examined their linkage to other known mutations in the CX3CR1 and CCR8 genes. Furthermore, a comparative analysis between human and mouse genes shows that the gene structure for CCR8 is conserved between mouse and human, but the gene structure for CX3CR1 has undergone significant mutation since the divergence of primates and rodents. Human and mouse CX3CR1 gene families but aids in the identification of conserved regulatory elements and potential differences in gene regulation.

**EXPERIMENTAL PROCEDURES**

**Phylogeny of Chemokines and Chemokine Receptors**—The amino acid sequence of human chemokines receptors along with three receptors we identified from the fugu genomics project (available on the World Wide Web at http://fugu.mrc.ac.uk; scaffold M001065) were aligned using ClustalW (34), and the alignment was edited by hand. Consensus trees were generated using the protein parsimony method with a bootstrap value of 1000 using the PHYLIP package (40).

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Fig. 1. Phylogeny of chemokine receptors. Known human chemokine receptors and three putative fugu chemokine receptors were aligned using ClustalW and a consensus tree generated by the protein parsimony method with a bootstrap score of 1000.

serum (Sigma). For transfections of CX3CR1 constructs, cells were spun down at 1600 rpm and resuspended in RPMI 1640 at a concentration of 20 × 10⁶ cells/ml. 750 μl of resuspended cells were added to micropuler cuvettes (Bio-Rad catalog no. 165-2089), and 15 μg of construct were added. Cells were electroporated at 200 V and 975 microfarads for THP1 cells and 400 V and 975 microfarads for Jurkat cells, using a Bio-Rad gene pulser II, and added to 3 ml of 37 °C complete RPMI medium in a 0.1-cm six-well plate (VWR, West Chester, PA). Cells were then incubated at 37 °C for 48 h. After incubation, luciferase activity was measured using the Bright-Glo luciferase assay system (Promega) according to the manufacturer’s directions. All transfections were performed four times, and triplicate samples were measured for each replicant. CCR8 construct transfections for THP1 cells were performed as above, whereas transfections into Jurkat cells were performed as follows. Jurkat cells were spun down at 1500 rpm and resuspended in Opti-MEM I reduced serum medium (Invitrogen) to a concentration of 10 × 10⁶ cells/ml. 200 μl of resuspended cells were added to each well of a six-well plate. 3 μg of DNA construct in 500 μl of Opti-MEM and 24 μl of GenePORTER reagent (GenePORTER Transfection Reagent, Gene Therapy Systems, Inc., San Diego, CA) in 500 μl of Opti-MEM were premixed and incubated at room temperature for 30 min before the complexes were added to the cells. Cells were then incubated at 37 °C for 4 h, followed by the addition of BoosterExpress reagent number 1 at a 1:50 dilution in Opti-MEM medium. Cells were then returned to the incubator for 48 h at 37 °C. After incubation, luciferase activity was measured using the Bright-Glo luciferase assay system (Promega) according to the manufacturer’s directions. All transfections were performed in triplicate samples for each construct. Potential transcription factor binding sites were identified using MatInspector Professional software (36).

Comparison between Species—The mouse genomic sequences for the CX3CR1 and CCR8 genes were obtained from the mouse genome data base of the Celera Discovery System (Celera Genomics, Rockville, MD), reference GA_x6K02T2NQG0:3500001.4000000. The chromosomal regions were compared with each other with the Blast2Sequences program (37). Individual exons and promoter regions were also compared using the water program from the European Molecular Biology Open Software Suite (38). Potential transcription factor binding sites were identified using MatInspector Professional software (36).

RESULTS

The Phylogenetic Relationship of CCR8 and CX3CR1 to the Chemokine Receptors on Chromosome 3—Despite broad classification of chemokine receptors based upon their ligand affinity, the relationship of the receptors based on amino acid composition clusters receptors into three broad phylogenetic trees. The vast majority of chemokine receptors fall within a large cluster on chromosome 3 (Fig. 1). This group is composed of XCR1, CX3CR1, CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, CCRL2, and CCBP2. Further subclassification indicates that CCR1 and CCR3 are most closely related, as is CCR2 with CCR5 and CX3CR1 with CCR8.

