

Genomic organization and promoter characterization of human CXCR4 gene

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Abstract CXCR4 is the receptor for the CXC chemokine SDF1 that has essential functions on embryo organogenesis, immunological functions and T lymphocyte trafficking. Recently, CXCR4 has drawn unexpected attention as it was recently identified as a co-factor required for entry of lymphotropic HIV isolates in CD4+ T lymphocytes. CXCR4 is the only SDF1 receptor identified so far. This suggests that CXCR4 expression is critical for the biological effects of SDF1. To investigate the mechanisms controlling both the constitutive and induced expression of CXCR4 receptors we have isolated and characterized the promoter region and determined the genomic structure of the human gene. The CXCR4 gene contains two exons separated by an intronic sequence. A 2.6 kb 5'-flanking region located upstream the CXCR4 open reading frame contains a TATA box and the transcription start site characteristic of a functional promoter. This region also contains putative consensus binding sequences for different transcription factors, some of them associated with the hemopoiesis and lymphocyte development.

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Key words: CXCR4 chemokine receptor; Promoter regulation; Transcription factor; HIV-1; Chemokine SDF1

1. Introduction

Chemokines are soluble mediators that control leukocyte migration into sites of inflammation and specific areas of lymphoid organs [1]. Activation of leukocytes is mediated by interaction of chemokines with specific receptors belonging to the family of the seven transmembrane domain, G-protein coupled receptors [2]. Chemokines carry conserved cysteines and fall into four families defined by a cysteine signature motif: CXC, CC, C or CX₃C where C is a cysteine and X any amino-acid residue [3]. Most of the chemokines belong to either CC or CXC families. Expression of CC chemokine receptors in T lymphocytes has proved to be dependent on the cell activation status and the nature of the signal used as stimulus. Thus, expression of CCR1, CCR2 or CCR5 are induced by IL2 [4,5], but are down-regulated by lectin or T cell specific activators [4,6]. Less information is available for the CXC family of receptors, although it has been reported that expression of IL8 receptors in lymphocytes is modified neither by IL2- nor CD3-mediated activation [4]. Among the

family of CXC receptors, CXCR4 has drawn unexpected attention as it was identified as a co-factor required for entry of lymphotropic HIV isolates in CD4+ T lymphocytes [7]. CXCR4 is the receptor for the CXC chemokine SDF1 [8,9] that plays a critical role in both embryonic B lymphopoiesis and organogenesis [10]. A chemoattractant for the pluripotent hematopoietic CD34+ cells [11] and a growth factor for pre- and pro-B murine lymphocytes in vitro [12], SDF1 is also known to promote the arrest on endothelium of lymphocytes rolling under flow conditions [13]. Moreover, SDF1 prevents HIV entry in host cells by both occupying [8,9] and promoting endocytosis of CXCR4 [14]. Nowadays, CXCR4 seems to be the only receptor capable of binding SDF1 selectively and permit cell signaling by this chemokine [8,15]. CXCR4 is expressed constitutively in a large number of human tissues and transformed cell lines [15]. Moreover, CXCR4 transcripts are very abundant in leukocytes [15].

To investigate the mechanisms controlling both the constitutive and induced expression of CXCR4 receptors, we have isolated and characterized the promoter region and determined the genomic structure of the human gene. The CXCR4 gene contains two exons separated by an intronic sequence. A 2.6 kb 5'-flanking region located upstream the CXCR4 open reading frame contains a TATA box and the transcription start site characteristic of a functional promoter. This region also contains putative consensus binding sequences for different transcription factors, some of them associated with hemopoiesis and lymphocyte development.

2. Materials and methods

2.1. Cells

The epithelial-like cell line HeLa was propagated in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), L-Gln and antibiotics. J.han cells were derived from the T lymphoblastoid cell line Jurkat and were grown in RPMI 1640 supplemented with 10% FCS. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient from healthy human donors.

2.2. DNA sequencing

Both DNA strands relative to the CXCR4 gene and the promoter region were sequenced by the dideoxy-chain termination method using the New England Biolabs cycle sequencing Kit and an automated sequencer ABI (Perkin Elmer). All sequencings were done in duplicate.

2.3. Library screening

A human BAC library from Research Genetics, Inc (2130 Memorial Parkway SW, Huntsville, AL 35801, USA) was screened according to the manufacturer's instructions by specific PCR for the CXCR4 gene. The primers used were CXCR45459: 5'-CCGAGGAAATGGGCT-

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The nucleotide sequence data reported in this paper will appear in the EMBL database with the accession number AJ224869.

