



## Molecular cloning and functional characterization of a mouse *ccl6* analog gene in the rat

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**ABSTRACT.** Suppression subtractive hybridization was used to analyze differential expression of genes in rat peritoneal macrophages after granulocyte macrophage colony-stimulating factor treatment. We identified and cloned the mouse C10 analog gene in the rat, and named it as *ccl6*. The full-length cDNA of rat *ccl6* was 467 bp, which contains a single-open reading frame and encodes 116 amino acid residues. Compared with other C-C chemokines, the rat *ccl6* gene had an unusual four-exon genome structure instead of the typical three exons, it had the highest homology with murine *ccl6*. The rat *ccl6* gene was localized on chromosome 10, where most of the C-C chemokine superfamily members are located. The recombinant rat C-C chemokine ligand 6 (CCL6) protein was expressed by the pGEX4T-1 plasmid in *Escherichia coli* BL21. The purified recombinant protein had bioactivity similar to that of mouse CCL6, which is a chemoattractant for macrophages and

lymphocytes, but not for neutrophils.

**Key words:** *ccl6*; Genome structure; Chemoattractant; Macrophages; Lymphocytes

## INTRODUCTION

C-C chemokine ligand 6 (CCL6, or C10) is a C-C chemokine that has been cloned from mouse (Berger et al., 1993). CCL6 is typically produced by activated macrophages, lymphocytes, and fibroblasts, and is stimulated by exposure to interleukin (IL)-3, IL-4, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF; Orlofsky et al., 1994). This novel chemokine shares high homology with human C-C chemokine, macrophage inflammatory protein-related protein 1 (MRP-1). Both of these chemokines are chemotactic for monocytes and T cells. Previous studies demonstrated that CCL6 had multiple activities (Olson and Ley, 2002). Furthermore, CCL6 is differentially regulated by T helper (Th)1 and Th2 cytokines (Orlofsky et al., 2000), and is also involved in a variety of chronic inflammatory disorders (Belperio et al., 2002).

Although mouse CCL6 has been extensively studied, its human or rat homologue is still unknown. In our previous study, we used the suppression subtractive hybridization (SSH) method to analyze the differential expression of genes in the rat peritoneal macrophages after stimulation with GM-CSF. Sequencing results showed that one positive clone, SSH-8, was the homologue of mouse *ccl6*. In this study, we cloned the homologue of *ccl6* from GM-CSF-treated rat macrophages. The novel chemokine was expressed in a prokaryotic system and purified. The purified protein had chemotactic effects on rat lymphocytes and macrophages, but not on neutrophils *in vitro*, which are similar to the effects of mouse CCL6 on these cells. These findings would be useful in the mechanistic study of inflammatory disorders using rat models.

## MATERIAL AND METHODS

### Database search and full-length cDNA deduction

SSH-8-related sequences were searched in dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/>) using the default parameters of the BLASTN program (<http://www.ncbi.nlm.nih.gov/blast>). A number of rat expressed sequence tags (ESTs) were identified using a method described elsewhere (Lou et al., 2003). These ESTs were retrieved and a contig was assembled from overlapping ESTs using the EST Assembly machine on the World Wide Web (<http://www.tigen.it>). The accession numbers of ESTs used for rat *ccl6* analog assembly were AW917998, AA848632, AI169984, AA800480, AI234153, BF555934, and BE095824.