CCR8 and CX3CR1 Are Closely Linked on Chromosome 3—To further determine the fine mapping and genomic organization of CCR8 and CX3CR1, we screened a human genomic library by PCR and obtained two overlapping BAC clones containing both the CX3CR1 and CCR8 genes. Sequencing revealed that the two genes are in a head to head orientation and their coding regions are separated by 66 kb (GenBank™ accession number AY016570). Within the context of the entire human chromosome 3, the CDS for CX3CR1 begins 6.3 megabase pairs after CCR4, and the CDS for CCR8 begins 6.5 megabase pairs before CCR9. Chemokine receptors on chromosome 3 are thus organized into three distinct clusters. CCR1, -3, -2, and -5 and CCRL2 constitute one minicluster all located within just over 200 kb flanked by the genes vascular endothelial growth factor receptor and lactotransferrin.

This is interesting, since CCR1 and CCR3, located beside each other, are most closely related (80% AA similarity, 63% AA identity), as are CCR2 and CCR5 (82% AA similarity, 72% AA identity). CX3CR1 and CCR8 are similarly situated side by side and are most homologous to each other (61% AA similarity, 40% AA identity) (Fig. 1). It would appear that these “pairs” of genes have arisen by gene duplication.

Gene Structure of CCR8 and CX3CR1—We next determined the exon intron structure of the CX3CR1 and CCR8 genes. We performed 5'-RACE on human peripheral blood mononuclear cells for both receptors. In the case of CCR8, we identified two species of transcripts, indicating two likely initiation sites and promoters (Fig. 2). The most abundant transcript begins at position −2626 relative to the start codon for CCR8 and has a single intron. The other transcript species begins at position −48 and has no intron. Similarly, three species of transcripts were identified for CX3CR1, indicating three putative promoters commencing at positions P1 (−15,191), P2 (−13,851), and P3 (−13,532) relative to the start codon. All three transcript species possess a single intron. The third species of transcript is most abundant followed by the first and second species, respectively. They all possess unique first exons but share exon 4 that contains the entirety of the coding region.

Analysis of the Promoters—To determine the functional organization of the putative promoter elements identified above, we created constructs for both putative CCR8 promoters as well as the first and third promoters of CX3CR1 containing sequentially longer fragments up to −500 bases from transcription start and a long promoter fragment (>1000 bp) cloned in
front of a luciferase reporter gene lacking promoter or enhancer elements. Constructs were transfected into THP-1 and Jurkat cells, and promoter activity was measured (Fig. 3). THP-1 cells are mononuclear in nature, most closely resembling monocytes, whereas Jurkat cells most closely resemble T-cells.

CX3CR1 P2 was omitted from the analysis due to the closeness of the third promoter (300 bp) and the low frequency of P2 transcript. The P1 promoter of CX3CR1, regions from /H11002 105 to /H11002 206 and /H11022/H11002 505, appear to contain positive promoter elements, whereas the region from /H11002 303 to /H11002 403 appears to contain a negative regulatory element. The third promoter contains positive elements in the /H11002 51 to /H11002 120 and /H11002 120 to /H11002 222 regions and negative elements in the /H11002 222 to /H11002 328 region. There also exist cell-specific differences in promoter expression. In particular, the /H11002 403 to /H11002 498 region of the CX3CR1 P3 promoter has a positive regulatory effect in THP-1 cells and a negative regulatory effect in Jurkat cells.

The CCR8 P1 promoter shows no appreciable activity in THP-1 or Jurkat cells. This may indicate that this promoter is only active in other cell types or that there are positive regulatory elements in the intron or at a significant distance from the start of transcription. The CCR8 P2 promoter, however, shows a strong positive element from 0 to −46 with a negative element between −46 and −96. The −196 to −296 region has a positive regulatory element in THP1 cells alone. The −196 to −296 region has a negative regulatory effect in THP1 cells but a positive regulatory effect in Jurkat cells and may be involved in cell-specific expression.

