Cloning and functional characterization of the human fractalkine receptor promoter regions

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We have previously shown that reduced expression of the fractalkine receptor, CX3CR1, is correlated with rapid HIV disease progression and with reduced susceptibility to acute coronary events. In order to elucidate the mechanisms underlying transcriptional regulation of CX3CR1 expression, we structurally and functionally characterized the CX3CR1 gene. It consists of four exons and three introns spanning over 18 kb. Three transcripts are produced by splicing the three untranslated exons with exon 4, which contains the complete open reading frame. The transcript predominantly found in leucocytes corresponds to the splicing of exon 2 with exon 4. Transcripts corresponding to splicing of exons 1 and 4 are less abundant in leucocytes and

splicing of exons 3 and 4 are rare longer transcripts. A constitutive promoter activity was found in the regions extending upstream from untranslated exons 1 and 2. Interestingly, exons 1 and 2 enhanced the activity of their respective promoters in a cell-specific manner. These data show that the CX3CR1 gene is controlled by three distinct promoter regions, which are regulated by their respective untranslated exons and that lead to the transcription of three mature messengers. This highly complex regulation may allow versatile and precise expression of CX3CR1 in various cell types.

Key words: chemokine receptor, CX3CR1, gene organization.

INTRODUCTION

CX3CR1 is the receptor of the chemokine CX3CL1 (previously known as fractalkine), the only member of a new class of chemokines characterized by three amino acids between the first two cysteines [1]. CX3CL1 and another chemokine CXCL16 [2] exist in both soluble and membrane-anchored forms. Soluble CX3CL1 was reported to recruit lymphocytes and monocytes [1,3]. The anchored CX3CL1 was initially described on the surface of interleukin-1- or tumour necrosis factor-activated endothelial cells. The anchored CX3CL1 was also observed in various organs such as the intestine, kidney, heart, brain, lung, skin, tonsils, skeletal muscle and pancreas [1,4–9]. The membrane-bound CX3CL1 directly mediates the capture and firm adhesion of CX3CR1-expressing leucocytes [10,11], thus providing a novel pathway for leucocyte activation. Along with strong expression on monocytes and natural killer (NK) cells [10], CX3CR1 is abundant on glial cells and astrocytes [12] and may directly modulate neuronal activity and survival [13,14]. CX3CR1's function remains elusive. Like a few other chemokine receptors, CX3CR1 has been subverted by HIV and serves as an HIV-1 co-receptor [15,16], its interaction with gp120 being blocked specifically by CX3CL1 [17]. However, the pattern of HIV strains that can use CX3CR1 is restricted, suggesting that this receptor may not play a key role in HIV infection.

Surprisingly, genetically manipulated mice lacking CX3CR1 [18] were indistinguishable from wild-type mice in both physiological and pathological conditions. However, recent data demonstrated that CX3CL1 knockout mice are less susceptible to cerebral ischaemia/reperfusion injury [19], confirming the importance of the CX3CR1/CX3CL1 axis in brain physio-

pathology. Recently we have reported that CX3CR1 polymorphisms provide a new marker for prognosis of HIV disease progression [20] and an independent genetic risk factor for human coronary artery disease [21]. In these studies, HIV disease acceleration and protection against acute coronary events were associated with a sharp decrease in the number of CX3CR1 molecules on the leucocyte cell surfaces. Thus, understanding mechanisms that control HIV co-receptor expression and adhesion of leucocytes to endothelial cells via regulation of CX3CR1 expression should lead to greater insight into disease control.

Here we describe the cloning and functional characterization of the promoter regions of human CX3CR1. Alternative splicing generates three transcripts that differ only by their untranslated regions (UTRs). We identified three functional promoter regions controlling the expression of each of the transcripts. These complex regions may allow conditional cell-specific CX3CR1 expression.

EXPERIMENTAL

Cells and culture conditions

Resting peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood taken from healthy volunteers by one-step centrifugation on a Ficoll separating solution (Biochrom KG, Berlin, Germany). CD4 T-lymphocytes, CD8 T-lymphocytes and NK cells were isolated by positive selection using, respectively, anti-CD4 monoclonal antibodies, anti-CD8 monoclonal antibodies or a mixture of anti-CD16 and anti-CD56 monoclonal antibodies coupled with magnetic beads (Miltenyi Biotech, Paris, France). Monocytes were enriched by negative selection using a mixture of anti-CD2, -CD7, -CD16,

Abbreviations used: AP1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; NFAT, nuclear factor of activated T-cells; NF1, nuclear factor-1; NK, natural killer; ORF, open reading frame; PBMC, peripheral blood mononuclear cell; 5'-RACE, 5'-rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR; SP1, stimulating protein-1; STAT, signal transduction and activators of transcription; UTR, untranslated region.

