Cloning and Functional Characterization of the 5′-Flanking Region of the Human Monocyte Chemoattractant Protein-1 Receptor (CCR2) Gene

ESSENTIAL ROLE OF 5′-UNTRANSLATED REGION IN TISSUE-SPECIFIC EXPRESSION* 

Keizo Yamamoto‡, Hideo Takeshima‡§, Kazuya Hamada‡, Mitsuyoshi Nakao§, Takeshi Kino‡, Toru Nishii, Masato Kochi, Jun-Ichi Kuratsu¶, Teizo Yoshimura**, and Yukitaka Ushio‡

From the Departments of *Neurosurgery and ¶Tumor Genetics and Biology, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan, |Department of Neurosurgery, Kagoshima University School of Medicine, Kagoshima 890-8520, Japan, and **Immunopathology Section, Laboratory of Immunology, NCI, National Institutes of Health, Frederick, Maryland 21702

The human monocyte chemoattractant protein-1 receptor designated hCCR2 is an essential co-receptor in cell entry by the human immunodeficiency virus as well as a receptor for monocyte chemoattractant protein-1, a member of the family of C-C chemokines that mediate monocyte chemotaxis. To elucidate the molecular mechanisms underlying the transcriptional regulation of hCCR2, we cloned and sequenced the hCCR2 gene; it was approximately 8 kilobase pairs in length and consisted of three exons divided by two introns. In the 5′-flanking region, there were the typical mammalian promoter consensus elements, a CAAT box and a TATA box, resulting in a single transcription initiation site. In addition, we found clustered tissue-specific cis-regulatory elements such as GATA consensus sequences, Oct-1 binding sequences, and CAAT/enhancer-binding protein binding sequences. Luciferase assays with various promoter deletions and gel mobility shift assays indicated that three cis-regulatory elements located within the region from −89 to +118 are required for basal activity in THP-1 cells. One element is an octamer sequence 36-base pair upstream from the TATA box; it binds mainly to Oct-1 and is capable of increasing transcriptional activity. The other two elements, which are tandem recognition sites of the CAAT/enhancer-binding protein family, are located in the 5′-untranslated region and account for the transcriptional activation as well as the tissue specificity of hCCR2.

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family that mediates leukocyte chemotaxis. We initially isolated it from the human malignant glioma cell line U-105 MG as a specific chemoattractant for monocytes (1, 2). Physiologically, MCP-1 is produced by endothelial cells, smooth muscle cells, and macrophages in response to a variety of mediators including platelet-derived growth factor, tumor necrosis factor α, interleukin 1, epidermal growth factor, and interferon-γ (3). The expression of MCP-1 is also increased under pathological conditions with monocyte-rich inflammatory processes such as atherosclerosis (4), rheumatoid arthritis (5), and certain malignant tumors (6, 7).

The cDNA of the specific receptor for human MCP-1, which is designated hCCR2, was recently cloned and shown to belong to seven-transmembrane domain receptor families (8) that couple via heterotrimeric G-proteins to affect cellular responses. The activation of hCCR2 is blocked by pertussis toxin, suggesting that hCCR2 couples to Gαi-class G-proteins (9). The hCCR2 gene has been cloned, and the mechanism of how it produces two alternatively spliced variants that differ only in their carboxyl-terminal tail has been elucidated (10). Human CCR5, one of the members of the C-C chemokine receptor family, acts as an essential cell surface co-receptor with CD4 in cell entry by macrophage-tropic human immunodeficiency virus type 1 strains (11, 12). In addition, hCCR2 and hCCR3 have been implicated as human immunodeficiency virus type 1 co-receptors in certain cell types (13, 14).