Identification and Characterization of Polymorphisms—Single nucleotide polymorphisms in CX3CR1 have been shown to alter susceptibility to both HIV infection and arteriosclerosis (11–15). To further characterize single nucleotide polymorphisms within the genes for CX3CR1 and CCR8, we screened both genes within the −15,192 to −17,242 regions, relative to the start codon, of human CX3CR1 and −16 to −4677 of human CCR8 (Table I). We identified nine additional polymorphisms within the CX3CR1 gene. Eight of these nine polymorphisms are located within the first putative promoter, with the ninth polymorphism being present within the first exon of the gene. Three of these polymorphisms have been described in the Celera data base, with one of them being present within the NCBI SNP data base as well. Within the human CCR8 gene, we identified four polymorphisms within the first promoter, one of which has been identified in the Celera data base and one within the second promoter (Table I). The /H11002 40T/G polymorphism in the second promoter lies within the /H11002 46 promoter construct (Fig. 3) that contains positive regulatory elements.

Two common nonsynonymous coding polymorphisms of human CX3CR1, namely V249I and T280M, were previously shown to result in altered ligand affinity and expression (12). Contrary to a previous study (14), we found both polymorphisms in all ethnic groups examined, although to varying degrees (Fig. 4D). In our samples, we found strong linkage disequilibrium between genotypes of these two coding SNPs across all ethnic groups. Since the most common chromosomal haplotype defined by CX3CR1 promoter and coding SNPs was different for each ethnic group (Fig. 4B), we performed a preliminary analysis of pairwise linkage disequilibrium relationships to determine whether there were differences between ethnic groups. Overall, there was weak linkage disequilibrium between the T280M and −16290T → C SNP and between V249I and the −15854A → G SNP (Fig. 4A). However, there
was a unique linkage relationship in Caucasians, with moderately strong linkage disequilibrium between both the \(\text{CX3CR1} \) V249I and T280M SNPs and the \(15430G\) 3C SNP (Fig. 4). This was not seen in other ethnic groups. Such interethnic differences were also seen for linkage relationships between \(\text{CCR8}\) SNPs (data not shown). This preliminary analysis indicates that there are interethnic differences in linkage relationships involving functional SNPs in the \(\text{CX3CR1}\) protein sequence and promoter. These genomic differences may contribute to interethnic disparities in associations with biochemical and/or clinical phenotypes. These preliminary findings suggest that future studies of genotype-phenotype association should have determinations of all possible SNPs at these loci. The functional consequences due to genomic differences at either \(\text{CX3CR1}\) or \(\text{CCR8}\) may be related to the ethnic-specific intact complement or haplotype block defined by all SNPs rather than to the putative functional consequence of any single SNP in isolation.

Comparison of the Human Gene Structure with Mouse—Since mice are frequently used as immunological models of human diseases, we examined the structures of the \(\text{CCR8}\) and \(\text{CX3CR1}\) mouse genes. We performed 5'-RACE on RNA from

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Fig. 3. Promoter analysis of human \(\text{CCR8}\) and \(\text{CX3CR1}\) genes. Promoter fragments of human \(\text{CX3CR1}\) P1 and P3 promoters and \(\text{CCR8}\) P1 and P2 promoters were cloned into an enhancerless luciferase reporter vector and transfected into THP1 cells or Jurkat cells, and activity was measured. Results are reported as the -fold increase over transfection of the luciferase vector alone ± S.E. from a representative experiment. Putative transcription factor binding sites are also indicated.
mouse spleen cells for CX3CR1 and thymocytes for CCR8 and mapped the corresponding 5' transcripts onto the murine genomic fragment identified in the Celera Mouse Genome Database (Fig. 5). The gene structure of CCR8 appears to be similar between humans and mice. Both have two promoters, where the first promoter initiates transcripts with a single intron and the second promoter commencing just prior to the coding sequence and lacking any introns. Sequence comparison of human and mouse exon 1 shows a substantial amount of sequence identity of 79.5% (Fig. 6).