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-CD19 and -CD56 monoclonal antibodies coupled with magnetic beads (DynaL France S.A., Compiègne, France). The enriched cell populations were 95 % pure, as confirmed by flow cytometric analysis. HL60, THP-1, U87, HeLa and HEK-293 cell lines were maintained in RPMI 1640 (Life Technologies SARL, Cergy Pontoise, France) supplemented with 10 % fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

RNA analysis by 5'-rapid amplification of cDNA ends (5' RACE)

Cellular mRNA was prepared from PBMCs and THP-1 cells using the small-scale µMACS mRNA Isolation Starting Kit (Miltenyi Biotech), using 30 units of DNase I to prevent genomic contamination. The 5'-RACE was performed according to the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). Briefly, after first-strand cDNA synthesis, reverse-specific primers, designed in the 5' region of the open reading frame (ORF; RACE-SP1, 5'-TGAGTCCAGAAGGGCAAAGTGGCTA-3'; and RACE-SP2 for nested PCR, 5'-GGCCAATGGCAAAGATGACGGAGTA-3'), were used in association with Universal Primers to selectively amplify CX3CR1 transcripts. PCR products were electrophoresed in a 1.5 % agarose gel, purified, cloned into PCR 2.1 Topo (Invitrogen, Leek, The Netherlands) and then sequenced on both strands using the BigDye Terminator kit (Applied Biosystems, Warrington, Cheshire, U.K.).

Isoform-specific reverse transcriptase PCR (RT-PCR)

After reverse transcription, cDNAs from different cell types were amplified using forward primers specific to the three alternative mRNAs obtained (SP1-Ex1, 5'-AGAGCTCTCTGGCTTCTGGGTGGAGAA-3'; SP1-Ex2, 5'-TGGCTGACTGGCAGATC-CAGAGGTT-3'; SP1-Ex3, 5'-TGGATGTGAAGCCAAAAGAGAGGGAGA-3') and reverse primers common to the three transcripts (RACE-SP1). The PCR conditions were as follows; denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Hypoxanthine phosphoribosyl-transferase was used to normalize the amount of mRNA between different cell types.

Genomic DNA analysis

Human CX3CR1 genomic clones were isolated using Genome Walker kits (Clontech) following the manufacturer's recommendations. Briefly, reverse-specific primers were designed in the 5'-UTR portion of the published cDNA upstream from the putative boundary between the two exons: GW-SP1 (5'-GAC-TGCCAAGGGAACCTCTGGATCT-3') was used for the first PCR, GW-SP2 (5'-CCTGTCTCTGCTCAGACTTTACCAGAGA-3') for the nested PCR. Primers were synthesized in both orientations to clone upstream and downstream from the UTR. The resulting amplicons were cloned into PCR 2.1 Topo. Two independent clones named Pr 0.6 and Pr 2 were obtained and sequenced on both strands. The promoter sequence was analysed by using the publicly available Promoter and Transcription Factor Binding Site Prediction facility at <http://transfac.gbf.de/cgi-bin/matSearch/matsearch2.pl> [22].

Construction of reporter plasmids

PCR was used to generate a series of truncated forms of the CX3CR1 promoter region, forward primers containing a *KpnI* tail and reverse primers containing a *XhoI* tail. PCR was performed on the Pr 2 genomic clone using standard conditions

(94 °C for 20 s, 55 °C for 20 s and 72 °C for 60 s) using 0.5 units of platinum *Taq* polymerase (Life Technologies SARL) complemented with a 10-fold lower concentration of *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The PCR products were double-digested and inserted into the luciferase basic plasmid vector pGL3-Basic (Promega, Charbonnières, France). The constructs were then sequenced on both strands to confirm their authenticity.