We previously reported that tumor-associated macrophages attracted by MCP-1 inhibit the growth of transplanted rat tumors in vivo (15). We posited that if we could stimulate the expression of MCP-1 in tumors and the expression of hCCR2 in monocytes, we would be able to obtain a greater inhibitory effect by a larger number of infiltrated macrophages against the growth of MCP-1-producing tumors such as human malignant glioma. The treatment of malignant tumors by the enhancement of an intrinsic immune system may be possible. The promoter region of the human MCP-1 gene has already been analyzed; it contains a distal nuclear factor κB binding site for induction by interleukin 1, tumor necrosis factor α, and a proximal GC box for basal transcriptional activity that is important for the transcriptional activation of MCP-1 (16). However, the promoter region for the hCCR2 gene remains to be characterized.

To elucidate the molecular mechanisms that regulate hCCR2, we cloned the hCCR2 gene, sequenced approximately 1.7 kbp of the 5′-flanking region, and mapped the transcription initiation site. We also assessed the promoter activity of the 5′-flanking region using luciferase (Luc) assays and gel mobility shift assays. We found that the Oct-1 binding sequence located 36 bp upstream from the TATA box and the tandem

* This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to H. T. and Y. U.). The costs of publication of this article were defrayed exclusively from the Ministry of Education, Science, Sports and Culture of Japan (to H. T. and Y. U.). The costs of publication of this article were defrayed solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF068265.

§ To whom correspondence should be addressed: Dept. of Neurosurgery, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Tel.: 81-96-373-5219; Fax: 81-96-371-8064; E-mail: m20406kaiju.mediekumamoto-u.ac.jp.

¶ The abbreviations used are: MCP-1, monocyte chemoattractant protein-1; CCR, C-C chemokine receptor; hCCR, human CCR; C/EBP, CAAT/enhancer-binding protein; UTR, untranslated region; Luc, luciferase; kbp, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction.
CAAT/enhancer-binding protein (C/EBP) binding sequences located at +50 to +77 within the 5'-untranslated region (UTR) are essential for the transcriptional activation and the tissue specificity of hCCR2 expression.

EXPERIMENTAL PROCEDURES

Cells and Cell Cultures—Two human monocytic leukemia cell lines, THP-1 and J-111, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The human malignant glioma cell line U-251 MG was obtained as described previously (7). THP-1 cells and U-251 MG cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and maintained at 37 °C in 5% CO2. J-111 cells were grown in Minimum Essential Medium with nonessential amino acids (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum and maintained at 37 °C in 5% CO2.

DNAs Used as Hybridization Probes—The cDNA encoding the hCCR2 type B (hCCR2b) was isolated by reverse transcription-polymerase chain reaction as described previously (17) from a larger number of infiltrated macrophages, using a set of primers (5'-CATCGGATCC-ATGCTGTCCACATCTCGTTCTCG-3' and 5'-GCTCAAGCTTTTATAAGTAGTGG-3') that contain BamHI and HindIII cloning sites, respectively, at their 5'-ends. The 1.1-kbp PCR product containing the entire coding sequence for hCCR2b was double-digested with BamHI and HindIII and subcloned into the pGEX-2TH vector (18) to generate the plasmid pGEX-2TH-CCR2b. The sequence of the insert was confirmed to be identical to the previously published sequences (8). To prepare the hCCR2b cDNA probe, the pGEX-2TH-CCR2b insert was double-digested with BamHI and HindIII, purified by low-melting point agarose gel electrophoresis, and labeled with [α-32P]dCTP using a random primer labeling kit (Takara, Otsu, Japan). We also used a 5'-end-labeled oligonucleotide probe (5'-AAAACGATCAGAGTAGTGG-TATTTCACCG-3') complementary to the 28 S ribosomal RNA to standardize the amount of RNA.

Isolation and Characterization of Clones Containing the hCCR2 Gene—A human genomic DNA library packaged in the phage vector EMBL3 SP6/T7 was purchased from CLONTECH (Palo Alto, CA). Plaques (1 × 10⁹) from this library were screened by the plaque hybridization technique (19), using 32P-labeled hCCR2b cDNA as a probe. Positive clones were re-screened at least twice, and a restriction enzyme mapping of the cloned DNA was carried out.