The human and mouse CX3CR1 genes contain several differences and similarities. Murine CX3CR1 has two putative promoters, with the transcript from first promoter containing a single intron and the transcript of the second promoter containing no introns. This structure is similar to the structure of the mouse and human CCR8 genes and differs from human CX3CR1, which contains three putative promoters that all produce transcripts with a single intron. Comparison of the genomic sequences also revealed two conserved nonrepetitive and noncoding elements in the human and mouse CX3CR1 genes. The first conserved region (Fig. 6, alignment 1) is located upstream of the third human and first mouse putative promoters. This region also overlaps with a positive regulatory region identified in our promoter analysis (Fig. 3). The second conserved region is contained within intron 3 in humans and intron 1 in mice. Furthermore, a region within exon 3 of mouse CX3CR1 has an 83% sequence identity with a region in exon 1 of mouse CX3CR1.

### TABLE I

| Relative to CDS | Absolute Position (AY016370) | Location | Polymorphism | Comments |
|----------------|-------------------------------|----------|--------------|----------|
| CX3CR1         |                               |          |              |          |
| -837           | 84,728                        | CDS      | C/T          | T280M Thr → Met |
| -744           | 84,635                        | CDS      | G/A          | V249I Val → Ile |
| -15,170        | 68,721                        | Exon 1, 5'-UTR" | T/G       | Celera (hCV7900512) |
| -15,430        | 68,461                        | Promoter P1 | G/C         |          |
| -15,850        | 68,041                        | Promoter P1 | G/A         |          |
| -15,854        | 68,037                        | Promoter P1 | T/C         |          |
| -16,253        | 67,638                        | Promoter P1 | A/G         |          |
| -16,290        | 67,601                        | Promoter P1 | T/C         | Celera (hCV15824069), NCBI dbSNP (2853707) |
| -17,182        | 66,729                        | Promoter P1 | G/A         | Celera (hCV11580347) |
| -17,184        | 66,707                        | Promoter P1 | A/G         |          |
| -17,186        | 66,705                        | Promoter P1 | T/C         |          |
| CCR8           |                               |          |              |          |
| +79³           | 17,982                        | CDS      | G/C          | Celera (hCV15824045) Gly → Ala at position 27 |
| -40            | 18,022                        | Promoter P1 | T/G         |          |
| -2994          | 20,976                        | Promoter P1 | G/T         |          |
| -3522          | 21,504                        | Promoter P1 | C/T         | Celera (hCV11541953) |
| -3571          | 21,533                        | Promoter P1 | C/T         |          |
| -3876          | 21,658                        | Promoter P1 | A/G         |          |

³ 5'-Untranslated region.

### FIG. 4. Analysis of human CX3CR1 single nucleotide polymorphisms.

Seven SNPs were identified in the human CX3CR1 P1 promoter, and one was identified in the human CX3CR1 first exon in addition to the previously described T280M and V249I coding polymorphisms. A, linkage disequilibrium between CX3CR1 SNPs was estimated using a maximal likelihood method with p value adjusted for multiple comparisons. B, most commonly found alleles in Caucasian, African, South Asian, Chinese, Inuit, and Native ethnic groups. C, linkage disequilibrium between CX3CR1 SNPs in the Caucasian population alone. D, frequency of Ile249 and Met280 coding polymorphisms in multiple ethnic groups. 60 subjects were studied for each ethnic group. *, p < 0.05, **, p < 0.001.
The CX3CR1/CCR8 Duplication Predates the Fish/Mammal Division—To date, a small number of chemokine receptors, namely CXCR1 and -4 and CCR1, -7, and -9, have been described in various fish species. The majority of receptors, however, do not currently have a fish homologue. Recently, the draft genome of the pufferfish _Fugu rubripes_, a teleost fish, has been released. This species appears to be unique in that whereas it maintains the same number of genes as similar species, the genome is only one-eighth the size. We screened the pufferfish genome and identified three putative chemokine receptors present within a single contig (Fig. 6). Whereas direct comparison with other receptors fails to give an unambiguous identity, phylogenetic analysis suggests that the three receptors represent CX3CR1, CCR8, and CCR2, respectively (Fig. 1). Interestingly, none of the receptors appear to possess the entire coding sequence on a single exon as is the case for their mammalian counterparts. Whether this is the result of the process by which fugu compacts its genome or is indicative of these genes being converted to pseudogenes has yet to be determined. Nevertheless, this appears to be a primitive locus corresponding to at least part of the primary chemokine cluster on chromosome 3 and indicates that the CX3CR1/CCR8 duplication predates the division of mammals and fish.