Promoter assay

Cells were transfected using Transfast technology (Promega), and were treated according to the manufacturer's instructions. Briefly, cultured cells seeded at 200 000 cells/well on a 24-well plate were grown overnight. The cells were co-transfected using 0.3 µg of Transfast reagent with 0.1 µg of pGL3 constructs that use firefly luciferase as a reporter of transcriptional activity and 0.01 µg of pRL plasmid (Promega) that use *Renilla* luciferase to monitor transfection efficiency. In each experiment, pRL, in which the *Renilla* luciferase gene is driven by the cytomegalovirus promoter, was used as a positive control. After 24 h, the cells were harvested and collected by centrifugation. The cell pellets were washed with PBS, lysed in 100 µl of lysis buffer and tested for luciferase activity using a single-tube assay (Dual-Luciferase Reporter Assay System; Promega) with a luminometer (Lumat LB9501, Berthold Technologies GmbH, Bad Wildbad, Germany).

Statistical analysis

Data handling, analysis and graphical representation was performed using Prism 2.01 (GraphPad Software, San Diego, CA, U.S.A.). Statistical analysis was performed by paired two-sample Student's *t* test for means.

RESULTS

Characterization of the 5'-UTR of CX3CR1 mRNA

To better characterize the 5'-ends of the transcripts coding for CX3CR1, we performed a 5'-RACE extension on mRNA isolated from PBMCs. When RACE products were analysed by gel electrophoresis, five bands were obtained (Figure 1A). Similar results were obtained with THP-1, a monocytic cell line that expresses CX3CR1 (results not shown). The two lower bands correspond to short messengers probably resulting from mRNA degradation. The three upper bands appeared at ≈ 250, 300 and 350 bp. Amplification products were cloned, and 10 clones of each size were randomly picked for sequence analysis. The sequenced products cloned from the ≈ 250 bp band represented the previously published CX3CR1 mRNA called V28 [23], an mRNA species presenting 87 nucleotides upstream from the start codon (Figure 1B). We could not extend V28, suggesting that the first nucleotide observed may be very close to the transcription start point. The two upper bands did not contain any V28 messengers but did contain two new transcripts of CX3CR1 mRNA, called clone 6 and clone 10. The longest 5'-UTRs for clones 6 and 10 were 183 and 136 nucleotides long respectively. Their sequences diverged from V28 and from a ≈ 4 kb CX3CR1 genomic sequence [24], upstream from nucleotide -9, thus defining the presence of an intron at that position.

These results indicate that the CX3CR1 gene is composed of an exon containing the full ORF and at least three untranslated exons. We could not detect transcripts containing a combination of the different 5'-UTRs described here, suggesting that either the long mRNAs containing multiple CX3CR1 exons were

Figure 1 5'-RACE performed from PBMC mRNAs

(A) 5'-RACE was performed on RNA isolated from PBMCs and PCR products were run on a 1.5% agarose gel. Arrows indicate the localization of human CX3CR1-specific products after nested PCR. The length of each PCR product is indicated in bp. M, 100 bp ladder. (B) Separate clones were sequenced. The longest sequences are represented here for the three alternative messengers found. The conserved sequence between cDNA and genomic sequences is underlined. Specific messenger primers used for semi-quantitative RT-PCR are underlined with a dotted line. The P4 genomic sequence has been described previously [24].

Figure 2 Semi-quantitative RT-PCR analysis of the three alternative human CX3CR1 mRNAs

(A) Poly(A)⁺ RNA from PBMCs (lanes 1) and THP-1 cells (lanes 2) were reverse-transcribed and cDNA product was subjected to specific messenger PCR amplification. Three sets of amplification conditions were performed (25, 30 and 35 cycles) and hypoxanthine phosphoribosyltransferase (HPRT) was used as the control. ND, not determined. (B) RT-PCR was performed on poly(A)⁺ RNA from NK cells (NK), CD4 T-lymphocytes (CD4), CD8 T-lymphocytes (CD8), monocytes (Mono) and B-lymphocytes (B).

expressed at a very low level or the transcripts may not arise from splicing events but from distinct transcriptional activities.

Expression of human CX3CR1 transcripts

We identified three CX3CR1 transcripts that differ only in their 5'-UTR. Primers were designed to specifically amplify each of the CX3CR1 transcripts to determine their relative level of expression in two cell types, PBMCs and THP-1 (Figure 2A). Interestingly, the three transcripts were present in each cell population tested but 25 amplification cycles were enough to show V28 mRNA in PBMCs and THP-1, whereas 35 cycles were needed to detect clone 6 expression. Clone 10 was slightly less abundant than V28. Besides, similar relative levels of expression of these transcripts were detected in each leucocyte subpopulation tested, CD4 T-lymphocytes, CD8 T-lymphocytes, NK cells and monocytes (Figure 2B). Whereas overall clone 6 expression was low, there was relatively greater expression of V28 transcripts than clone 10 transcripts in these cell types. Interestingly, no expression of CX3CR1 could be detected in B-lymphocytes. These results show that three distinct CX3CR1 transcripts are produced in leucocytes, at different levels.