1	caattctgaa	tcttgccttt	tgcacttaat	gtttcataag	tatttcccca	tgtcactaaa
61	aattctttcca	aataacattc	acgatgtcca	tatggaattt	cagatgtgga	tgaacccaaa
121	tcttgtcaac	tattccacta	acagtggtta	tttagggatg	ttcagacatt	tcactattta
181	aaaaaaaatg	ttccacaaa	tacctttgtg	gcataagttt	ttatgagtgg	agttactggt
	<u>Lvf1</u>					
241	ctgaagttcc	tgtcgaatag	aaaatgcttt	ccagtgaaggc	tgtcccaagc	cacattccca
301	tcagtgacaa	gcgagagaca	gctggctctt	tcaaatccgg	agaccaaata	ttatctttga
361	aaaaaaatgg	atttttgcct	aatttggtag	tcaccaaata	gcattctcatt	gttcttttaa
	<u>C-ets2</u>					<u>C-ets2</u>
421	ttatctgctt	ccttttagta	gagatcccta	aaaagatctg	aaaggagtct	tcagataaag
481	gaaggagctt	tcttttgcct	gtctacaatc	aacaaatatt	tattatgcaa	accattttgc
541	tccgagtttt	ctcctctttc	cctttttgga	cagatttgga	agatctcacc	tttcaggttt
601	tagacatcgt	gcaggagga	gttttgaggt	agggtgcagc	ttacggtcca	ggataaaaac
661	tactgattct	gccactacca	ggctttgtga	aaagcaagtc	atgaaaacgc	tctgaaattc
					<u>HoxD</u>	
721	tagaccttca	gtagatagga	tctaccgtgt	ctataaaaaat	atgaagatcc	tttaagtttta
				<u>HoxD</u>		
781	ttaaagattc	gaaaaaagta	aaagtgtttt	tacgggtttta	ttttcatttt	tatttctttac
841	cgttatcgtt	tattataaag	gatattataa	aggatacaga	tgaagagata	cgtaatgcaa
	<u>SP1</u>					
901	ggcctgtgag	aaggggctg	gagcttccga	aacctcttcc	agccaccacc	ctccaagaac
961	ctggagtttc	tttttttttt	tttaattcta	caaatgtaat	attagatttg	attttatctg
1021	gccattagtg	tgtgtcctaa	ctcgttcggt	tctgagagtc	ccatctcccg	gcccgggata
	<u>PEA3</u>	<u>C-Jun</u>				
1081	tcactctttc	tgtgtcagtg	aaagtgcaga	gtagatgaga	acctttaacc	accaacatta
	<u>MAZ</u>					
1141	gggaggggtc	ccagacaaag	ggggtaagtc	atgctctgta	gagaaaaggt	tccctgcctc
			<u>PEA3</u>			
1201	cgaactacct	ctggaacact	ccagtaaatg	tttctctctt	tgatatagaa	aagagggatc
1261	gtgtgtagag	tgcagtcagg	gcaatccctc	tctctgggac	catttcgggg	taggggcctc
1321	tggggctcgt	gtcgcgacgc	acgcgcctcg	gtcccagcta	tctccgcagc	gggcccaccc
		<u>SP1</u>		<u>SP1</u>		
1381	gcctgcggac	gcagtttctc	ggccccgcgc	cacactcgct	ccccgcgcgc	accagctctc
1441	gcgcgcggag	ggaagtggcg	cgagggggaa	agcactgtct	gcgcgcgcac	tgcaaacctc
1501	agccagtcgt	agatcgcttt	aaacgtctga	ccccaccccc	cactccgcgc	cgcccggttc
1561	ttcaacctaa	tttctgattc	gtgccaaagc	ttgtcctctg	ctcaaaatcg	tggaagacgc
	<u>AP2</u>					
1621	cgagtatggg	gaccgaagac	ctgggttcaa	gcccggcttg	gaatccctgc	ccatccctgg
	<u>SP1</u>					
1681	catttcatct	ctccgggctt	atttgcctgt	ttctccgaat	gcgggccttg	tctgggtcac
	<u>AP2</u>					
1741	gctggatccc	caacgcctag	aacagtgcgt	ggcacgcagt	tcgtccttct	ataaatatcg
1801	gactaaatgc	atctctgtga	tggttaatac	cacacgggtg	tgtgagaatg	aatgagtgat
1861	tctgtgcaag	ttcctagtga	tctgttaca	aaagtactgg	tcgctaaatt	actcttataa
1921	taaagcatac	tttttagata	ataaagcact	attcggaat	tggttaccgc	tattatgaaa
1981	ttactgagca	atcacatctc	acatctgctc	agtcctccaa	attatgcaa	atcctactct
2041	cttctgaaag	tatctcctaa	ttatctgcac	ctgaccctag	tgatgctgtg	aatgtgcaag
	<u>PEA3</u>	<u>C-ets2</u>				
2101	tatagctaca	tcctccgaag	gaaaggatct	ttactccttt	tacctcctga	atgggctgcg
		<u>SP1</u>				<u>SP1</u>
2161	tctgctgaaa	gcgcggggga	atgggctgtt	ggaagcttgg	ccctacttcc	agcattgccg
			<u>C-ets2</u>			
2221	cctactgggt	gggttactcc	agcaagtcac	tccccttccc	tgggcctcag	tgtctctact
2281	gtagcattcc	caggtctgga	attccatcca	ctttagcaag	gatggacgcg	ccacagagag
		<u>C-ets2</u>				
2341	acgcgttctc	agccccgcgt	tcccacctgt	cttcaggcgc	atccccgttc	cctcaaaact
	<u>Lvf1</u>					
2401	aggaaatgcc	tctgggaggt	cctgtccggc	tccggactca	ctaccgacca	cccgcaaaaa
	<u>C-ets2</u>				<u>SP1</u>	<u>PU.1</u>
2461	gcagggtccc	ctgggcttcc	caagccgcgc	acctctccgc	CCCCCctctg	cgccctcctt
			<u>SP1</u>	<u>MAZ</u>		
2521	cctcgcgtct	gcccctctcc	cccacccgc	cttctccctc	cccgcgccag	cggcgcctgc
		<u>TATA BOX</u>				
2581	gccgcgctcg	gagcgtgttt	ttTATAAaagt	ccggccgcgg	ccagaaactt	cagtttgttg
	↓Transcription start		5'UTR REGION			
2641	gctGCGGCAG	CAGGTAGCAA	AGTGACGCCG	AGGGCCTGAG	TGCTCCAGTA	GCCACCGCAT
		<u>CDS 1° Exon</u>		<u>Intron</u>		
2701	CTGGAGAACC	AGCGGTACC	ATGGAGGGGA	TCAGTGTAA	Tccagtttca	acctgctttg
2761	tcataaatgt	acaaacgttt	gaacttagag	cgcagccctt	ctccgagcgg	gcagaagcgg
2821	ccaggacatt	ggaggtaacc	tgaccatcct	atatagtgcg	gggtgggtgg	ggggggagcag
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3181	cggggttaag	cgcctggtga	ctgttcttga	cactgggtgc	gtgtttgtta	aactctgtgc
3241	ggccgacgga	gctgtgccag	tctcccagca	cagttaggcag	agggcgggag	agggcggtgg
3301	accacccgcg	ccgatcctct	gaggggatcg	agtgttgcca	gcagctagga	gttgatccgc
3361	ccgcgcgctt	tgggttttag	ggggaaacct	tcccgcgctc	cgaagcgtgc	ctcttcccca
3421	cgccgcgcgag	tgggtcctgc	agttcagagag	tttggggctg	tgcaagagtc	agcgaggtgg
3481	tttgacctcc	cctttgacac	cgcgagctgc	ccagccctga	gatttgcgct	ccggggatag
3541	gagcgggtac	ggggtgaggg	gcggggcgcg	ttaaaccgca	cctgggctgc	caggtcgccg

