*Original Article*

# The Monocyte Chemotactic Protein-1 Gene May Contribute to Hypertension in Dahl Salt-Sensitive Rats

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**In a previous study, we performed a genome-wide quantitative trait loci (QTLs) analysis for blood pressure using F2 rats derived from Dahl salt-sensitive (DS) and Lewis (LEW) rats and identified two QTLs that influenced blood pressure levels. Although we determined that one of the causative genes in the chromosome (Ch) 1 region seemed to be *Klk1*, we did not perform detailed analyses on the Ch10 QTL region. The purpose of the present study was to identify candidate genes that influence blood pressure in the Ch10 QTL region. Using microarray analysis, we compiled a list of the genes that are differentially expressed between the two strains and that were localized to the Ch10 QTL region. Subsequent reverse transcription–polymerase chain reaction (RT-PCR) and Northern blot analysis identified that, while the expression levels of *Ccl2* mRNA were not different between the kidneys of DS and LEW rats fed a normal diet, those in DS were 10-fold higher than those in LEW under a high-salt diet. Although the promoter reporter assay failed to identify causative nucleotide changes that led to the differential expression, monocyte chemotactic protein-1 (MCP-1) release from isolated monocytes were significantly higher in DS than in LEW. Intriguingly, this Ch10 QTL for blood pressure was also a possible QTL for urinary albumin excretion. Since *Ccl2* is well known to be involved in various types of renal injury, it is likely that a higher expression of *Ccl2* might aggravate macrophage infiltration, which in turn could aggravate tubulointerstitial injury, and thereby accelerate salt-sensitive hypertension. Thus, *Ccl2* appears to be a interesting candidate gene for salt-sensitive hypertension in DS. (*Hypertens Res* 2007; 30: 185–193)**

***Key Words*: Ch10, *Ccl2*, candidate gene, salt-sensitive hypertension**

## Introduction

Dahl salt-sensitive (DS) rats have been widely used to study salt-sensitive hypertension. Sixteen genomic regions containing quantitative trait loci (QTLs) for blood pressure (BP) regulation have been reported in this strain (*1–3*). However, all of the major genes responsible for this salt-sensitive hypertension have not yet been confirmed, despite numerous genetic studies.

In a previous study, we performed a genome-wide QTL analysis for BP using 107 male F2 rats derived from DS and Lewis (LEW) rats (*4*). BP was measured by a telemetry system, and the genotypes of the F2 rats were determined with >400 genetic markers throughout the genome. We identified two QTLs that influenced BP levels: a broad chromosome

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(Ch) 1 region around *Klk1* and a broad Ch10 region around *Pex12*. Although we determined that one of the causative genes in the Ch1 region seemed to be *Klk1*, we did not perform detailed analyses on the Ch10 QTL region. The purpose of the present study was to identify candidate genes that influence BP in the Ch10 QTL region. For this purpose we performed microarray analyses in the kidneys of DS and LEW rats. We compiled a list of the genes that are differentially expressed between the two strains that localized to the Ch10 QTL region, and identified an intriguing candidate gene, the *Ccl2* gene, the product of which is known as the monocyte chemoattractant protein-1 (MCP-1).

## Methods

### Experimental Animals

DS and LEW rats were purchased from Sunplanet (Tokyo, Japan) and Charles River Japan (Yokohama, Japan), respectively. Rats were housed in a temperature-controlled room with the light on from 7:00 AM to 7:00 PM (daytime) and fed normal rat chow (0.5% NaCl; Clea Japan, Tokyo, Japan) and tap water *ad libitum*.

The details of F2 analysis have been described previously (*4*). Briefly, male DS rats were mated with female LEW rats to produce F1 rats, and F1 rats were then intercrossed to produce an F2 population consisting of 107 male rats. F2 rats were started on an 8% NaCl diet (Oriental Yeast, Tokyo, Japan) at 5 weeks of age according to the protocol described by Garret *et al*. (*1*). Radiotelemetry devices (Data Sciences International, Arden Hills, USA) were implanted into the lower abdominal aorta of F2 rats at 9 weeks of age using sodium pentobarbital as an anesthetic agent (25 mg/kg i.p.). Just 2 days before BP levels were assessed by telemetry, rats were housed in metabolic cages and their urine was collected for 48 h for the assessment of urinary albumin (UA) levels. At 14 weeks of age, BP and heart rate were continuously measured for 44 h (daytime 7:00 AM to 7:00 PM; nighttime 7:00 PM to 7:00 AM), and the data obtained during the latter 24 h were used for analyses. The results were analyzed using Fluclet TM software (Dainippon Pharmaceutical, Osaka, Japan) (*5*). The present study was conducted in accordance with the current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