Genomic organization of CX3CR1

A genomic library was screened to determine CX3CR1 gene organization using Genome Walker technology. Initially we based our research on the most abundant transcript described that corresponded to the V28 mRNA. We cloned a 2 kb insert (called Pr 2) that included the V28 5'-UTR from nucleotide 1 to 74 (Figure 3). Analysis of this sequence revealed that it also contained the 5'-UTR of clone 10 from nucleotide 1 to 127. However, we could not localize clone 6 on this genomic clone. We were also unable to produce, by PCR, a genomic fragment using forward primers sitting in the 3'-end of the 2 kb clone and reverse primers in the 5'-end of CX3CR1 ORF, suggesting that the intron was probably too long to be amplified. Recently, a contig of ≈ 86 kb was released to the GenBank

taaagaggggtaaaatcacgtcctggacaggacagccaggtgagttgggaaggaagagagcctgccacgggacagggcatgttggggga
TATA **c/EBP**
 agtggaaagtggcgagagcacagtaggaagtgagaaGGGGCGGGCCGTGCTTACCAGGCCGTGGACTTAAACCAGGATGAGAGAACCCCTG
EXON 1 (127 bp)
GAGGCGTTTAAGTTGGCAGACTTGGATTTCAGGAAGAGCTCTCTGGCTTCTGGGTGGAGAATGGCCAGTGGGgt aagtggtgagaggaaa
NFAT
 gacagagaacgggagaaggttagatgggcttgggaaatccaggccctggatggaggtagagatgtgtgctcatgaacacggaggggat
c/EBP
 tactgatgtggggtggatgagactgtcgtcaagagtgtgggacaggaagagaggagagtccttggccagatccaagaaaggagccctcag
INTRON 1 (1577 bp)
 aagaggaggggagtcagaggcaaggaaagggtgagggcagccagccagctgagtgagaccaggagaggtatcacagggtggtgtgggggt
SP1
ggggagggggccagtgtcagaaagtggatggggagcggcctgactctgcttttgcctgtggccttctggccaaaggcagggaaagggtggc
SP1 **NFAT**
 caaacactgagaccaagaacaaagaagaaactgctggtggacttctccaccacgagcagggccaccaagcccgcagcactgcacttgc
CAAT **NFAT**
 agccccagctctgcccgtggggttggggaggtgaggaggggcaaggtgggagcacacagagcaccgcgtgtcctcggaaccccacagcg
SP1 **GRE**
 actagaggttaagggagcaccggatgtggctgggatgtgggcagcaagggggccagaggggccttgaaggggtcacagaccatttaataag
CAAT
 gtgtattgaaggccaccatgggcccagccctagttaggatggatcagaattatagcatatgccaggggtcaggcaggttaataagtg
 atcggaaggtgatgaggcagtgagcttgagattcacgttgagtcgccccaaagctggccaggccaggagcagaagcatggctggatgc
 cggagcccaccaggctccccactgcagggcaagagtggcagggggagagactgtgaaaggagcataggccaggtcctgggtgaaagctgt
 gtcctcagccttgactgatgggtataggagccactaaatgccttggggcagagaggtgaggaaaaaaatattaccgagcatctacaag
CAAT **c/EBP**
 gtgcaaggtactcactagatgccttcagtagcacaagcttctcaaaacttagtatgcataatcactcttctaagaatttcattaaatgcaga
 ttctaatcagcagatatagggcagggccttgaggtgctgtctttaaataagctcccagtgccctgggactgcactttgaggagaagagctgt
AP1 **GATA**
 gtgtgcccagctgtggtcctgtgagtactctgggtccctctcatgggagggaagctgaggggcccatgagctctccagcttccctgaa
SP1
 ggctccccattaatgagagctgactgtgtgtgtgttctgactgcagggcctgctccctgccccccacctccaggttggggtaagtggc
c/EBP
 acctctctccctccagctccgcagttctccctgaggtttagatctccaggtttataaaagtcaggccctcctgttgccagctggcctcca
TATA
 ccctggagtatctgagcttgcctgtggcagcatctaaagatagctcccttacaggaacaagatactattggctaactctgcaataaaa
GATA **STAT** **NFAT** **GATA** **OCT1** **TATA**
 atgctcttagaggaaggaaggaaggaatACTCGTCTCTGGTAAAGTCTGAGCAGGACAGGGTGGCTGACTGGCAGATCCAGAGGTTCCCT
EXON 2 (78 bp)
TGGCAGTCCACGCCAGgt aggtgcacaggactagttgggtacctgtggg-----accagactgaacctcaaacccagctcttcaactttac
INTRON 2 (7298 bp)
 taaaaagccataaacagagaccaGGAGGGTAAAACTACCAGAAGATACACTGGATTAGAAAAACAGTAAGCTGGATGTGAAGCCAAAAG
EXON 3 (174 bp)
AGAGGGAGAAAGCAACATAAAAGAATGTGAGATTGAAGAAGAAATGGATTCTGGCCACCTAAAGAAGAAGGGAAGTATAGAAAAGGGAAG
CTGATTGGAAACAATGGgtaatgatgagtgctcctccctaaaggttaaaaaataaatgagcaggcatgagaaaaggaagtagggcaga
 ggaagtagaccaggatcagccacggatgtgagtggggaattttacaaatttc-----tttgtttctctgataactaacacagttacttgt
INTRON 3 (5959 bp)
 tttttttctgatccagGCCTTCACCATGGATCAGTTCCTGAATCAGTGACAG
EXON 4 ORF