### Cell culture and cloning of rat *ccl6*

Rat macrophages were purified from the parietal peritoneum and cultured in Dulbecco's modified Eagle's medium (supplemented with 100 U/mL ampicillin and 100 µg/mL streptomycin). Cultured cells were treated with 50 ng/mL GM-CSF for 24 h, and total RNA was extracted from cells using Trizol (lot: 1148608, Invitrogen, Carlsbad, USA), according to manufacturer instructions. For reverse transcriptase (RT)-PCR, total RNA was

reverse transcribed using 600 U Moloney murine leukemia virus reverse transcriptase and oligo (dT; Takara) at 37°C for 60 min. Nested PCR was performed using the following primers: P1: 5'-AGACCAGCTGGGCCTGTCCT-3'; P2: 5'-ATGTCAGGCTGAATCCATGTGAA-3'; P3: 5'-CAGGATGATGAGACATTCC-3'; P4: 5'-GGAGACAGAAGAATTCAGG-3'. After gel extraction, the PCR product was inserted into the pMD18-T vector (Takara) and named pMD18-T/*ccl6*. The accuracy of pMD18-T/*ccl6* was confirmed by enzyme digestion and sequencing analysis by Genecore Corp. (Shanghai). The characteristics of the cDNA fragments and their putative amino acid sequences were analyzed using the DNAsis software (HITACHI).

### Recombinant rat CCL6 production and purification

The coding region of rat *ccl6* was amplified from the vector pMD18-T/*ccl6* using primers P3 (5'-ATGCGGATCCATGATGAGACATTCCAAGAC-3') and P4 (5'-ATGCGAATTCAAGCAATGGCACTG-3'), in which a *Bam*HI site was inserted at the 5'-region and an *Eco*RI site was inserted at the 3'-region. The PCR product and pGEX4T-1 (Pharmacia) expression vector were digested with *Bam*HI and *Eco*RI. After extraction from gels, the *ccl6* fragment was inserted into the vector. The recombinant plasmid was pGEX4T-1/*ccl6*, and the accuracy of insertion was verified by restriction enzyme digestion.

The pGEX4T-1/*ccl6* vector was transformed into *Escherichia coli* (BL21 strain) by a standard protocol. The recombinant protein was linked to glutathione S-transferase (GST), named GST-CCL6 protein. The GST-CCL6 protein was extracted from bacterial cells by sonication in phosphate-buffered saline (PBS) containing 1% Triton X-100 and purified by glutathione-Sepharose 4B column (Pharmacia; Ikadai et al., 1999).

The purified fusion protein was cleaved with thrombin (10 U/mg fusion protein, Amersham Pharmacia Biotech) overnight (12 h) at room temperature, and then eluted by PBS (Williamson et al., 2000). The solution was purified by glutathione-Sepharose 4B column again to remove the fusion GST tag. The solution was loaded onto a Sephacryl-G75 column (2.6 x 80 cm) to obtain a purified CCL6 protein. Protein samples were analyzed by 15% polyacrylamide gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### Chemotaxis assay

Purified neutrophils were resuspended at  $1 \times 10^6$  cells/mL in Hank's balanced salt solution (HBSS), and macrophages and lymphocytes were resuspended at  $2 \times 10^6$  cells/mL and  $5 \times 10^6$  cells/mL in Roswell Park Memorial Institute medium (RPMI) 1640 (Life Technologies) supplemented with 0.5% low endotoxin bovine serum albumin (BSA) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), respectively. Cell migration was measured using a 48-well chemotaxis chamber as previously reported (Sarafi et al., 1997). The lower wells contained the purified rat CCL6 protein, and the medium without the purified rat CCL6 protein was set as the untreated control. The pore size of the polycarbonate membrane (Millipore) for the neutrophils was 3  $\mu$ m and the incubation time was 30 min. For monocytes and lymphocytes, a 5- $\mu$ m pore membrane was used and the incubation time was 90 min. The cells that passed through the pore of membrane were fixed and stained. Stained cells were counted in 3 random fields (400X magnification) under light microscope. Results were represented as means  $\pm$  standard deviation from triplicate samples.

## RESULTS

### SSH screen and the deduced sequence for rat *ccl6*

Using the SSH method, we found that 26 positive clones were homologous to mouse *ccl6*. The searching sequences from the GenBank database revealed that the fragment of SSH-8 might be a novel gene. Using this sequence to search the EST database, a number of ESTs were identified. With help of the EST assembly machine, we spliced a full-length rat gene (Figure 1). It included a single polyadenylation signal AATAAA and a poly(A) tail. Based on rat genome information, it was located on chromosome 10, where most of the rat chemokines are located.