DNA Sequence Analysis—The nucleotide sequence of the 5'-flanking region and that of the entire exon-intron boundary were determined by the dideoxynucleotide chain termination method using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA) and a PE 373A sequencer (Perkin-Elmer). Sequence data were analyzed using the MatInspector V.2.1 computer program via the publicly available World Wide Web server (http://transfac.gbf-braunschweig.de/cgi bin/matSearch/matsearch.pl) to find specific sequences, including some transcriptional factor binding elements.

Northern Blot Analysis—Total cellular RNA (10 µg) extracted by the guanidinium thiocyanate-phenol chloroform extraction method (20) was subjected to 1% agarose gel electrophoresis and blotted onto a nitrocellulose filter (Schleicher & Schuell, Keene, NH). After baking at 80 °C for 2 h, the filters were hybridized with a hCCR2 probe and a 28 S RNA oligonucleotide probe at 42 °C in 50% formamide, 5× SSC, 1× Denhardt's solution, 50 µm sodium phosphate buffer (pH 7.0), and 100 µg/ml heat-denatured salmon sperm DNA. After hybridization, the filter was washed twice for 10 min with 2× SSC and 0.1% SDS at room temperature and washed once for 45 min with 0.1× SSC and 0.1% SDS at 56 °C. The filters were subsequently exposed to Fuji RX film at −80 °C for 24–72 h.

Primer Extension—The primer extension reaction was conducted using the avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, WI). For the primer, 5'-end-labeled synthetic 30-nucleotide-long DNA (5'-CTTATGCAACCTTTGAGTTGGAGTCAGGGCAA-3') that corresponds to the anti-coding strand cover-
ing nucleotides –57 to –86 downstream from the TATA box was used. Polyadenylated RNA (1 μg) extracted from THP-1 cells and 100 fmol of \(^{32}P\)-labeled primer were annealed using 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM dithiothreitol, 1 mM each deoxynucleotide triphosphate, and 0.5 mM spermidine, and the primer extension reaction was started by adding 1 unit of reverse transcriptase. The reaction mixture was incubated for 30 min at 42 °C. The primer-extended product was analyzed by electrophoresis on a 6% denaturing acrylamide gel. Autoradiography was performed for 24 h at 280 °C.

**FIG. 2.** Nucleotide sequences of the 5′-flanking region of the hCCR2 gene. Noncoding first exon sequences are shown in *lowercase letters*. The transcription initiation site is boxed and assumed to be at position +1. A TATA box, a CAAT box, and GATA binding sites, and GATA consensus sequences are underlined.
and pGL3-0.14KAE was the same as that for pGL3-0.23K, and the sense primer for pGL3-0.12KAE and pGL3-0.1KAE was the same as that for pGL3-0.26K. These PCR products were double-digested with KpnI and HindIII and ligated into the Kpn/HindIII sites of the promoterless Luc vector pGL3-Basic (Promega) immediately upstream from the firefly Luc gene. For the construction of other deletion mutants, the pGL3-5K was double-digested with KpnI and PvuII (pGL3-3.1K), SaeI (pGL3-1.8K), SmaI (pGL3-1.7K), and PstI (pGL3-1.4K), blunt-ended with T4 DNA polymerase, and self-ligated. To obtain the mutated constructs pGL3-OM, pGL3-CM1, pGL3-CM2, and pGL3-CMd, site-specific mutagenesis was carried out by PCR with the following mutated primers: for pGL3-OM, sense primer 5'-TCCAGGTATCGTGTCTCTAAGCTTCAGGAACATTGTACATTGGGTTGAGGTCTCCAGAATAGGATTAATTTTGAAATCT-3'; for pGL3-CM1, antisense primer 5'-TCATAAGGTCCTGGAACATTGTGAGGTGTTGAGGTCGTCAGAGGTTGTA-3'; for pGL3-CM2, sense primer 5'-TCATAACGTTCCAGAACTGATGATTCGTCAGAGGTTGAGGTCGTCAGAGGTTGTA-3'; and for pGL3-CMd, antisense primer 5'-TCATAAGGTCCTGGAACATTGTGAGGTGTTGAGGTCGTCAGAGGTTGTA-3'. The entire sequences of DNA fragments generated by PCR were confirmed to be identical to the original 5'-flanking sequences.