Fig. 1.

CAGGGGA-3' and CXCR45462: 5'-CATTGGGGTAGAAGCG-GTCACA-3'. The PCR mixtures contained 5 µl of DNA from the BAC library pool, 1 µl of each primer at a concentration of 500 ng/µl, 200 mM dNTPs, 1 mM of MgCl₂, 0.2 units of Taq DNA polymerase (Pharmacia), 5 µl of DMSO and water to 100 µl. Amplification was conducted for 30 cycles at 93°C 1 min, 62°C 2 min, 72°C 2.30 min, followed by a final elongation step at 72°C for 15 min. The PCR products were visualized on a 2% agarose gel stained with ethidium

bromide. This procedure led to the identification of a BAC clone (24D3) that contained the entire CXCR4 gene and adjacent sequences. The identification of a putative TATA box and consensus binding sites for transcription factors were performed using the program TESS (Transcription Element Search Software) (<http://agave.humgen.upenn.edu/teess/index.html>). Only sequences showing total homology with the canonical consensus sequence for transcription factors were considered.

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3601 cccgcgaagac tggcaggtgc aagtggggaa accgtttggc tctctccgag tccagttgtg
3661 atgtttaacc gtcggtggtt tccagaaacc ttttgaaacc ctcttgctag ggagtttttg
3721 gtttctctga cggcgcgcca attcaaaagac gctcgcggcg gagccgccca gtcgctcccc
3781 agcaccctgt gggacagagc ctggcgtgtc gccacgcgga gccctctgag cgtgcttgcc
3841 gggcggttgg cgtgggtgta gtgggcagcc gcggcgcccc ggggctggac gaccggcccc
3901 cccgcgtgcc caccgcctgg aggtctccag ctgcccacct ccggccgggt taactggatc
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4021 cacgggtgca gaccccgtaa cttctccac ccaggaaaat gcccgctccc ctaacgtccc
4081 aaacgcgcca agtgataaac acgaggatgg caagagaccc acacaccgga ggagcgcccc
4141 cttgggggag gaggtgccgt ttgttcattt tctgacactc ccgcccaata taccccaagc
4201 accgaagggc cttcgtttta agaccgcatt ctctttaccc actacaagtt gcttgaagcc
4261 cagaatggtt tgtatttagg caggcggtggg aaaattaagt ttttgcgctc taggagaatg
4321 agtcttttga acgcccccg cctccccccc tgatcctccc tctccccctc ttccccctct
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4501 ttttttcttc cctctagtgg gcggggcaga gggattagcc aagatggtga ctttgaaacc
4561 ctcagcgtct cagtgccctt ttgttctaaa caaagaatgt tgtaattggt tctaccaaaag
4621 aaggatataa tgaagtcact atgggaaaag atgggggaga aaagatgggg aggaagattg
4681 taggattcta cattaatctt cttgtgccct tagccacta cttcagaatt tcttgaagaa
4741 agcaagcctg aattggtttt ttaaattgct ttaaaaaatt tttttaactg ggtaaatgct
↓2° Exon
4801 tgctgaattg gaagtgaatg tccattcctt tgcctctttt gcaGATATAC ACTTCAGATA
4861 ACTACACCGA GGAATAGGGC TCAGGGGACT ATGACTCCAT GAAGGAACCC TGTTTCCGTG
4921 AAGAAAATGC TAATTTCAAT AAAATCTTCC TGCCCAACCAT CTACTCCATC ATCTTCTTAA
4981 CTGGCATTGT GGGCAATGGA TTGGTCATCC TGGTCATGGG TTACAGAAG AACTGAGAA
5041 GCATGACGGA CAAGTACAGG CTGCACCTGT CAGTGGCCGA CCTCCTCTTT GTCATCACGC
5101 TTCCCTTCTG GGCAGTTGAT GCCGTGGCAA ACTGGTACTT TGGGAACCTC CTATGCAAGG
5161 CAGTCCATGT CATTTACACA GTCAACCTCT ACAGCAGTGT CCTCATCCTG GCCTTCATCA
5221 GTCCTGGACG CTACCTGGCC ATCGTCCACG CCACCAACAG TCAGAGGCCA AGGAAGCTGT
5281 TGGCTGAAAA GGTGGTCTAT GTTGGCGTCT GGATCCCTGC CCTCTGCTG ACTATTCCCG
5341 ACTTCATCTT TGCCAACGTC AGTGAGGCAG ATGACAGATA TATCTGTGAC CGTTTCTACC
5401 CCAATGACTT GTGGGTGGTT GTGTCCAGT TTCAGCACAT CATGGTTGGC CTTATCCTGC
5461 CTGGTATTGT CATCTGTGCC TGCTATTGCA TTATCATCTC CAAGCTGTCA CACTCCAAGG
5521 GCCACCAGAA GCGCAAGGCC CTCGAAGCCA CAGTCATCCT CATCTGGCT TTCTTCGCC
5581 GTTGGCTGCC TTACTACATT GGGATCAGCA TCGACTCCTT CATCCTCCTG GAAATCATCA
5641 AGCAAGGGTG TGAGTTTGAG AACACTGTGC ACAAGTGGAT TTCCATCACC GAGGCCCTAG
5701 CTTTCTTCCA CTGTTGTCTG AACCCCATCC TCTATGCTTT CCTTGGAGCC AAATTTAAAA
5761 CCTCTGCCCA GCAGCAGCTC ACCTCTGTGA GCAGAGGGTC CAGCCTCAAG ATCCTCTCCA
5821 AAGGAAAGCG AGGTGGACAT TCATCTGTTT CCACTGAGTC TGAGTCTTCA AGTTTTCAC
5881 CCAGCTAACA CAGATGTAAA AGACTTTTTT TTATACGATA AATAACTTTT TTTTAAGTTA
5941 CACATTTTTT AGATATAAAA GACTGACCAA TATTGTACAG TTTTATTGCT TTGTGGATT
6001 TTTTCTTTGT GTTTCTTTAG TTTTGTGAA GTTTAATTGA CTTATTTATA TAAATTTTTT
6061 TTTTTCATTA TTGATGTGTG TCTAGGCAGG ACCTGTGGCC AAGTTCCTAG TTGCTGTATG
6121 TCTCGTGGTA GGAAGGAACT AAAGGGAAGT GAACATTCCA GAGCGTGTAG TGAATCACGT
6181 AAAGCTAGAA ATGATCCCA GCTGTTTATG CATAGATAAT CTCCTCCATC CGGTGGAACG
6241 TTTTTCCTGT TCTTAAGACG TGATTTTGCT GTAGAAGATG GCACTTATAA CCAAAGCCCA
6301 AAGTGGTATA GAAATGCTGG TTTTTCAGTT TTCAGGAGTG GGTGATTTC AGCACCTACA
6361 GTGTACAGTC TTGTATTAAG TTGTTaataa aagtacatgt taaacttact tagtggtatg
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6481 tgcaagcac tatcctaggt