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GATCCCCAGACCAGCTGGGCCTGTCCTTCAGGATGATGAGACATTCCAAGACTGCCATT      60
TCATTCTTTATCCTTGTGGCCGTCCTTGGGTCCCAGGCTGGGCTCATACAAGATACGGTA    120
AAAGAAGATCGTCCCTTTAATCCTACAATAATTACCAAGGCTTCAAGACTCTTCAGAC      180
TGCTGCTTCTCCTATGCCTCACAGATCCCATGTTCAAGATTTATATATTATTTTCCAAC    240
CAGTGGTGGGTGCACCAAGCCAGGCATCATCTTTGTCAACCAGGAAGAGGAAGCGGGTCTG   300
TGCCAACCCGAGTGATCAGAGAGTTTACAGCGTGCATAAGCACCCCTGAAGCTAGGCCCAAG  360
ATCTGGGAACAGTGCCATTGCTTGAGAAGGAGGGCGTACTGCCACCTGCATTCTTCTGTC   420
TCCCCAGTGACCACCTAGGAAGCGCTAAGCATTGTGTTTTATAGATATTTAAAGCACGTA    480
TTCTTCTGTACAGGTTTTAAAGCAGCACATGTAGTCCACAGTATTCACATGGATTTCAGCCT  540
GACATGATTAGAGCCATCTCGAGGTGTAACCATCATGAGTGTCTTTGGGTAACCTGTTGGG   600
TTTTCTTGATAGTTCCCTCAGCAGATTATAAATGGATAAACTACTAGTCTTTGGGACTTT   660
GGAATGTGCTCGGTTTTGATGCAAGCTTAAGCCAGGTGTATCCAGCTGAGATGAAATCAA    720
TTTTTGCCCTAAGCCATACATATGTCCAACCTTTGTGGGGACCCAGCTGTTCTTCTGCCA   780
CCATAGAGCAGTAAGTGACCCCAATAAAGTGCACCTCCATGTAAAAAAAAAAAAAAAAAAAA  838