Figure 3 Genomic sequence of the human CX3CR1 gene

Exon sequences are capitalized in bold, while intron and flanking sequences are in lower case. Single underlines indicate potential transcription-factor-binding sites and double underlines indicate exon boundaries. The dotted underline represents sequences common to several chemokine receptor genes. The length, in nucleotides, of each gene region is indicated in parentheses. Exons 1, 2, 3 and 4 correspond to clone 10, V28, clone 6 and ORF respectively. C/EBP, CCAAT/enhancer-binding protein; GATA, GATA-binding site; AP1, activator protein-1; SP1, stimulating protein-1; NF1, nuclear factor-1; NFAT, nuclear factor of activated T-cells; OCT1, octamer-binding factor 1; STAT, signal transduction and activators of transcription; GRE, glucocorticoid response element.

Nucleotide Sequence Database (accession number AY016370) describing the organization of the CX3CR1 gene as being composed of two untranslated exons of 84 and 80 bp and the ORF coding exon. This sequence allowed us to localize the 2 kb of genomic clone from nucleotide 68530 to 70442, as well as the entire 5'-UTR of clone 6 in the 77742–77928 bp region. The ORF resides at positions 83882–86172. Thus the CX3CR1 gene contains three introns and four exons spanning a region of over 18 kb; the three exons forming the 5'-UTR of the gene are located over a region of 9 kb upstream from exon four, which

codes for the ORF (Figure 4). The 5'-end of clone 10 extends transcript 1, described in AY016370, by 43 nucleotides, whereas transcript 2 is two nucleotides longer than the 5'-end of V28.

The three transcripts correspond to the splicing of each of the three 5' exons with exon 4; i.e. clone 10 combines exons 1 and 4, V28 combines exons 2 and 4 and finally clone 6 combines exons 3 and 4 (Figure 4). The splice junctions for these transcripts obey the consensus rules for 5' intron/exon boundaries: GT/intron/AG. Surprisingly, the combination of two or three untranslated exons was not detected by either RACE analysis nor

Figure 4 Genomic sequence and mRNA splicing of human CX3CR1

(A) The scheme depicts genomic organization of CX3CR1 with exons in black boxes and the ORF in a white box. Exons are labelled E1–E4 and introns I1–I3. The sizes of the introns are indicated. (B) Corresponding transcripts are described with angled lines indicating splicing patterns. The name of each mRNA is shown.

exon-specific PCR, suggesting that these splicings are rare events or that these transcripts may not be synthesized. Mutual exclusion of the untranslated exons would suggest that transcription of each species is under the control of a specific promoter.