### Genotyping and QTL Analysis

Genotyping was performed by polymerase chain reaction (PCR) using appropriate PCR primer pairs (custom-made by Amersham Pharmacia Biotech, Piscataway, USA), based on information from the Rat Genome Database (http://rgd.mcw.edu/) and Ratmap (http://ratmap.gen.gu.se/). We found sequence variations between DS and LEW rats in several genes, and these polymorphisms were also included in the genotyping data. We separately analyzed QTLs for daytime systolic BP (D-SBP), diastolic BP (D-DBP), nighttime systolic BP (N-SBP), diastolic BP (N-DBP), and UA levels using MapManager QTLb20.6 (*6*). UA excretion per day was logarithmically transformed to attain a normal distribution ($\log_{10}$[UA/day]). We first performed a Quick Test to obtain significant thresholds for BP and UA values. The likelihood ratio statistics (LRSs) for suggestive, significant, and highly significant loci were calculated to be 12.3, 17.7, and 25.9, respectively. Next, we performed a marker regression for these 4 BP and UA values. The most significant QTL was added to the background, and a second marker regression was performed to obtain the second QTL, which was added to the background to obtain the third QTL.

### Microarray Analysis

Male DS and LEW rats ($n$=3 each) were fed an 8% NaCl diet starting at 5 weeks of age for 8 weeks. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, USA) from DS and LEW kidneys, and subjected to microarray analysis. Procedures for the microarray analysis were essentially identical to those reported previously (*7*). The rat genome GeneChip® Rat Genome 230 2.0 arrays were used in the present study. The resulting data were analyzed with GeneChip Operating Software. Three independent experiments were performed to compare expression levels of mRNAs between salt-loaded DS and salt-loaded LEW rat kidneys (the raw data have been submitted to NCBI; GEO accession number: GSE 4800). We selected genes whose expression levels were modulated more than 2-fold or less than 0.5-fold in all three independent assessments. Genes with expression levels below 100 in both strains were excluded due to unreliability. We then selected genes that were localized to the Ch10 QTL region between D10Rat32 and D10Rat93.

### Northern Blotting

Male DS and LEW rats ($n$=6 each) were fed an 8% NaCl diet starting at 5 weeks of age for 8 weeks, while control groups ($n$=6 each) were fed an 0.3% NaCl diet for the same period. Total RNA samples were prepared from kidneys as described above and subjected to Northern blot analyses. Aliquots of total RNA (10 μg) were separated by 1% denatured agarose gel electrophoresis and transferred to a Zeta-Probe membrane (Bio-Rad, Hercules, USA). After prehybridization for 1 h at 65°C, the membranes were subjected to hybridization for 16 h at the same temperature and in the same solution containing $^{32}$P-labeled *Ccl2* or *Gapdh* cDNA fragments as probes. The cDNA fragment was prepared by reverse-transcription (RT)-PCR with the following amplimers: *Ccl2*-forward, 5′-agtcgg ctggagaactacaa; *Ccl2*-reverse, 5′-taccttggactctcaaacac; *Gapdh*-forward, 5′-ccactcagaagactgtgga; *Gapdh*-reverse, 5′-cctctcttgctctcagta. It was then labeled by using a Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, USA). After hybridization, the membranes were washed twice for 5

**Table 1. Primers for Sequence Analysis of the 5'-Flanking Region of *Ccl2* Gene**

| Forward primer | Reverse primer |
|---|---|
| PS1: 5′-gccagacattccagatggctctctc | PA1: 5′-cagtgagagttggctggtttctggg |
| PS2: 5′-ggaagtagcagtgagatgctctgg | PA2: 5′-gagagagccatctggaatgtctggc |
| PS3: 5′-gactctgctcagccactggcatg | PA3: 5′-ctgtgccaggagtgtttggaaccc |
| PS4: 5′-ccaagtcagagctcagactatgcc | PA4: 5′-agcactaacaagttgagagatgcca |
| PS5: 5′-agtggaagtagcaaaggccacat | PA5: 5′-tagtcactgacctcctcctggct |

Primers used for sequence analysis of the 5′-flanking region of *Ccl2* gene are shown.