**Gel Mobility Shift Assay**—Nuclear extracts from THP-1 cells, J-111 cells, and U-251 MG cells were prepared according to the method of Dignam et al. (22). The probe and competitor sequences used are shown in Figs. 6A and 7A. Complementary single-stranded oligonucleotides were synthesized, mixed with TE, heated briefly, and cooled to room temperature for more than 1 h. Probes were end-labeled using 50 mCi of [γ-32P]ATP and T4 polynucleotide kinase (Takara); unincorporated radiolabel was removed by Sephadex G-25 chromatography. Nuclear extracts (4 μg) were preincubated with or without unlabeled competitor (used in 100-fold molar excess over the radiolabeled probe) in gel shift binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 4% (v/v) glycerol, and 50 μg/ml poly(dI-dC)-poly(dI-dC)) for 10 min at room temperature. Radiolabeled probe (175 fmol) was added to this mixture and incubated for an additional 20 min before the binding reaction was loaded onto a native 4% polyacrylamide gel, dried under a vacuum, and exposed to X-ray film. For the supershift assay, 3 μg of each specific polyclonal antibody against Oct-1, Oct-2, C/EBPα, C/EBPβ, and C/EBPδ (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the binding reaction, and the mixture was incubated at room temperature for 10 min before the addition of radiolabeled probe.

**RESULTS**

**Isolation and Genomic Organization of the Human CCR2 Gene**—Using hCCR2b cDNA as a probe, we screened 1 × 10⁶ plagues from a human genomic DNA library and isolated two independent positive clones designated AMCP-1R1 and AMCP-1R2. Most of the genomic organization of the hCCR2 gene was determined by the phage clone AMCP-1R1, which covers the entire coding sequence of the hCCR2b cDNA but lacks the carboxyl-terminal intracellular domain for hCCR2a (Fig. 1).
The hCCR2 gene consists of three exons divided by two introns, spanning a minimum of 8 kbp (Fig. 1). All splice junction sequences for the donor and the acceptor are in agreement with the GT/AG rule (data not shown). The sequence of the entire coding region was identical to that reported previously (8). The first exon encodes a 5'-untranslated region, and the second exon encodes the amino-terminal extracellular domain, the seven-transmembrane domain, and the hCCR2b carboxyl-terminal intracellular domain. The third exon contains the carboxyl-terminal intracellular domain of hCCR2a. The second intron is optionally used to create the hCCR2a isoform, whereas the first intron is essentially spliced.

**Structure of the 5’-Flanking Region and Transcription Initiation Site of the CCR2 Gene**—Several features of the nucleotide sequence 1.7 kbp upstream from the transcription initiation site of the hCCR2 gene are notable (Fig. 2). First, the putative promoter region contains the typical mammalian promoter consensus element TATAA at -231 to -227, and CCAAT at -212 to -208 (numbering the transcription initiation site as +1). Second, we found clusters of several well-known tissue-specific transcriptional factor binding sites, i.e. seven GATA consensus binding sites, three consensus C/EBP binding motifs, and three Oct-1 binding sequences. Third, the region lacks Sp1 and nuclear factor κB binding sites, which are important for the regulation of the MCP-1 gene.

The transcription initiation sites of the hCCR2 gene were determined in a primer extension analysis. A single prominent band was observed 26 bp downstream from the TATA box; this is commonly observed in nonhousekeeping mammalian genes (Fig. 3).

**Functional Analysis of the 5’-Flanking Region of the hCCR2 Gene**—To determine which cell line(s) expressed hCCR2 mRNA, we performed a Northern blot analysis using extracts of total RNA prepared from THP-1, J-111, and U-251 MG cells. The expression of hCCR2 mRNA was clearly detectable only in THP-1 cells; it was at undetectable levels in J-111 and U-251 MG cells (Fig. 4).