Analysis of the CX3CR1 promoter sequences

The sequences upstream from each of the untranslated exons were analysed using a publicly available engine (see the Experimental section), allowing prediction of transcription start sites and transcription factor-binding sites (Figure 3). Potential TATA boxes were detected 125 nucleotides upstream from exon 1 and 28 nucleotides upstream from exon 2, but no consensus TATA sequence was detected upstream from exon 3. Only the TATA box present upstream from exon 2 appeared to be at the suitable position to initiate transcription of exon 2. In contrast, the position of the TATA box for exon 1 was too distant from the potential 5'-end of this exon: either the box may be irrelevant or the proposed 5'-boundaries of exon 1 may be upstream of the one described in Figure 3. Several other potential transcription factor-binding sites were found, such as CCAAT/enhancer-binding protein (C/EBP), GATA-binding site (GATA), activator protein-1 (AP1), stimulating protein-1 (SP1), nuclear factor-1 (NF1), nuclear factor of activated T-cells (NFAT), octamer-binding factor 1 (OCT1) and signal transduction and activators of transcription (STAT), but their functional characterization still remains to be assessed.

The cloned sequences were compared with other chemokine receptor gene sequences using the LALIGN program for best local sequence alignments. This analysis identified an ≈ 80 nucleotide sequence located 495 bp upstream from exon 2 sharing $\approx 60\%$ identity with promoter regions for CCR2 (accession number AF068265), CCR5 (accession number AF032132), CXCR1 (accession numbers U11866 and U11878) and CCR3 (accession number AF237381). This sequence, thought to be common to a few chemokine receptor genes, has been observed by other groups [25], but its role is not yet clear.

Characterization of the functional CX3CR1 promoters

Structural analysis suggested that regions upstream from exons 1, 2 and 3 might contain functional promoters for each of the

Figure 5 Comparison of firefly luciferase activities of promoters P1, P2 and P3 with or without their respective exon

Chimaeric firefly luciferase promoter constructs without (A) or with (B) exons were co-transfected with *Renilla* luciferase control vector in HeLa, HEK-293, HL60 and U87 cell lines. Constructs are termed according to the exon tested and the numbers in brackets are relative to bp +1 of each exon. A dual-luciferase assay was performed, and the activity of firefly luciferase was normalized to that of *Renilla* luciferase. The relative promoter activities were arbitrarily calculated by defining the activity of the pGL3 basic vector as 1. Data are representative of three independent experiments carried out in triplicate, and are expressed as means $\pm$ S.D. In (A) promoter construct activities were compared with pGL3 basic vector activity; * $P < 0.05$. In (B) P1 and P2 activities were compared and significances are indicated; * $P < 0.05$.

transcripts of CX3CR1. To test this hypothesis, 5' sequences extending upstream from exons 1, 2 and 3, respectively called P1 (–631/0), P2 (–601/0) and P3 (–520/0), were subcloned upstream from the luciferase ORF in the plasmid pGL3-basic. The constructs were transfected into a promyeloid cell line HL60, astroglial cell line U87, fibroblastic-type cell line HEK-293 and the cervical adenocarcinoma HeLa cell line, and 24 h after transfection the cell lysates were tested in luciferase assays. A low constitutive promoter activity was observed only for P1 and P2 within HL60 and U87 cells (Figure 5A). We could not detect any constitutive activity for P3 in any cell line tested. These data suggest cell-specific constitutive activity for promoter region P1 and P2. However, the activity of these promoter regions remained weak. Because some untranslated exons are known to have a potential role in promoter regulation, as described for CCR3 [25] and CCR2 [26], we added to each promoter its respective untranslated exon and tested these new extended constructs in luciferase assays. Interestingly, exon 2 enhanced

Figure 6 Deletion analysis of P2 in different cell lines

Chimaeric firefly luciferase promoter constructs without (white bars) or with (black bars) exon 2 were co-transfected with *Renilla* luciferase control vector in the HeLa, HEK-293, HL60 and U87 cell lines. Constructs numbered C1–C7 correspond to sequential deletions of promoter 2 starting at nucleotides –601, –540, –410, –325, –235, –150 and –73 relative to bp +1 of exon 2 and extending to nucleotide +1 (white bars) and +74 (black bars). A dual-luciferase assay was performed and the activity of firefly luciferase was normalized to that of *Renilla* luciferase. The relative promoter activities were calculated arbitrarily by defining the activity of the pGL3 basic vector as 1. Data are representative of three independent experiments carried out in triplicate, and are expressed as means $\pm$ S.D.