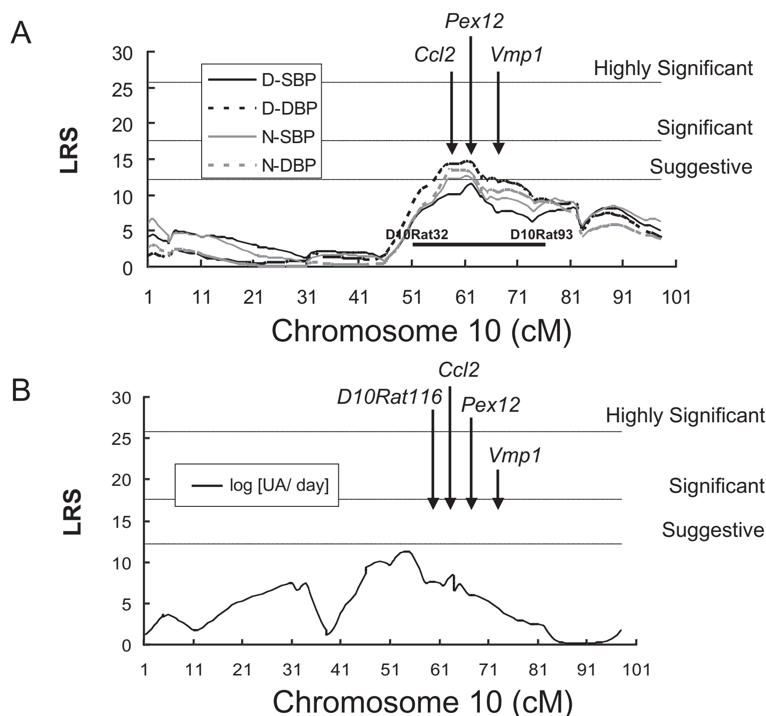


**Fig. 1.** *Interval mapping analyses of blood pressure (BP) and urinary albumin (UA) QTL on chromosome 10. Interval mapping analyses of 4 BP values were performed on chromosome 10 controlled by D1Rat27. Likelihood ratio statistics (LRS) plots for BP (upper) and UA (UA excretion per day) (lower) are shown.*

min each time at room temperature with 2× SSC and 0.1% SDS, and twice for 30 min each time at 60°C with 1× SSC and 0.1% SDS. Hybridized signals were detected and quantified by a BAS-2500 imaging system (Fuji Film, Tokyo, Japan).

## Culture of Rat Mononuclear Cells and MCP-1 ELISA

Mononuclear cells from peripheral blood of 10-week-old DS and LEW rats ($n=5$ each) were isolated using NycoPrep 1.077A (AXIS-SHIELD, Oslo, Norway) and cultured in RPMI1640 with 10% fetal calf serum in an atmosphere of 5% $CO_2$ and 100% relative humidity at 37°C. One hour later, the medium was aspirated and the cells were washed twice with 1× PBS to remove floating cells. Adherent cells were cultured for 24 h in the presence or absence of 10 μg/ml lipopolysaccharide (LPS) (Sigma, St. Louis, USA). The culture media were centrifuged at 1,100 rpm for 5 min and the supernatants were frozen at −80°C until use. Adherent cells (monocytes) were harvested in 250 mmol/l Tris-HCl (pH=7.4) and the freeze/thaw procedure was repeated three times. The cell suspension was sonicated for 2 min and the protein levels were determined using a BCA Protein Assay Kit (Pierce, Rockford, USA). The concentrations of MCP-1 (the product of the *Ccl2*) in the culture medium were determined using an Endogen Rat MCP-1 ELISA Kit (Pierce). ELISA was performed according to the manufacturer's protocol. The concentrations of MCP-1 were corrected by total cellular protein levels. Statistical analysis was performed by two-way ANOVA followed by Turkey's HSD test.