In summary, CCR8 and CX3CR1 appear to have been duplicated prior to the branching of mammals and fish. Throughout evolution, the human and mouse genes have maintained many similar features both within coding and noncoding regions, although differences, such as the loss of one promoter and the addition of two others in human CX3CR1, have resulted and are likely to contribute to the interspecies variation in gene regulation. Both genes possess coding and promoter polymorphisms that may alter susceptibility to various diseases.

**DISCUSSION**

In the present study, we have determined the human and mouse genomic organization of the genes for CX3CR1 and CCR8. In humans and mice, these two genes are located within 66 and 24 kb of each other, respectively, in a head to head fashion. In pufferfish, the genes are located 4 kb from each other in a tail to head manner (Fig. 6). Based on amino acid composition, CX3CR1 and CCR8 are more closely related to each other than they are to any other chemokine receptor. The close proximity and high amino acid identity indicates that these genes probably arose through a duplication event prior to the divergence between fish and mammals. Structurally, the gene for mouse _CCR8_ is similar to the structure of the mouse gene for _CX3CR1_ and the human gene for _CCR8_. This suggests that the duplication event encompassed a region larger than the coding sequence alone and included additional promoter elements. In fact, the entire chemokine receptor cluster on chromosome three appears to be a result of sequential duplication events. The premise that at least some of these duplications include more than just the coding region of genes is supported by the fact that several other genes appear to have also been duplicated within the cluster, including a series of krueppel-related C2H2-type zinc finger proteins, elongation factors, keratin genes, ribosomal proteins, and WD domain-containing proteins interspaced within the chemokine receptors (data not shown) in both mice and humans.

Despite the close amino acid homology of CX3CR1 and CCR8, the ligands for these receptors are phylogenically different. This raises interesting questions about the evolution of chemokine receptors and their ligands. External binding domains of the CX3CR1 and CCR8 receptors are conserved, since both can bind vMIP-II; the receptors have obviously di-
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verged to accommodate binding of very different ligands. CCR8 binds CCL1 (I309), and CX3CR1 binds CX3CL1 (fractalkine). Phylogenetic chemokine analysis shows that these two chemokines fall within different subtrees (data not shown). This probably indicates that chemokine receptor evolution through duplication does not parallel the duplication of the chemokines themselves and that duplicated receptors undergo significant changes in their ligand binding properties. Since the majority of the receptor is not directly involved in ligand binding, these nonbinding regions provide a more accurate evolutionary history of the molecule. The extracellular binding portions of divergent receptors may have convergently evolved in some cases to bind similar ligands. For example, CXCR3 resembles two other receptors more closely than CXCR4, but SDF1 and ITAC (ligands for the two respectively) are most closely related to each other.

Of the chemokine receptor genes examined in detail, multiple promoters have been found in all cases (28–33). In fact, the 5’-untranslated regions of chemotactic receptor genes tend to be heterogeneous with multiple exons often accompanied by alternate splicing. CXCR2, for example, has 12 alternately spliced first exons (29, 32). CCR5 has three alternately spliced exons transcribed by two promoters preceding the fourth exon that contains the entire coding region (28). Typically, the coding sequencing is contained in a single exon, although the receptor CCR7 is coded for in multiple exons (6). Our work confirms this trend, showing that CCR8 and CX3CR1 possess multiple promoters in both humans and mice, although there appear to be significant differences between mouse and human CX3CR1. Mouse CX3CR1 as well as mouse CCR8 and human CCR8 all have promoters (P2 promoters) that produce transcripts containing the coding sequence (and flanking 5’- and 3’-untranslated regions) but contain no introns. This is not the case in human CX3CR1. The corresponding promoter appears to have been lost following the divergence between human and mouse lineages. However, the P3 region and third exon of human CX3CR1 are highly homologous to the P1 region and first exon of mouse CX3CR1 gene. Human CX3CR1 possesses two additional promoters (P1 and P2) and exons located upstream of P3. These promoters are not present in mouse CX3CR1, mouse CCR8, or human CCR8 genes. It is possible, however, that the transcripts we used to define the promoter regions for each of these genes do not represent the full complement of transcripts, and cryptic promoters may be functional in alternate tissues.