the activity of P2 in every cell line tested by a factor of 2–5 (Figure 5B). Although exon 1 increased P1 activity in U87 and HeLa cells by 2–3-fold, it had no effect in HEK-293 and HL60 cells. Furthermore, we noticed that P1-exon 1 activity was lower than P2-exon 2 within each cell line tested ($P < 0.05$). Nevertheless, the presence of exon 3 had no effect on promoter activity. These results show that non-coding exons induced a cell-specific enhancement of the promoter activity. An algorithm predicting transcription factor-binding sites revealed a potential C/EBP-binding site within exon 2 that may be responsible for non-cell-specific P2 activity enhancement. In addition, several potential binding sites for NF1, AP1 or SP1 are present within exon 1, which could explain cell-specific regulation. These potential transcription factor-binding sites remain to be functionally characterized.

Because V28 is the main form found in leucocytes, we focused our analysis on the P2 promoter region. To better delineate the regulating elements and identify the minimal promoter sequences, 5'-end truncations of a 675 bp fragment including or not exon 2 were constructed, were transfected into various cell lines and tested for promoter activity (Figure 6). Within the six deletion constructs of P2 (–601/0) assayed, only the shortest P2 (–73/0)

did not show any luciferase activity whatever the cell line tested, suggesting that the minimal promoter is present in the –150/–73 region. Surprisingly, all the constructs containing exon 2, even the P2 (–73/+74) construct, displayed strong luciferase activity, suggesting that exon 2 provides sufficient constitutive enhancement to activate this region.

DISCUSSION

In the present work, we identified new CX3CR1 transcripts, defined the structural organization of the CX3CR1 gene and delineated regions with high constitutive promoter activity in various cell types. The CX3CR1 gene is, therefore, multi-exonic with the ORF residing entirely in a single exon, like most chemokine receptors. However, unlike previously published chemokine receptor gene organization, three functionally independent promoter regions direct CX3CR1 transcription, giving rise to three different transcripts that may be expressed selectively in different cell populations.

Based on structural organization, the CX3CR1 gene appears extremely complex but nonetheless retains features common to most chemokine receptor genes, as depicted by Mummidi et al.

[27]: (i) the ORF is intronless, (ii) the 5'-UTR is complex, (iii) the AG-acceptor site used for intron splicing resides a short distance upstream from the translation initiation site, (iv) a large intron separates the ORF from UTRs and (v) the CX3CR1 gene is located in the same chromosomal region as most of the CC chemokine receptors.

To date, the CX3CR1 gene is one of the largest chemokine receptor genes, described as having four exons and three introns spanning over 18 kb. A large intron (≈ 6 kb) resides a very short distance upstream from the previously described ORF and thus separates the coding sequence from the untranslated exons. The reasons why multiple 5'-UTRs are maintained, especially when no protein isoforms are generated, remain to be elucidated. However, we have clearly demonstrated the differential regulation of promoter regions P1 and P2, which are cell- and exon-specific. The P3 region was not constitutively active, but the presence of messengers specific to that promoter indicates that some regulatory events could enhance P3 activity. Surprisingly, the presence of exon 2 reveals promoter activity of the short construct, P2 ($-73/0$), suggesting that the minimal promoter element may be located in the $-73/0$ region. However, to be fully active, this region would need enhancer factors to bind in the exonic region. Another possible role for the multiple 5'-UTRs is to modulate the stability of the 5' ends of the transcripts. Thus variations in the secondary structures of the 5'-UTRs may influence translation efficiency. For instance, it has been shown that a hairpin structure ($\Delta G = -30$ kcal/mol) located at the start of the preproinsulin coding sequence does not reduce proinsulin production, whereas a more stable stem-and-loop structure ($\Delta G = -50$ kcal/mol) reduces proinsulin yield [28]. When the 5'-UTRs of CX3CR1 were analysed using an algorithm predicting RNA secondary structure (<http://www.bioinfo.rpi.edu/applications/mfold/>), the Gibbs free energy of formation for exons 1, 2 and 3 was respectively -47.4 , -25.2 and -31.3 kcal/mol. This analysis suggests that exons 2 and 3 may promote a more efficient translation, whereas exon 1 may resist unfolding when scanned by the migrating 40 S ribosome subunit.