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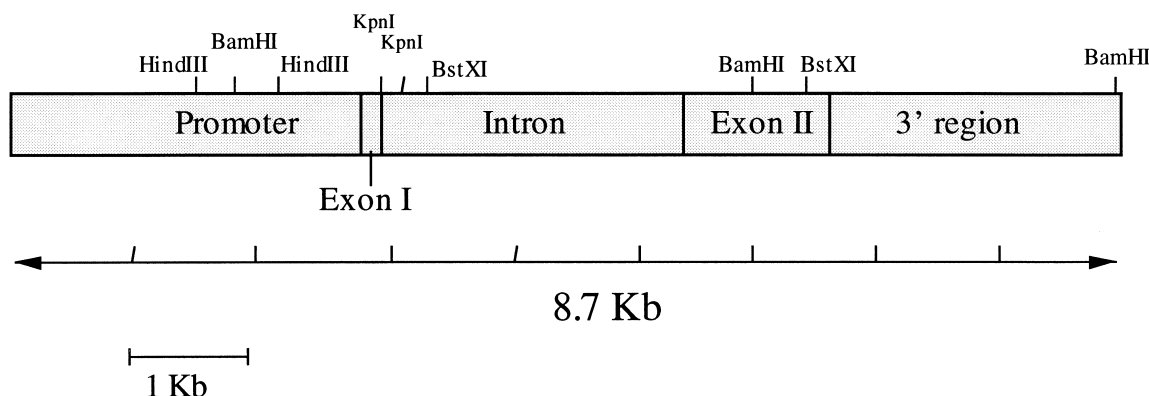


Fig. 1. Sequence of the CXCR4 gene and promoter region. The consensus TATA box sequence and putative transcription factor consensus sites are indicated above the sequence. The two coding regions are represented in bold capital letters and the 5' UTR in italic capital letters. A schematic representation of the CXCR4 gene structure with reference to the principal hallmarks of the sequence and the restriction pattern is represented at the bottom of the figure.

2.4. Southern blotting

Ten micrograms of BAC 24D3 DNA were digested with *EcoRI*, *BamHI*, *XbaI*, *HindIII*, *SstI*, *PstI*, *XhoI*, *KpnI* and *SpeI*, run on a 0.8% agarose gel, and blotted onto a nitrocellulose membrane. A radioactive probe was synthesized by PCR amplification of a CXCR4 cDNA plasmid in the presence of 0.5 mM of $\alpha^{32}\text{P}$ -dATP and the previously described primers. This probe was hybridized to the nitrocellulose membrane. This procedure led to the identification of 3.5 and 3.7 kb bands of *BamHI* digested BAC DNA that contained the CXCR4 gene. These fragments were subcloned in pBluescript SK \pm (Stratagene) and sequenced. Further direct sequencing of the BAC clone permitted the identification of additional 1.8 kb more relative to the 5' end of the sequence characterized previously.