This raises some interesting questions in regard to gene regulation. Our data would suggest similarities and differences in the regulation of human CX3CR1 and mouse CX3CR1. Whereas the homologous promoter/ exon between human and

![Fig. 6. Interspecies comparison of the CX3CR1 and CCR8 genes in humans, mice, and pufferfish (fugu). In humans and mice, the CX3CR1 and CCR8 genes are located in a head to head fashion in their respective genomes. Their order relative to other genes in the cluster is also shown. Sequence comparison was performed using the Blast2Sequences program between the corresponding genomic regions premasked for repetitive elements. Four regions of homology outside of the coding sequences were identified: 1) first exon of CCR8 in both human and mouse genes, 2) the P3 promoter region of the human CX3CR1 gene and the P1 promoter region of the mouse CX3CR1 gene, 3) third and first exons of the human and mouse CX3CR1 genes, respectively, and 4) an intronic element conserved between human and mouse CX3CR1. For conserved sequences that are nontranscribed, putative transcription factor binding sites shared between mouse and human sequences are also identified.](image)
mouse CX3CR1 may provide for an overlapping function, the distinct promoters in both species may contribute to species-specific roles for this gene.

Interestingly, Garin et al. (41) utilized our initial report (GenBank™ accession number AY016370) of sequencing the CX3CR1 locus, defining the CX3CR1 P1 and P3 promoters, to identify three CX3CR1 promoters. They were able to confirm our location of the P1 and P3 promoters but additionally identified a unique third promoter distinct from the P2 promoter we report in the present study. They did not examine the organization of CCR8 or SNPs located within the promoter regions of either gene.

We also observed an additional highly conserved region (33 bases, 94% conserved) within the large intron of human and mouse CX3CR1. This region does not appear to be a repetitive element as determined by RepeatMasker (available on the Internet at ftp.genome.washington.edu/RM/RepeatMasker.html).

Neither of these conserved regions within CX3CR1 have reported gene polymorphisms, either from our observations or data present in the NCBI dbSNP or Celera databases. Nevertheless, we found eight polymorphisms within the first putative promoter of CX3CR1. To date, two identified human CX3CR1 coding polymorphisms have been implicated in altered expression and function of the receptor, namely the V249I and T280M mutations (11–14). Defining the individual contributions of these polymorphisms is complicated because they are in linkage disequilibrium, and homozygotes for both Ile249 and Thr280 are rare. Nevertheless, initial studies suggest that the Ile249 polymorphism correlates with a 35% decrease in cell surface receptor number (12) and the Met280 polymorphism is associated with decreased affinity for the CX3CL1 ligand (14). The Ile249 has been associated with decreased availability of the CCR5 and the second producing a transcript consisting of the coding sequence and the second producing a transcript consisting of the coding exons exclusively. The CX3CR1 receptor, however, appears to have undergone subsequent evolution in the human, leading to the addition of two other noncoding exons and the loss of the P2 promoter in mice. Despite all nontranscribed regions in both the P3 promoter (P3 human, P1 mouse) and intron have persisted to the present day, highlighting both the overlapping function of these receptors and their interspecies diversity. Within humans alone, polymorphisms within the receptor lead to altered phenotypes and changes in disease susceptibility. Within different racial groups, the distribution and linkage of these polymorphisms has led to interethnic diversity that may well contribute to a RACE-specific impact of a polymorphism block defined by all SNPs rather than a single polymorphism in isolation.