**Table 2. A List of Genes between D10Rat32 and D10Rat93**

| | | | | | |
|---|---|---|---|---|---|
| Myh3 | RGD1563179_predicted | Olr1489-ps | RGD1566149_predicted | LOC688910 | Trim25_mapped |
| LOC691644 | RGD1308134_predicted | Olr1490_predicted | Slc13a2 | Slfn3 | LOC497978 |
| Myh4_predicted | RGD1305687_predicted | Olr1491_predicted | RGD1309472 | LOC688925 | Nog |
| Myh8 | Alox12_predicted | Olr1492_predicted | Sarm1_predicted | RGD1564411_predicted | Ankfn1_predicted |
| Myh13 | Alox12e_predicted | LOC680253 | Vtn | LOC287569 | Pctp |
| LOC691936 | Alox15 | Olr1495-ps | Og9x | Pex12 | MGC108778 |
| Gas7 | Pelp1 | Olr1496 | LOC303332 | Ap2b1 | Maf |
| Rcvrn | Arrb2 | Olr1497-ps | Poldip2_predicted | Rasl10b_predicted | LOC690286 |
| Glp2r | RGD1563202_predicted | Olr1498_predicted | Tnfaip1 | Gas2l2_predicted | Stxbp4_predicted |
| RGD1564224_predicted | Cxcl16 | Olr1499_predicted | RGD1309400_predicted | LOC689039 | LOC690300 |
| Dhrs7c_predicted | Zmynd15_predicted | Olr1500 | Tmem97 | Mmp28_predicted | RGD1562626_predicted |
| Usp43_predicted | Tm4sf5_predicted | Olr1501_predicted | RGD1561602_predicted | Taf15_predicted | RGD1564550_predicted |
| Stx8 | RGD1559861_predicted | LOC680358 | RGD1305651 | RGD1559875_predicted | LOC303448 |
| Ntn1 | RGD1560459_predicted | Olr1503-ps | RGD1562012_predicted | Ccl5 | Kif2b |
| RGD1563261_predicted | Psmb6 | Olr1504 | LOC685475 | Ccl9 | |
| RGD1560850_predicted | Pld2 | Olr1505_predicted | Nos2 | Ccl6 | |
| RGD1307767_predicted | LOC303259 | Olr1507_predicted | Lgals5 | Ccl3 | |
| Ccdc42_predicted | Chrne | Olr1508-ps | LOC497963 | Ccl4 | |
| LOC691948 | LOC691991 | Olr1509_predicted | Lgals9 | LOC689133 | |
| RGD1564148_predicted | LOC691992 | Olr1510-ps | Ksr1_predicted | Expi | |
| Myh10 | Slc25a11 | LOC497952 | RGD1565317_predicted | LOC360228 | |
| Ndel1 | Rnf167 | Olr1511_predicted | LOC685523 | RGD1566204_predicted | |
| RGD1310573_predicted | Pfn1 | Olr1512_predicted | Wsb1 | Tcf2 | |
| LOC287416 | Eno3 | Olr1513_predicted | LOC685546 | LOC363658 | |
| Rpl26 | Spag7_predicted | Olr1514_predicted | Nf1 | Ddx52 | |
| Odf4 | Camta2_predicted | Olr1515_predicted | Omg | Ap1gbp1 | |
| LOC691957 | RGD1306656_predicted | Olr1516_predicted | LOC685433 | Dusp14_predicted | |
| Arhgef15_predicted | Kif1c | Olr1517_predicted | Rab11fip4_predicted | Tada2l | |
| LOC497933 | RGD1565881_predicted | Olr1518-ps | LOC685597 | Acaca | |
| RGD1563195_predicted | LOC691993 | Olr1519_predicted | LOC363652 | Aatf | |
| Pfas_predicted | Rabep1 | RGD1563134_predicted | LOC303341 | Lhx1 | |
| RGD1563106_predicted | Nup88 | Olr1520_predicted | RGD1311017_predicted | RGD1566232_predicted | |
| Aurkb | RGD1308492 | Garnl4_predicted | Taok1 | RGD1307935 | |
| LOC497934 | C1qbp | RGD1562276_predicted | RGD1304598_predicted | Zfp403 | |
| LOC691750 | Dhx33_predicted | RGD1307222_predicted | Trp53i13_predicted | Pigw | |
| Vamp2 | LOC691956 | Pafah1b1 | Git1 | RGD1559961_predicted | |
| Per1 | LOC501706 | LOC360568 | RGD1564005_predicted | LOC497975 | |
| Hes7_predicted | LOC691994 | Mnt_predicted | Coro6 | Ca4 | |
| Aloxe3_predicted | LOC691995 | Rutbc1_predicted | Ssh2_predicted | LOC689401 | |
| Alox12b | LOC691996 | Srr | RGD1309650_predicted | Usp32_predicted | |
| Alox15b | Nalp1_predicted | LOC287522 | LOC685780 | RGD1310166_predicted | |
| Gucy2e | LOC691998 | Hic1_predicted | LOC287551 | Appbp2 | |
| Cntrob_predicted | RGD1310602_predicted | LOC680922 | LOC685811 | Ppm1d_predicted | |
| Trappc1 | RGD1308212 | LOC680936 | LOC685842 | RGD1560788_predicted | |
| Kcnab3 | Aipl1 | RGD1564623_predicted | RGD1309863 | Tbx2_predicted | |
| RGD1563441_predicted | RGD1308747_predicted | RGD1562694_predicted | Slc6a4 | Tbx4_predicted | |
| Chd3 | Pitpnm3_predicted | Rtn4rl1 | Blmh | Brip1_predicted | |
| RGD1559567_predicted | LOC679548 | Rpa1 | Tmigd_predicted | RGD1305547_predicted | |
| RGD1559617_predicted | RGD1304728_predicted | Smyd4_predicted | LOC363655 | Thrap1_predicted | |
| Jmjd3_predicted | Txnl5_predicted | LOC680846 | Cpd | LOC689568 | |
| DLP2 | Med31_predicted | Serpinf1 | Gosr1 | Tmem49 | |
| Efnb3_predicted | LOC679576 | Serpinf2 | Tusc5 | RGD1306819 | |
| Wdr79 | Slc13a5 | RGD1311334_predicted | RGD1311234_predicted | Cltc | |
| Tp53 | LOC679600 | RGD1562631_predicted | Abr_predicted | Dhx40 | |
| Atp1b2 | Fbxo39 | Prpf8 | Timm22 | RGD1311564_predicted | |
| Shbg | Tekt1 | Rilp_predicted | Nxn_predicted | Gdpd1_predicted | |
| Sat2_predicted | LOC679629 | Scarf1_predicted | RGD1561464_predicted | RGD1306862_predicted | |
| Fxr2h_predicted | Ggt6 | Slc43a2_predicted | RGD1306595 | RGD1561541_predicted | |
| Sox15_predicted | Mybbp1a | Pitpna | RGD1560575_predicted | RGD1307084 | |
| Mpdu1 | RGD1561884_predicted | LOC287533 | LOC686099 | Trim37_predicted | |
| Cd68 | Ube2g1 | Myo1c | Centa2 | Ppm1e | |
| Eif4a1 | Ankfy1_predicted | Crk | Rnf135 | RGD1563765_predicted | |
| Senp3 | Cyb5d2 | LOC363643 | LOC686138 | Tex14_predicted | |
| Tnfsf13 | Zzef1_predicted | Ywhae | Rhbdl4_predicted | LOC689764 | |
| Tnfsf12 | Atp2a3 | Doc2b | RGD1310429_predicted | Sep4 | |
| LOC691974 | P2rx1 | Rph3al | Zfp207 | Mtmr4_predicted | |
| Polr2a_mapped | Camkk1 | RGD1565611_predicted | Psmd11_predicted | LOC689785 | |
| LOC691976 | RGD1308139_predicted | RGD1359691 | Cdk5r1 | Rnf43_predicted | |
| Zbtb4_predicted | P2rx5 | Vps53_predicted | Myo1d | Supt4h2_predicted | |
| Chrnb1 | Tmem93_predicted | RGD1563715_predicted | Tmem98 | Bzrap1 | |
| Fgf11 | Tax1bp3 | Dbil5 | Spaca3_predicted | Mpo_mapped | |
| RGD1559450_predicted | Ctns_predicted | RGD1307010 | Accn1 | Lpo_predicted | |
| LOC497938 | Carkl | RGD1309077_predicted | LOC688465 | Mks1 | |