2.5. Primer extension

After RNA extraction from HeLa cells using the PolyATtract mRNA isolation system (Promega), CXCR4 cDNA synthesis was performed with 100 ng of $\gamma^{32}\text{P}$ -ATP end-labeled primer complementary to the coding strand of the 3' end of exon 1 (5'-GGTTCTCCAGATCGGGTGGCTACTGGAGCACTCAGGCCCT-3'). The same primer was used as sequencing primer of the genomic DNA and both products were electrophoresed in 6% polyacrylamide gel.

2.6. Construction of reporter plasmids

The larger promoter region of CXCR4 and the deletion variants were cloned from the genomic DNA by PCR into the eukaryotic expression plasmid pGEM luc (Promega). To reduce the likelihood of PCR generated mutations, all reactions were performed with Pfu DNA polymerase (Stratagene) and the amplified material was verified by sequencing. To facilitate subcloning in the expression plasmid, the primers used included the *HindIII* and the *BamHI* site at the 5' and 3' ends, respectively, for the construction smaller than 1 kb and the *BamHI* and *NotI* for constructing the 2.6 kb promoter fragment. All PCR amplifications were carried out using the antisense primer 5'-TCGAGGATCCCCAACAACTGAAGTTTCTG which encompasses the transcription start site (+1). Specific sense primers for amplification of the different promoter regions were: -2643/+1, 5'-GATCGCGGCCGCACTTAATGTTTCATAAGTATTTTC-3'; -888/+1, 5'-TCGAAAGCTTGGATCCCCACGCCTAGAAC-3'; -417/+1, 5'-TCGAAAGCTTATTGCGCGCTACT-3'; -110/+1, TCGAAAGCTTCTCGCGTCTGCCC-3'; -63/+1, 5'-TGGAAAGCTTAGCGGCGCATGCGC-3'; -48/+1, TCGAAAGCTTGCCTCGGAGCGTGTGTTTTTA-3'.

2.7. Transfection and luciferase assay

Transfections of cells were performed by electroporation. The conditions used were: 320 V and 1500 μF for PBMC; 260 V and 1500 μF for J.jhan; and 200 V, 900 μF for HeLa. Twenty-four hours upon transfection cells were stimulated for 18 h with the different stimuli. Phorbol myristate acetate (PMA, Sigma) was used at 20 ng/ml. Ionomycin (Sigma) and Phytohemagglutinin (PHA, Welcome) were used at 250 ng/ml and 1 $\mu\text{g/ml}$, respectively. Final concentrations of recombinant TNF, IL2 and synthetic SDF1 α were 5 ng/ml, 30 IU/ml and 100 nM, respectively. Stimulation with either anti-CD3 or anti-CD3+anti-CD28 antibodies were performed as described previously [16]. The luciferase activity generated in cell cultures was assessed using a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions. Results are expressed as relative light units (RLU) per milligram of protein after subtracting the background signal (lysate from non-transfected cells).

3. Results and discussion

A fragment of 8724 base pairs (bp) containing the totality of the CXCR4 coding region and 2.6 and 2.3 kb flanking sequences relative to 5' and 3', respectively, was isolated from a human genomic BAC library (the accession number in EMBL database is AJ224869). The analysis of the sequence revealed that the CXCR4 open reading frame is encoded as two exons separated by a 2.1 kb of intronic sequence. The first exon carries the sequence corresponding to the five first CXCR4 amino acids and a 5' UTR region. The second

exon codes for the last 247 amino acids and a 3' UTR sequence. The entire nucleotidic sequence corresponding to the CXCR4 ORF and flanking regions, along with a schematic representation of the CXCR4 gene, is shown in Fig. 1. Six base pairs downstream last codon of the first exon, we noted the presence of an alternative canonical splice donor site (GT). This is reminiscent of an alternative splice donor site found in the mouse CXCR4 gene which permits expression of two different transcripts [17,18]. In contrast with these results, we failed to prove that the hypothetical, alternative splicing donor site found in human CXCR4 gene we identified was efficiently used to generate two mRNA species in normal (PBMC) and transformed (J.jhan, HeLa) human cells.

To identify the promoter region boundaries, we performed a computer-assisted analysis of the sequence data. A consensus TATA box sequence was recognized 30 bp 5' upstream transcription start site (Fig. 2), suggesting that this region was the promoter of the CXCR4 gene. The CXCR4 promoter region herein described is identical to that recently described by Morihuchi et al. [19] and contains an additional and previously unidentified 5' regulatory region of 1.4 kb. The CXCR4 promoter region carries different consensus binding sites for transcriptional factors. Some of these factors are supposed to be associated with hemopoiesis or lymphocyte maturation (MAZ, C-ets2, PU.1, PEA3, Lyf1) [20–23]. Seven putative consensus binding sites for the ubiquitous SP1 sequence were also identified.

To functionally characterize the promoter region of CXCR4, a series of 5' truncations of this region were subcloned into a plasmid driving the expression of a luciferase gene. Upon transfection into different human cell types, both the constitutive and the induced expression of luciferase was measured in cytoplasmic lysates obtained from disrupted cells. Spontaneous transcriptional activity from the CXCR4 was observed from the promoter in the three cell types, J.jhan, HeLa (Fig. 3) and PBMC (Fig. 4B). In both HeLa and J.jhan cells, the maximal transcription activity corresponded to the construct carrying the longest region (-2643/+1) of the promoter. Truncation of sequences progressing from the 5' end reduced basal activity of the promoter in all three cell types. The effect was more striking in HeLa cells where removal of 5' sequences between -2643 and -888 led to a five-fold reduction of promoter activity (Fig. 3). The region spanning from -48 to +1 retained a significant level of transcriptional activity, roughly comparable to that displayed by constructs encoding upstream sequences up to position -417. This finding suggests that sequences from -48 to +1 contain the proximal element accounting for the basal activity of the promoter. We could not confirm that removal of the NRF1 consensus site for the transcription factor (-73/-62) which has been described [19] as controlling the activity of the CXCR4 minimal promoter, affected significantly the basal activity of the promoter. Differences in the type of cells used in this study might account for the divergent results.