To define the minimal promoter region important for the basal and cell-specific expressions of the hCCR2 gene, we subcloned a series of 5’- and 3’-nested deletion mutants of the hCCR2 promoter into the Luc reporter vector pGL3-Basic for transient transfection assays using the three cell lines. Remarkable Luc activity was observed only in THP-1 cells; little activity was found across these constructs in J-111 cells and U-251 MG cells (Table I). This result was consistent with the Northern blot analysis, suggesting that these regions contained essential cis-regulatory elements important for the cell-specific expression of the hCCR2 gene.

Deletion from -242 to -185 (pGL3-0.5K versus pGL3-0.3K) reduced the level of expression by nearly 50% (Fig. 5). Further truncation to -122 restored the Luc activity to the previous level, suggesting the possible presence of inhibitory sequences that contain the CAAT box in this region. Although deletion from -122 to -89, which removed the distal Oct-1 binding sequence, did not change the Luc activity, deletion to -58 reduced the level of expression by nearly 60%. This suggests that the region from -89 to -59 that contains the proximal Oct-1 binding site is important for the transcriptional activation of hCCR2.

Curiously, pGL3-0.17K, which consisted of only a TATA box
downstream from a TATA box and a part of the 5'-UTR, retained the cell-specific promoter activity at a 6-fold increase relative to the activity of the pGL3-Basic vector. To determine which region functioned as a cell-specific cis-regulatory element, we constructed a series of 39 deletion mutants. Deletion from 1118 to 1175 (pGL3-0.23K versus pGL3-0.18KDE) reduced the level of expression by nearly 60%. Further deletion of more than 40 bp (pGL3-0.14KDE, pGL3-0.12KDE, and pGL3-0.1KDE) completely abolished the Luc activity. These observations suggest that the region from 136 to 1118 contained tissue-specific cis-regulatory elements and that the sequence near position 175 is responsible. Finally, highly probable C/EBP binding sites were identified in tandem at residues 150 to 163 (site 1) and 164 to 177 (site 2) by consensus search.

Binding of octamer factors to the hCCR2 promoter—The identification of the 31-bp region (289 to 259) adjacent to the TATA box and the 83-bp region (136 to 1118) within the 5'-UTR prompted us to determine which nuclear factors could bind to these sites, because the former region contained a fully matched octamer sequence, ATGCAAAT, and the latter region retained C/EBP binding sequences, i.e. AGGTTGCATAAGCA (site 1; 150 to 163) and AGATTTCAAAATTA (site 2; 164 to 177). First, we used the gel mobility shift assay to test nuclear extract from THP-1 cells for its ability to bind to a 30-bp radiolabeled probe corresponding to nucleotides 289 to 260 of the hCCR2 promoter (Fig. 6A). At least three protein-DNA complexes (B1, B2, and B3) representing specific binding to this 30-bp probe were identified (Fig. 6B, lane 2). B1 and B2 are efficiently competed by the same unlabeled oligonucleotide or the oligonucleotide composed of the octamer sequence itself (Fig. 6B, lanes 3 and 4). B3 was reduced even when nonspecific competitor was used (Fig. 6B, lane 5), suggesting that this band represents a nonspecific DNA-protein complex. The addition of a specific antibody against Oct-1 to the reaction abolished B1 and resulted in the formation of a supershifted complex (Fig. 6C, lane 3). In contrast, the inclusion of a specific antibody against Oct-2 resulted in the formation of a small amount of supershifted complex but apparently did not change the intensity of any of the bands. Thus, this octamer sequence is recognized mainly by Oct-1 in THP-1 cells, and Oct-1 may constitutively activate the expression of hCCR2 to the basal level.