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REFERENCES

1. Sallusto, F., Mackay, C. R., and Lan泽vechia, A. (2000) Annu. Rev. Immunol. 18, 593–620
2. Murphy, P. M., Bagnoli, M., Charn, I. F., Hebert, C. A., Horuk, R., Matsuhashi, K., Miller, L. H., Oppenheim, J. J., and Power, C. A. (2000) Pharmacol. Rev. 52, 145–176
3. Nelson, P. J., and Kreysky, A. (2001) Immunity 14, 377–386
4. Randolph, D. A., Huang, G., Carruthers, C. J., Bromley, L. E., and Chaplin, D. D. (1999) Science 286, 2159–2162
5. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Gac, D. R., Zlotnik, A., and Schall, T. J. (1997) Nature 385, 640–644
6. Schwickart, V. L., Rapoport, C. J., Godiska, R., Byers, M. G., Eddy, R. L., Jr., Shares, T. B., and Gray, P. W. (1994) Genes Dev. 8, 643–650
7. Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., Streit, W. J., Salafarantz, M. N., Adhikari, S., Thompson, D. A., Botti, P., Bacon, K. B., and Feng, L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10896–10901
8. Chen, S., Bacon, K. B., Li, L., Garcia, G. E., Xia, Y., Lo, D., Thompson, D. A., Siani, M. A., Yamamoto, T., Harrison, J. K., and Feng, L. (1998) J. Exp. Med. 188, 193–198
9. Combesadre, C., Salzwedel, K., Smith, E. D., Tiffany, H. L., Berger, E. A., and Murphy, P. M. (1998) J. Biol. Chem. 273, 23799–23804
10. Fossati, A., Bouchet-Delbos, L., Berrebi, D., Durand-Gasselin, I., Courbe, L’Hermine, A., Kreyssig, R., Galanau, P., Levy, Y., and Emile, D. (2001) Blood 98, 1678–1686
11. Hendel, H., Winkler, C., An, P., Roemer-Binns, E., Nelson, G., Haumont, P., O’Brien, S., Khallili, K., Zagury, D., Rapoport, J., and Zagury, J. F. (2001) J. Acquired Immune Defic. Syndr. 26, 507–511
12. Moatti, D., Faure, S., Fumeron, F., Amara, M., Seknadji, P., McDermott, D. H., Debro, P., Aumont, M. C., Murphy, P. M., de Porest, D., and Combesadre, C. (2001) Blood 97, 1925–1929
13. McDermott, D. H., Calla, J. S., Kleeberger, C. A., Plankey, M., Rosenberg, P. S., Smith, E. D., Zimmerman, P. A., Combesadre, C., Leitman, S. F., Kaslow, R. A., Goedert, J. J., Berger, E. A., O’Brien, T. R., and Murphy, P. M. (2000) Science 290, 2031
14. Faure, S., Meyer, L., Costagliola, D., Vandenbergh, C., Genin, E., Aturan, B., Delfs, E. J., McDermott, D. H., Murphy, P. M., Debré, P., Theodorou, I., and Combesadre, C. (2000) Science 287, 2274–2277
15. McDermott, D. H., Halcox, J. P., Schenke, W. H., Walsawia, M. A., Merrell, M. N., Epstein, N., Quayumi, A. A., and Murphy, P. M. (2001) Circ. Res. 89, 401–407
16. Jinno, A., Shirimizu, N., Soda, Y., Haraguchi, Y., Kitamura, T., and Hoshino, H. (1998) Biochem. Biophys. Res. Commun. 243, 497–502
17. Goya, I., Gutierrez, J., Baraona, R., Kremer, L., Zaballos, A., and Marquez, G. (1998) J. Immunol. 160, 1975–1981
18. Horuk, R., Besselsgerg, J., Zhou, Y., Paulds, D., Halke-Miller, M., Harvey, S., Toub, D., Samson, M., Parmentier, M., Rucker, J., Deran, J. B., and Doms, R. W. (1998) J. Biol. Chem. 273, 386–391
19. Roos, R. S., Loetscher, M., Legler, D. F., Clark-Lewis, I., Baggiolini, M., and Clark, L. J. (1997) J. Biol. Chem. 272, 17251–17254
20. Tiffany, H. L., Lautens, L. L., Gao, J. L., Pease, J., Locati, M., Combesadre, C., Modi, W., Bonner, T. I., and Murphy, P. M. (1997) J. Exp. Med. 186, 165–170
21. D’Ambrosio, D., Ilem, A., Bonecchi, R., Mazzeo, D., Sozanni, S., Mantovani, A., and Sinigaglia, F. (1998) J. Immunol. 161, 5111–5115
22. Zingoni, A., Soto, H., Hedrick, J. A., Stoppacciaro, A., Storlazzi, C. T., Sinigaglia, F., D’Ambrosio, D., Garra, A., Robinson, D., Rochi, M., Santoni, A., Zlotnik, A., and Napolitano, M. (1998) J. Immunol. 161, 547–551
Characterization of the CX3CR1/CCR8 Locus