Our findings indicate that the 1.4 kb region that we have identified and is located upstream the previously characterized promoter region [19], also contains regulatory elements involved in the control of CXCR4 constitutive transcription activity. The participation of distal regions in the control of the basal activity was more marked in epithelial cells as compared to lymphoid cells, thus suggesting some degree of tissue specificity in the regulation of the CXCR4 promoter.

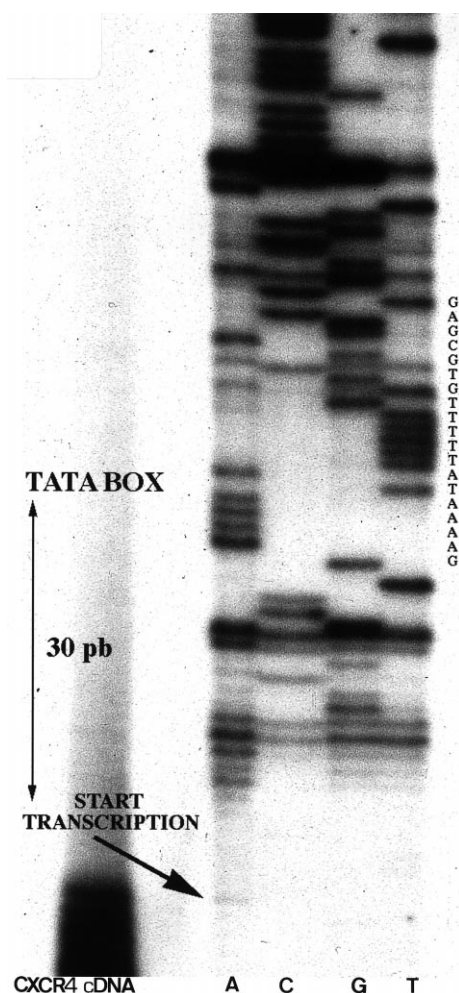


Fig. 2. Identification of transcription start site of the CXCR4 mRNA by primer extension in HeLa cells. The first line represents the major CXCR4 cDNA extension product, electrophoresed in the same gel as the sequencing reaction of the genomic DNA right four lines.

The capacity of the CXCR4 promoter to undergo transactivation upon stimulation by cell activation signals was assessed using the regulatory sequences between positions -888 and $+1$. Among the different stimuli used, only combined addition of PMA and ionomycin proved to be an efficient signal to enhance transcription activity from the promoter in the three cell types tested (Fig. 4A and B). Thus, the capacity of different regions of the promoter to be induced and transactivated by cell activation signals was investigated by using simultaneous addition of PMA and ionomycin. We show that the promoter regions responding to cell activation differ in HeLa and J.jhan cells (Fig. 5A and B). Indeed, in HeLa cells promoter transactivation requires the presence of the more distal regions (upstream position -417). In contrast, in lymphoblastoid J.jhan cells proximal promoter elements retained significant reactivity to activation of cell signals. Thus, similar to the constitutive expression of the CXCR4 promoter, induced transcription upon cell activation may have some degree of tissue specificity that would depend on selective protein/DNA interactions.

The capacity of stimuli mimicking physiologic activation of T lymphocytes to transactivate the CXCR4 promoter, was investigated. Upon transfection with the $(888/+1)$ -driven luciferase construct, J.jhan or HeLa cells (Fig. 4A) were treated with either TNF or SDF1 α . The transcription activity of the promoter was not modified by either stimuli despite they induced efficiently cells as indicated by I κ B α degradation (TNF) or CXCR4 endocytosis and calcium mobilization (SDF1 α) in both cell types (data not shown). Using PBMC (Fig. 4B) we also investigated the capacity of T lymphocyte mitogenic signals or IL2 to enhance transcriptional activity from the CXCR4 promoter. The constitutive activity of the promoter was not modified significantly by stimulation of PBMC with anti-CD3, anti-CD3+anti-CD28 antibodies or incubation with IL2, although these stimuli induced CD25 expression and cell proliferation (data not shown).

The constitutive expressions of the CXCR4 promoter in different cell types are in keeping with the constitutive expression of CXCR4 gene observed in human tissues [15]. CXCR4