Relative expression of the transcripts has been approached using an isoform-specific RT-PCR strategy, and suggests that V28 and clone 10 are similarly expressed in PBMCs, whereas clone 6 species are relatively rare. The semi-quantitative assessment of the different transcript expression performed here may be biased in that isoform-specific PCRs may not have the same efficiency. However, amplicon sizes are similar and the annealing temperature of the primers is identical, suggesting that PCR efficiency may be equivalent. Thus the differences between PCR products observed in Figure 2 may reflect different levels of expression of CX3CR1 transcripts. Still, this question of differential expression needs to be confirmed by using a more quantitative method, such as Northern blot analysis. In addition, further investigation is needed to characterize transcript expression in solid organs such as the brain.

This question of cell-specific expression is even more puzzling because the two main transcripts are under the control of different promoter regions, each containing different transcription-factor-binding sites. A cluster of potential transcription factor-binding sites resides in the close vicinity of the transcription start site of exon 2 and, notably, three tissue-specific *cis*-regulatory elements (GATA- and STAT-binding sites), suggesting cell-specific regulation of CX3CR1 expression. Thus regulation of the expression of these transcripts may differ depending on the cellular context. However, our results correlated the activities of the three promoters with the presence of their respective messengers. For instance, in the presence of their re-

spective exons, P2 has strong and constitutive activity, which was higher than that of P1 in every cell line tested. In parallel, V28 messengers are present in higher quantities than clone 10 messengers, as shown by semi-quantitative RT-PCR.

Multiple promoters regulating the transcription of different splice variants coding for the same protein have already been described for the chemokine receptors CCR5 [27] and CXCR2 [29]. For instance, in the case of CCR5, two promoter regions have been described, P_v and P_d . Interestingly, P_d had a strong constitutive promoter activity in leucocyte cell types contrary to P_v . These two regions are in close proximity (less than 1 kb apart) and may be part of a single complex regulatory unit. In contrast, CX3CR1 promoters are spread over 10 kb, and clearly regulate CX3CR1 transcription independently.

One of the last peculiar characteristics of chemokine receptor genes is that they can be clustered [30,31]. In fact, chemokine receptor family members may be the result of the duplication of an ancestral gene. Thus most of the CC chemokine receptors colocalize on chromosome 3p21 [32], whereas several of the chemoattractant receptors cluster on chromosome 19q13 [33]. CX3CR1 is the only member of the CX3C chemokine receptor class but it is more related to CC chemokine receptors. Not surprisingly, the CX3CR1 gene has been described on chromosome 3p21 in the vicinity of a main cluster of six genes coding for chemokine receptors (CCR1, CCR3, CCR2, CCR5, CCR4 and CXCR1) and is only 50 kb away from CCR8 [34]. Until recently, substantial relatedness had only been observed in the coding sequences of these genes. We and others [25] have now identified a short region in the 5'-UTR (dotted lines in Figure 3) that may be a relic of a regulatory element controlling this hypothetical ancestor. This sequence is located in a functional proximal promoter region of each of the chemokine receptors tested, although it was not found in the CXCR4 promoter. This region, corresponding to nucleotides -408 to -310 relative to the CCR3 transcription start site, was described as part of an Alu family repeat [25], and may have been used for gene duplication or translocation. In the case of CCR5, this region is located in intron 1 ($+120/+185$) relative to the numbering of Mummidi et al. [27] and in the proximal promoter ($-690/-635$) relative to the numbering of Guignard et al. [35]. In both studies, this sequence resided in a region with strong constitutive promoter activity. The homologous sequence in CXCR1 ($-236/-160$) was also functional and was described as containing two NFAT sites [29]. This sequence was more distal in the CCR2 promoter ($-1100/-1000$), and a chimaeric construct containing this sequence was constitutively active although the sequence was not essential. A detailed analysis is needed to delineate clearly the properties and origins of this element.

In summary, the proposed structure of the CX3CR1 gene contains three introns and four exons; exons 1, 2 and 3 may be used alternatively as cell-selective 5'-UTRs, and exon 4 codes for the entire ORF. The regulation of the CX3CR1 gene is highly complex and the transcription combines numerous levels of complexity, especially multiple promoter regions, some of which are composed of independent functional elements and multiple transcripts. This provides a molecular framework aimed at identifying transcription factors involved in selective tissue expression. In addition, this characterization will allow investigations into the polymorphisms of this gene. We have shown previously that polymorphisms in the coding region of this gene affect HIV disease progression and risks of acute coronary disease. These effects were correlated with a decrease in the total number of CX3CR1 proteins present at the cell surface. This impairment may be due to mutations in the promoter region affecting gene transcription.

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