23. Haque, N. S., Fallon, J. T., Taubman, M. B., and Harpel, P. C. (2001) Blood 97, 39–45.

24. Haque, N. S., Zhang, X., French, D. L., Li, J., Poon, M., Fallon, J. T., Gabel, B. R., Taubman, M. B., Kaschinsky, M., and Harpel, P. C. (2000) Circulation 102, 786–792.

25. Chensue, S. W., Lukacs, N. W., Yang, T. Y., Shang, X., Frait, K. A., Kunkel, S. L., Kung, T., Wiekowski, M. T., Hedrick, J. A., Cook, D. N., Ziegus, A., Narula, S. K., Zlotnik, A., Barrat, F. J., O’Garra, A., Napolitano, M., and Lira, S. A. (2001) J. Exp. Med. 193, 573–584.

26. Luttichau, H. R., Stine, J., Boesen, T. P., Johnsen, A. H., Chantry, D., Gerstoft, J., and Schwartz, T. W. (2000) J. Exp. Med. 191, 171–189.

27. Dairaghi, D. J., Fan, R. A., McMaster, B. E., Hanley, M. R., and Schall, T. J. (1999) J. Biol. Chem. 274, 21569–21574.

28. Mummidi, S., Ahuja, S. S., McDaniel, B. L., and Ahuja, S. K. (1997) J. Biol. Chem. 272, 30662–30671.

29. Ahuja, S. K., Shetty, A., Tiffany, H. L., and Murphy, P. M. (1994) J. Biol. Chem. 269, 26381–26389.

30. Moriuchi, M., Moriuchi, H., Turner, W., and Fauci, A. S. (1997) J. Immunol. 159, 4322–4329.

31. Zimmermann, N., Daugherty, B. L., Kavanaugh, J. L., El Awar, F. Y., Moulton, E. A., and Rothenberg, M. E. (2000) Blood 96, 2346–2354.

32. Sprenger, H., Lloyd, A. R., Lautens, L. L., Bonner, T. I., and Kelvin, D. J. (1994) J. Biol. Chem. 269, 11065–11072.

33. Yamamoto, K., Takushima, H., Hamada, K., Nakao, M., Kino, T., Nishii, T., Kochi, M., Kuratsu, J., Yoshimura, T., and Ushio, Y. (1999) J. Biol. Chem. 274, 4646–4654.

34. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680.

35. Hegele, R. A., Plaetke, R., and Lalouel, J. M. (1990) Genet. Epidemiol. 7, 69–81.

36. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884.

37. Tatusova, T. A., and Madden, T. L. (1999) FEMS Microbiol. Lett. 174, 247–250.

38. Rice, P., Longden, I., and Bleasby, A. (2000) Trends Genet. 16, 276–277.

39. Deal, C., Ma, J., Wilkin, F., Paquette, J., Rosen, F., Ge, B., Hudson, T., Stampler, M., and Pollak, M. (2001) J. Clin. Endocrinol. Metab. 86, 1274–1280.

40. Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package), Version 3.5c, University of Washington, Seattle.

41. Garin, A., Pellet, P., Deterre, P., Debre, P., and Combadiere, C. (2002) Biochem. J. 15, 753–760.