Binding of the C/EBP family to the 5'-UTR of hCCR2 Gene—To investigate the possible transcriptional factor(s) that interact with the 5'-UTR of the hCCR2 gene, we subjected a 73-bp radiolabeled probe covering the region from +46 to +118 to gel mobility shift assays. Three retarded complexes (C1, C2, and C3) were observed with nuclear extracts from THP-1 cells (Fig. 7B, lane 2). The formation of all complexes was abolished by unlabeled competitors that contain two C/EBP binding sequences, i.e. AGGTTGCATAAGCA (site 1; 150 to 163) and AGATTTCAAAATTA (site 2; 164 to 177). First, we used the gel mobility shift assay to test nuclear extract from THP-1 cells for its ability to bind to a 30-bp radiolabeled probe corresponding to nucleotides -89 to -60 of the hCCR2 promoter (Fig. 6A). At least three protein-DNA complexes (B1, B2, and B3) representing specific binding to this 30-bp probe were detected (Fig. 6B, lane 2). B1 and B2 are efficiently competed by the same unlabeled oligonucleotide or the oligonucleotide composed of the octamer sequence itself (Fig. 6B, lanes 3 and 4). B3 was reduced even when nonspecific competitor was used (Fig. 6B, lane 5), suggesting that this band represents a nonspecific DNA-protein complex. The addition of a specific antibody against Oct-1 to the reaction abolished B1 and resulted in the formation of a supershifted complex (Fig. 6C, lane 3). In contrast, the inclusion of a specific antibody against Oct-2 resulted in the formation of a small amount of supershifted complex but apparently did not change the intensity of any of the bands. Thus, this octamer sequence is recognized mainly by Oct-1 in THP-1 cells, and Oct-1 may constitutively activate the expression of hCCR2 to the basal level.

FIG. 6. Binding of octamer factors to the hCCR2 promoter. A, sequence and position of the probe and competitor DNAs used in the gel mobility shift assay. B, results of a gel mobility shift assay using a radiolabeled probe that covers -89 to -60 (O1) and nuclear extracts from THP-1 cells, as described under “Experimental Procedures.” Unlabeled competitors, which were present at a 100-fold molar excess, are as indicated in A (lanes 3–5). Complex co-migrations with bands B1, B2, and B3 formed on the O1 probe are indicated. C, antibodies against Oct-1 and Oct-2 were preincubated with nuclear extracts from THP-1 cells before the addition of radiolabeled O1. Antibody against C/EBPβ was used for the negative control.
transcriptional factors that recognize the C/EBP binding motifs.

To further corroborate the presence of C/EBP family members in the respective complexes, we performed antibody perturbation experiments. Antibodies against C/EBPα, C/EBPβα, and C/EBPδ were tested for their ability to interact with the complexes described above. The presence of C1 was due to C/EBPα binding, because anti-C/EBPα antibody decreased C1, resulting in a supershifted band (Fig. 7C, lane 3). The presence of C2 was due to C/EBPδ recognition, and the presence of C3 was due to C/EBPδ recognition, because they were reduced by anti-C/EBPδ or anti-C/EBPδ antibodies, respectively, resulting in supershifted bands (Fig. 7C, lanes 4 and 5).

Roles of Oct-1 Binding and C/EBP Binding in the Expression of hCCR2—To determine whether the cell-specific expression of CCR2 is actually dependent on the expression of Oct-1 or the C/EBP family, we performed a gel mobility shift assay using nuclear extracts from CCR2-negative J-111 and U-251 MG cells with a radiolabeled O1 probe or N1 probe (Figs. 6A and 7A). These transcriptional factors were almost absent in U-251 MG cells and were considerably decreased in J-111 cells (Fig. 8). It was particularly interesting that C/EBPα (indicated by the C1 complex) was not detected in either cell line. These results suggest that the expression of hCCR2 is regulated by the tissue-specific expression of Oct-1 and the C/EBP family.

The role of the particular octamer sequence and the two C/EBP binding sites was confirmed by the Luc assay using promoter reporter constructs containing single or multiple mutations of each motif (Fig. 9). Alteration of the octamer sequence by substitution decreased promoter activity by 68%, which is consistent with the results of deletion analysis (Fig. 5).