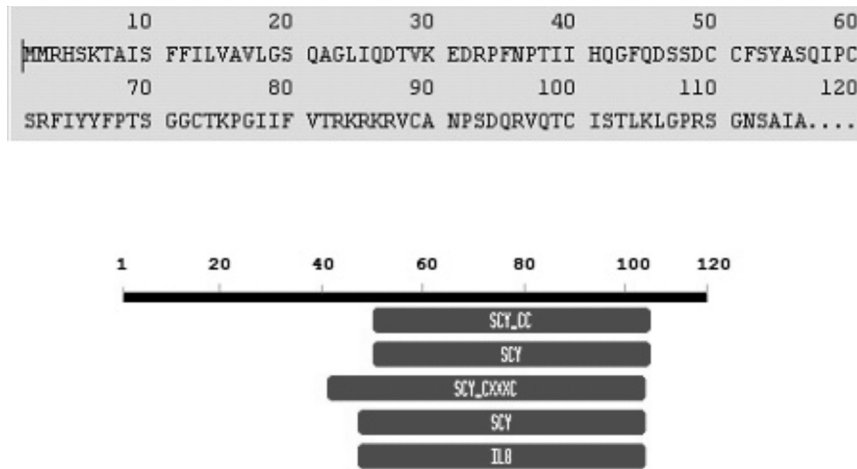
```

**Figure 1.** Complete assembled contig sequence of ESTs. The letters in bold are the encoding region.

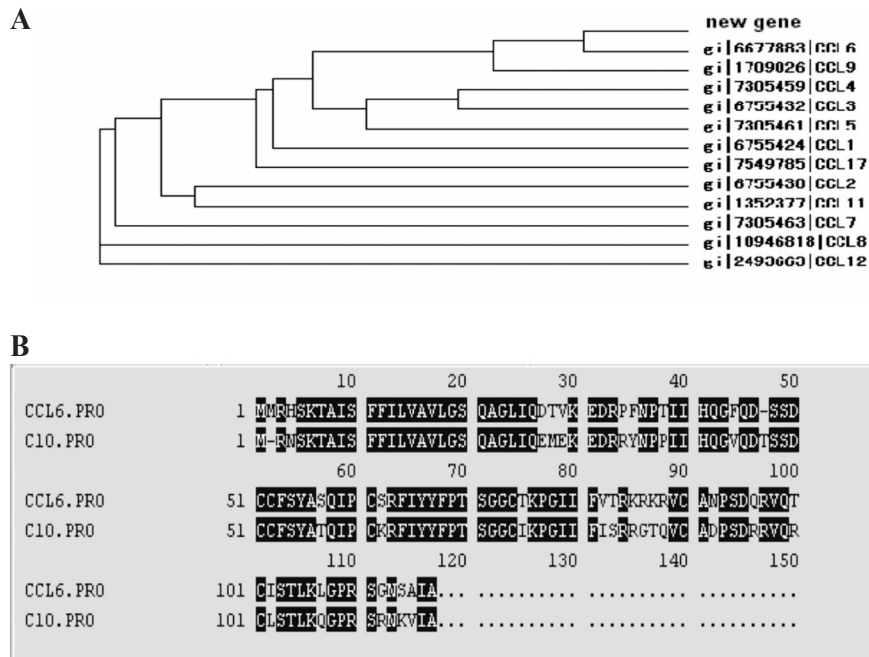
### Cloning and sequencing of rat *ccl6*

We used the nested primers to localize the upstream start codon and the downstream stop codon, and the full-length cDNA of rat *ccl6* was successfully obtained from GM-CSF-stimulated rat macrophages by RT-PCR. The cDNA fragment was successfully cloned into the pMD18-T vector and checked by sequencing. It was demonstrated to be a novel gene by the sequencing results. The full-length cDNA was uploaded to GenBank (accession No. AY620903). The predicted amino acid sequence and NCBI Conserved Domain Search results are shown in Figure 2. There was only 1 open reading frame with an ATG initiation codon at nucleotide 26. It encoded a polypeptide of 116 total residues with a calculated molecular mass of 12.8 kDa. The peptide sequence had a typical C-C chemokine motif compared to Conserved Domain Database at GenBank (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd>; Petrenko et al., 1995). We compared it with mouse C-C chemokines using CLUSTALW (<http://www.biosino.org/pages/lab.htm>; Figure 3A). It shared 79% sequence homol-

ogy with mouse CCL6 (Figure 3B). The deduced rat CCL6 protein did not contain the putative glycosylation site and typical signal cleavage site, based on the searching on the Signal P server (Nielsen et al., 1999); thus, we chose the prokaryotic system to express it.



**Figure 2.** Predicted amino acid sequence and NCBI Conserved Domain Search result.



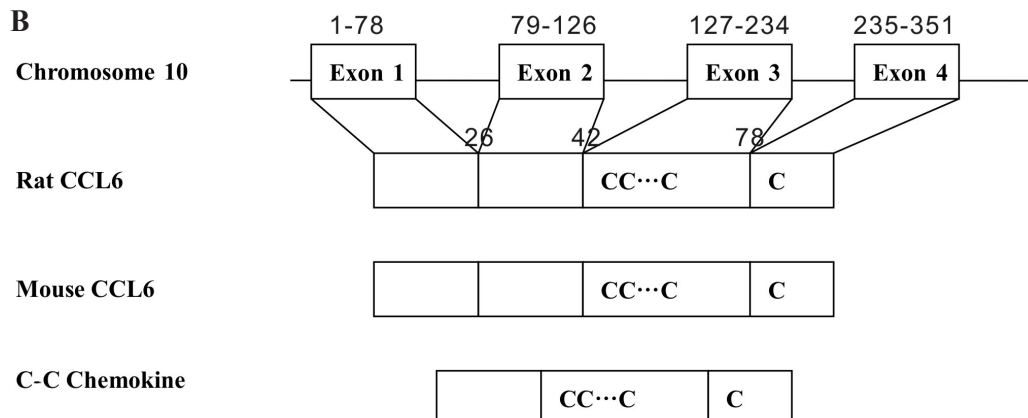
**Figure 3.** Alignment of the new gene with other chemokines. **A.** Alignment of the new gene with other mouse C-C chemokines using CLUSTALW. **B.** Alignment of the new gene with mouse CCL6; rat CCL6 shares 79% homology with mouse CCL6. Conserved amino acids are shown in black background.

We also revealed that the new gene sequences were similar to 4-interval fragments in the chromosome. The mouse *ccl6* gene has 4 exons, and exons 3 and 4 of *ccl6* contain 4 cysteines in the same manner as those in exons 2 and 3 of other beta-chemokine family members. In this study, we observed that the structure of rat *ccl6* was very similar to that of mouse *ccl6* (Figure 4A and B).