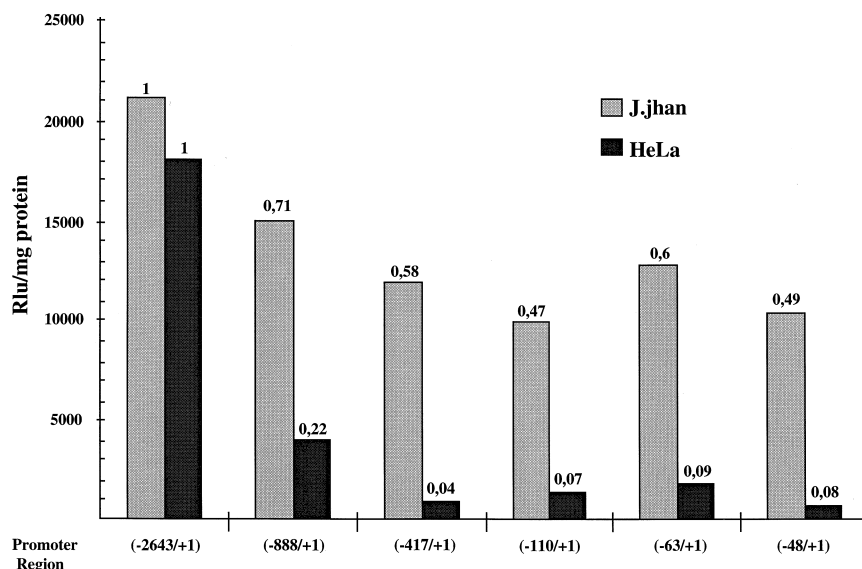


Fig. 3. Basal activity of the 2643 bp CXCR4 promoter and different deletions in transfected HeLa and J.jhan cells. Values on top of columns are relative to these of construct $(-2643/+1)$ which we set arbitrary as 1.

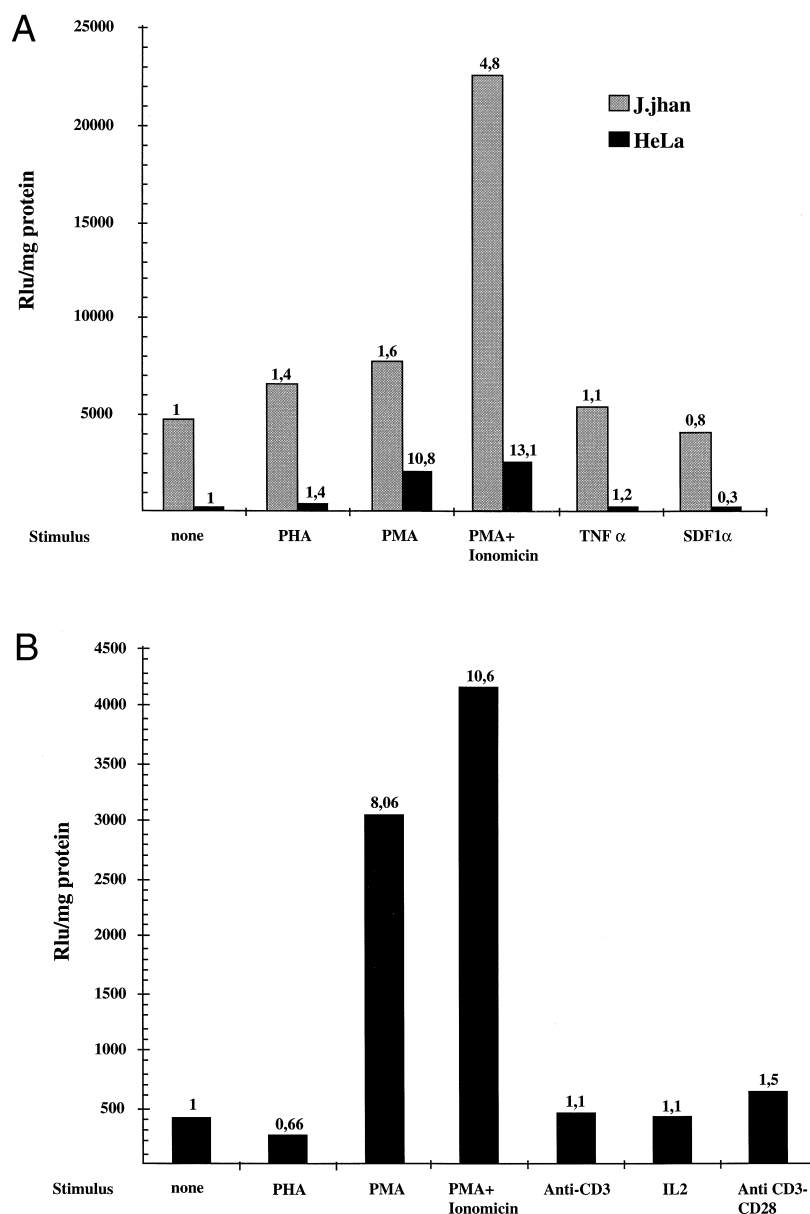


Fig. 4. Induction of the $-888/+1$ promoter region with different stimuli in HeLa and J.jhan cells (A) and healthy donor PBMC (B). The fold increases with respect to the promoter without stimulus are indicated on top of each column.

transcripts are expressed in a large diversity of tissues and transformed cell lines [14,15]. The expression of CXCR4 is also constitutive in cells of hematopoietic origin. Indeed, CXCR4 is expressed in most human resting T lymphocytes isolated from blood although the percentage of positive cells shows some degree of variability among blood donors [14,24]. CXCR4 is also expressed in most of 60% freshly isolated, human blood monocytes [24]. Interestingly in Langerhans and endothelial cells, constitutive expression of CXCR4 is detected and the protein accumulates in the cytoplasm [25,26]. Langerhans cells when kept in culture for some hours after isolation show CXCR4 at the cell membrane thus suggesting that the lack of cell surface expression could be related to microenvironmental influences [25].