The introduction of mutations at site 1 or both C/EBP binding sites completely abolished the Luc activity, whereas the mutagenesis of site 2 reduced the promoter activity by 66%. Accordingly, the Oct-1 binding site and both C/EBP binding sequences are actually active in the transcriptional regulation of hCCR2 in THP-1 cells. Among them, site 1 was the most important for basal and tissue-specific transcriptional activity.

DISCUSSION

In the present work, we isolated and functionally characterized the 5′-flanking region of the hCCR2 gene to elucidate its regulatory mechanism. The structural analysis disclosed that the proximal 5′-flanking region of this gene contained a classic TATA box, a CAAT box, and clustered consensus sequences for tissue-specific cis-regulatory elements, i.e. the GATA binding
sequence, Oct-1 binding sequence, and C/EBP binding motifs. This is compatible with the fact that the hCCR2 gene is not a housekeeping gene and is regulated in a cell-specific manner.

Among the members of the CCR family, only the promoter region for hCCR5 has been isolated and functionally characterized to date (23). The transcription of the hCCR5 gene was initiated by two different promoters, and both proximal and distal promoters lack the canonical TATA or CAAT box. Instead, they contain the consensus sequence for several transcriptional factors such as Oct-1 and multiple motifs for GATA-1, which are also observed in the hCCR2 gene. It is interesting that these closely related genes have rather different transcriptional initiations, although they are thought to share the same ancestral gene.

The Luc assay using various deletion mutants of the hCCR2 promoter region demonstrated that at least four regions are important for the constitutive expression of hCCR2 in THP-1 cells: (a) −429 to −186 for activation, (b) −185 to −123 for repression, (c) −89 to −59 for activation, and (d) +36 to +118 for cell specificity and activation. Among these four regions, we focused on the latter two, because the deletion mutant containing only these regions (−89 to +118) was sufficient for the basal expression and tissue specificity.

The gel mobility shift assay and mutagenesis demonstrated that Oct-1 bound mainly to the octamer consensus sequence (ATGCAAAT) located in the third region (from −89 to −59) and was probably responsible for the trans-activation of this region. Although Oct-1 is ubiquitously expressed (24), it is reduced or under the detectable level in CCR2-negative cell lines (J-111 and U-251 MG cells) (Fig. 8). This suggests that Oct-1 plays a certain role in the tissue-specific expression of hCCR2.

The most important cis-acting element was located at a rather unusual position, the 5′-UTR of the hCCR2 gene (+36 to +118). It has been reported that the 5′-UTR could regulate the expression of genes in two different ways, one of which is that the 5′-UTR can work as a tissue-specific active translational enhancer taking a stable stem and loop structure; examples are the human γ-glutamyl transferase gene (25), the thymidine kinase gene (26), the glutathione peroxidase gene (27), and the ornithine decarboxylase gene (28). Stable secondary structures were predicted between +12 and +118 of the hCCR2 gene, presenting the free energy formation of −43.6 kcal/mol. However, the present Northern blot analysis indicated that the hCCR2 gene is regulated at least at the transcriptional level in various cell lines (Fig. 4). Therefore, we should focus on the other mechanism, the transcriptional activity of the 5′-UTR, which has been demonstrated in the human plasminogen gene (29) and the ⁿγ-globin gene (30), indicating that the transcriptional factor bound to a specific region within 5′-UTR.

We particularly noted the position near +75, where two putative C/EBP binding sequences were located in tandem, because pGL3-0.18KΔE, which partially disrupted the C/EBP binding consensus, reduced the Luc activity by nearly 60%. The gel mobility shift assay using a 73-bp fragment containing tandem C/EBP binding sequences as a probe detected three prominent bands. Competition using the C/EBP consensus sequence and the supershift assay demonstrated that each band was related to C/EBPa, C/EBPβ, or C/EBPδ. Finally, the introduction of mutation in each motif disclosed that site 1 was the most important site for the tissue-specific expression of hCCR2.