## A

<b>Exon1</b>		<b>Intron1</b>		<b>Exon2</b>
<u>CTC ATACAA</u>	ctgagctctc ...	(3643 bp)...	cctcaccttg	AGATACGGT
<b>Exon2</b>		<b>Intron2</b>		<b>Exon3</b>
ATT CAC CAA	taaacgacca...	(385 bp) ...	ctcttctac	GCTTTC AAG
<b>Exon3</b>		<b>Intron3</b>		<b>Exon4</b>
CAG GCA TCA	gtaagtgtc ...	(332 bp) ...	tttctcttc	ATC TTT GTC

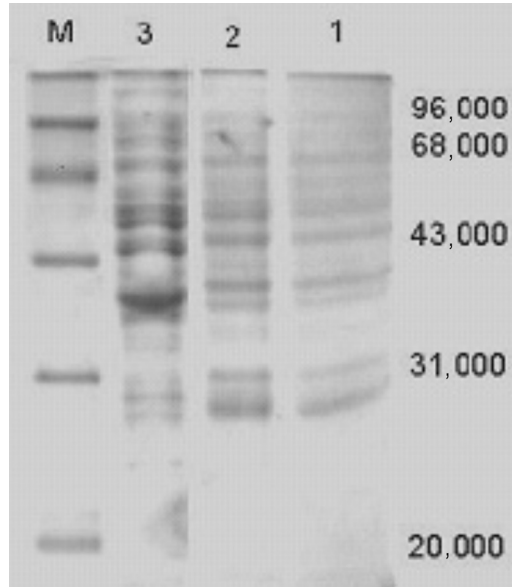
## B



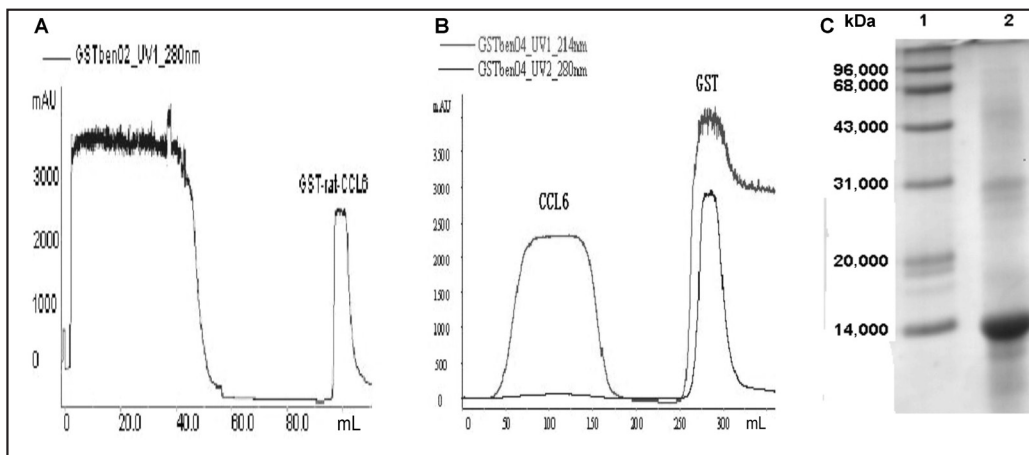
**Figure 4.** A. Sequences of the intron-exon junctions in rat *ccl6*. Exons are shown in capital letters; intron sequences are shown in lower case letters. The length of each intron is shown in parentheses. B. Sketch map of rat CCL6, mouse CCL6 and other C-C chemokines. Rat CCL6 and mouse CCL6 have four exons (the third exon contains the first three of four conserved cysteines, and the fourth exon contains the last conserved cysteine), while most other C-C chemokines only have three exons.

## Expression and purification of rat CCL6

The recombinant rat CCL6 protein was expressed in a soluble form and obtained as a fusion protein with GST. The protein had an apparent molecular size of 39 kDa, which was equal to the predicted molecular weight of the fusion protein (Figure 5). The recombinant fusion protein constituted approximately 15% of total cellular proteins in the bacterial cells. The purification procedure for rat CCL6 polypeptide production is shown in Figure 6. The final optimized yield of rat CCL6 was approximately 3-4 mg/L culture broth, which was higher than the typical yield of 2.5 mg/L for GST fusion proteins (Amersham Pharmacia Biotech).



**Figure 5.** SDS-PAGE analysis for total proteins from *Escherichia coli* bacterial cells: *lane 1* = crude lysate of BL21 cells with IPTG induction; *lane 2* = crude lysate of BL21 cells that are transformed with pGEX4T-1 and induced by IPTG; *lane 3* = crude lysate of BL21 cells that are transformed with GEX-4T-1/*ccl6* and induced by IPTG; *lane M* = molecular weight marker.

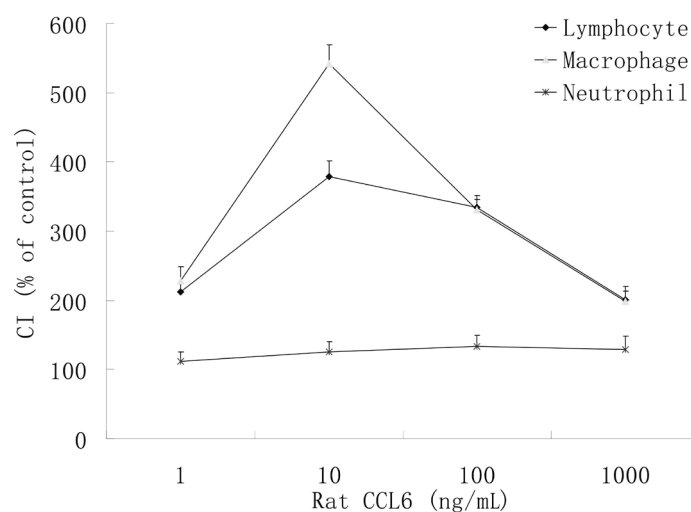