However, we have not found cell signals induced by either cytokines or growth factors, or mediated through membrane receptors involved in specific T lymphocyte activation, capa-

ble to up-regulate the constitutive transcription activity of the CXCR4 promoter. Our data diverge from those of Moriuchi et al. [19] who found a discrete enhancement of CXCR4 promoter transcription activity in PBMC activated by IL2 or via CD3. Differences in the status of cell activation and differentiation might explain these divergent data. Indeed, while the experiments described by Moriuchi et al. were performed in blast T lymphocytes expanded with IL2, we transfected and induced resting PBMC. In keeping with the results published by Moriuchi et al., the enhancement of CXCR4 expression at the cell membrane in PHA-activated human T lymphocytes has been reported previously [6]. Nevertheless and in sharp contrast with these data, evidence supporting down-regulation of CXCR4 expression in PHA-stimulated T lymphocytes has been provided by Loetscher et al. [15]. Findings obtained in our laboratory confirmed Loetscher et al.'s observations and show that PHA activation induces a rapid and sustained re-

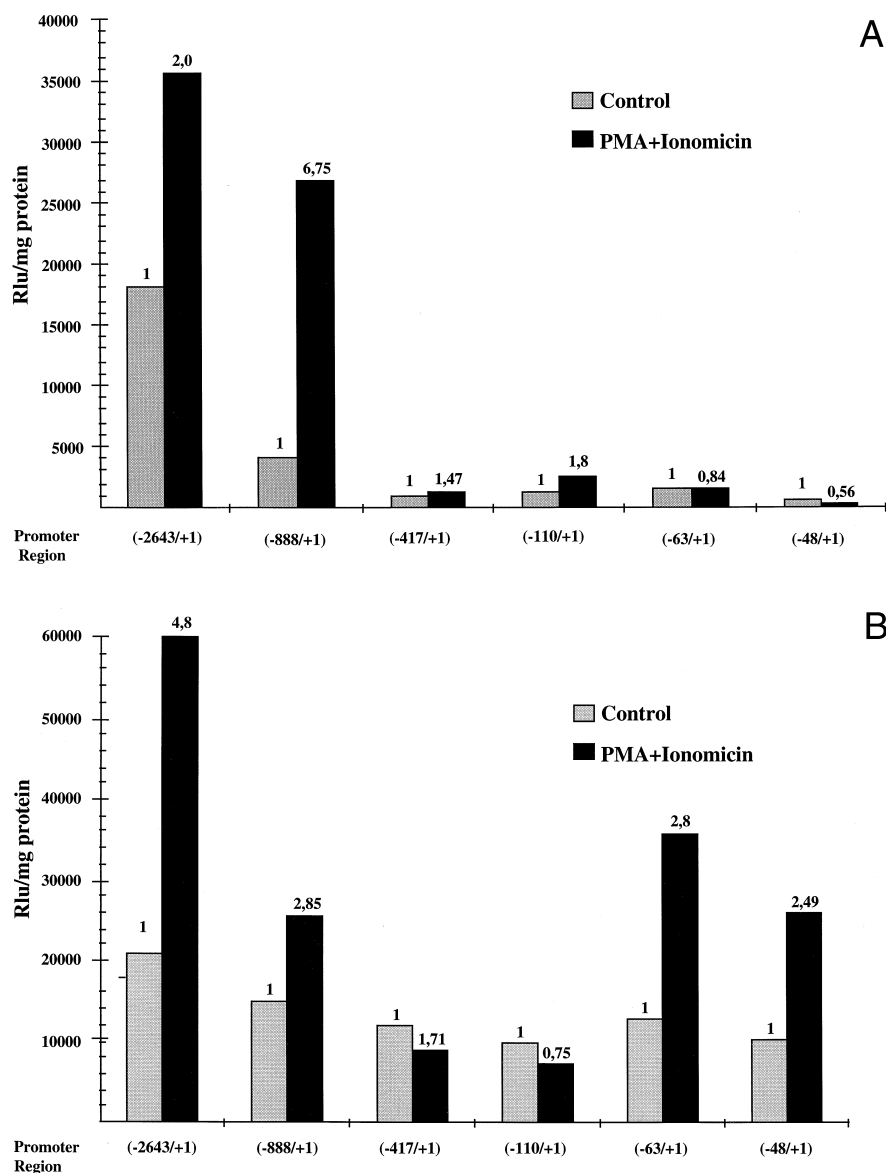


Fig. 5. Induction of CXCR4 promoter constructions by PMA and ionomycin compared with the basal level in Hela (A) or J.jhan (B) cells. The fold increases with respect to the control without stimulus are represented over each column.

duction in the amount of CXCR4 transcripts and impair CXCR4 cell membrane expression in PBMC (Caruz and Alcamí, unpublished results). This observation could reflect either induction of a transcription inhibition mechanism or more likely a reduced stability of the CXCR4 messenger. Down-regulation of chemokine receptor after cell activation is not without precedent, and has been described previously for the chemokine receptors CCR1 and CCR2. Indeed, mitogenic activation of fully responsive lymphocytes dramatically reduced the amount of transcripts for both receptors and lead to the loss of responsiveness to RANTES and MCP-1, respectively [4]. Moreover, it has been shown recently that IFN γ , a cytokine secreted by PBMC lymphocytes in response to mitogenic stimuli [27], profoundly down-regulates CXCR4 mRNA expression in endothelial cells, a monocytoid cell line and PBMC [28,29]. The apparently divergent findings described above should be resolved by a more detailed analysis of the

physiologic signals regulating the expression of the CXCR4 gene both at pre- and post-transcriptional phases in different cell types.

Taking into account the important role played by SDF1 in embryo development and immunological functions, on one hand, and given that CXCR4 is the only SDF1 receptor identified so far, the analysis of mechanisms regulating expression of the CXCR4 gene appears like a topic of outstanding interest for future work.

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