The C/EBP family of transcriptional factors is involved in tissue-specific gene expression in adipocytes, hepatocytes, and monocyte/macrophages (31). In particular, target genes for C/EBP include acute phase response genes in liver cells and

---

**Fig. 8.** Differential expressions of octamer factors and the C/EBP family in THP-1 cells, J-111 cells, and U-251 MG cells. Results of gel mobility shift assays using nuclear extracts from THP-1 cells, J-111 cells, and U-251 MG cells, as described under “Experimental Procedures.” Equal amounts (4 μg) of nuclear extracts from each cell line were mixed with either radiolabeled O1 probe or N1 probe to confirm the binding of octamer factors and the C/EBP family.

**Fig. 9.** Inhibition of the hCCR2 promoter activity by the introduction of mutations at the octamer factor binding sequence and C/EBP binding sequences. Schematic representation of hCCR2 reporter constructs. The TATA box, proximal octamer factor binding site (−81 to −68), and two C/EBP binding sites (Site 1 and Site 2) are indicated by closed box, open oval, and closed ovals, respectively. The transcription initiation site is indicated by a bent arrow. These sites were mutated (indicated by X) in some of the constructs. These constructs were transfected into THP-1 cells as indicated in Fig. 5, and the relative promoter activities were calculated by arbitrarily defining the activity of pGL3-Basic vector as 1. Columns indicate the mean ratio of the Luc activities. Error bars, the standard deviation.
cytokine genes in monocytes/macrophages (31). Our present results demonstrate that CCR2-negative cells (J-111 and U-251 cells) had considerably reduced expression of the C/EBP family (Fig. 8). Therefore, it is reasonable to speculate that the C/EBP binding sequence located in the 5′-UTR can regulate the expression of hCCR2 in a monocyte-specific manner.

It is interesting that C/EBPβ and C/EBPδ contribute to the lipopolysaccharide response of MCP-1, the ligand of CCR2 (32). MCP-1 and CCR2 are probably coordinately regulated at the transcriptional level by the C/EBP family for effective immune responses. The tumor cytotoxicity of macrophages from C/EBPβ knockout mice was severely impaired (33). Our previous study showed that the growth of MCP-1-producing tumors was inhibited by the infiltration of tumor-associated macrophages (15), indicating that MCP-1 and CCR2 may play an important role in the cytotoxicity of macrophages in cancer. Although we were unable to identify the stimulation that effectively induces the expression of hCCR2, our present findings provide a useful foundation for further studies.

Acknowledgment—We thank Ursula Petralia for editorial assistance.

REFERENCES

1. Kuratsu, J., Leonard, E. J., and Yoshimura, T. (1989) J. Natl. Cancer Inst. 81, 347–351
2. Yoshimura, T., Robinson, E. A., Tanaka, S., Appella, E., Kuratsu, J., and Leonard, E. J. (1989) J. Exp. Med. 169, 1449–1459
3. Yoshimura, T., and Leonard, E. J. (1992) in Cytokine (Baggiolini, M., and Sorg, C., eds) Vol. 4, pp. 131–152, Karger, Basel, Switzerland
4. Takeya, M., Yoshimura, T., Leonard, E. J., and Takahashi, K. (1993) Hum. Pathol. 24, 534–539
5. Koch, A. E., Kunkel, S., Harlow, S. L., Johnson, L. A., Evanoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M., and Strieter, R. M. (1992) J. Clin. Invest. 90, 772–779
6. Walter, S., Botazzi, B., Govoni, D., Colotta, F., and Mantovani, A. (1991) Int. J. Cancer 49, 431–435
7. Takeshima, H., Kuratsu, J., Takeya, M., Yoshimura, T., and Ushio, Y. (1994) J. Neurosurg. 80, 1056–1062
8. Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J., and Coughlin, S. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2752–2756
9. Myers, S. J., Wong, L. M., and Charo, I. F. (1995) J. Biol. Chem. 270, 5786–5792

...and more references follow.