**Figure 6.** Purification of CCL6 protein. **A.** Glutathione Sepharose 4B affinity column to purify fusion protein. **B.** Gel filtration pattern on G75 of eluent from Glutathione Sepharose 4B after thrombin digestion. **C.** SDS-PAGE analysis for purified rat CCL6. *Lane 1* = molecular weight marker; *lane 2* = purified rat CCL6. GST = glutathione S-transferase.

### Chemotactic activities of recombinant rat CCL6

To confirm the chemotactic activity of rat CCL6, the effect of rat CCL6 on the migra-

tion of macrophages and lymphocytes was examined using a chemotaxis chamber (Aarvak et al., 1999). The chemotactic activity results were represented by the percentage obtained by the number of migrated cells in the medium containing rat CCL6 divided by the number of migrated cells in the control medium without rat CCL6. Compared with the control medium, the medium containing rat CCL6 induced an obvious chemotactic response in rat macrophages and lymphocytes that peaked at 10 ng/mL, although the response was reduced at higher concentrations in a bell-shaped chemotactic response curve (Han et al., 2001b). Rat CCL6 had no obvious effect on the migration of neutrophils in this experiment (Figure 7).



**Figure 7.** Chemotaxis of leukocyte subsets by rat CCL6. Leukocyte subsets were exposed to rat CCL6 (1~1000 ng/mL) and cell migration was determined. The medium without rat CCL6 was set as the untreated control. Values are means  $\pm$  standard deviation from three independent experiments. CI = (number of migrated cells in the medium with rat CCL6  $\div$  number of migrated cells in the control medium)  $\times$  100%.

## DISCUSSION

Chemokines comprise a broad family of chemoattractant cytokines that play important roles in several physiological and pathological processes (Li et al., 2000; Gerard and Rollins, 2001). Based on the appearance of 4 conserved cysteine residues, 4 subfamilies are distinguished. Chemokines are thought to have evolved from 1 ancestral gene, thus their sequences share high homology. Chemokines belong to a single subfamily and are clustered in 1 chromosome. Hundreds of chemokine genes have been reported, and they are highly conserved among humans, mice, and rats.

Herein we were interested in determining the mouse *ccl6* analog gene in rat. Although all initial attempts were unsuccessful to clone the mouse *ccl6* analog gene from human cDNA libraries, the search for its rat homologue was successfully conducted. In this study, SSH, a comprehensive and large-scale gene expression-based approach, was used to unbiasedly discover the genes that are induced or suppressed by a particular treatment. Furthermore, SSH allows one to identify differentially expressed cDNAs without prior knowledge of their sequence. The SSH-8 clone that contains the typical chemokine characteristic sequence was



identified as a new sequence after comparing it with all known chemokine sequences. A number of rat ESTs were identified after searching this sequence in the EST database.

Most members of the C-C chemokine subfamily have 3 exons, and the second exon contains the first 3 of the 4 conserved cysteines, and the third exon contains the last (Han et al., 2001a). In this study, we found that the rat *ccl6* gene contained 4 exons that are similar to the mouse *ccl6* gene. Thus, we named the new gene rat *ccl6*.

Chemokine C10, also called MRP-1 or CCL6, is a C-C chemokine that was first isolated in 1991 from mouse bone marrow (Orlofsky et al., 1991). It is a macrophage chemoattractant both *in vitro* and *in vivo*. It is upregulated in experimental allergic bronchopulmonary aspergillosis during chronic peritoneal inflammation (Steinhauser et al., 2000), and in mouse models of cutaneous wound repair (Kaesler et al., 2002). Our chemotactic assays suggested that rat CCL6 was a major chemoattractant for macrophages and lymphocytes but not for neutrophils, which is similar to the chemotactic function of mouse CCL6. Mouse CCL6 can be selectively induced by IL-10 and GM-CSF, thus we cloned our new gene from GM-CSF-treated macrophages. The assembled ESTs are primarily derived from liver, kidney, heart, brain, and eye, which indicates that CCL6 may be expressed in these tissues. In addition, GM-CSF is probably an important inducer for rat CCL6.

In summary, we cloned the novel rat cytokine gene *ccl6* by the SSH method. Furthermore we showed that rat CCL6 and mouse CCL6 had similar characteristics and bioactivity.

## ACKNOWLEDGMENTS

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