**Table 3. The Possible Differentially Expressed Genes in the Ch10 QTL Region**

| Gene name | Gene symbol | GenBank ID | Alignments | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | LEW | DS | log ratio | LEW | DS | log ratio | LEW | DS | log ratio |
| Transcribed locus (1) | | AA942816 | chr10:59349777–59350732 (−)//99.37//q24 | 238.5 | 585.8 | 1.3 | 315.3 | 677 | 1.3 | 234.7 | 506.9 | 1.1 |
| Transient receptor potential cation channel, subfamily V, member 1 | *Trpv1* | AB041029 | chr10:60127226–60148312 (+)//99.7//q24 | 91.3 | 404.9 | 2.1 | 170.3 | 395 | 2.1 | 124.4 | 359.8 | 1.5 |
| Transcribed locus (2) | | AI146250 | chr10:62798395–62799026 (+)//23.81//q24 | 157.8 | 726.4 | 2.2 | 291.6 | 816.7 | 2.2 | 253.3 | 781.5 | 1.6 |
| Transcribed locus (3) | | AA944136 | chr10:63765057–63765909 (−)//88.07//q24 | 33 | 633.8 | 4.3 | 78.8 | 689.2 | 4.3 | 58.1 | 576.8 | 3.3 |
| Oligodendrocyte-myelin glycoprotein | *Omg* | BI279325 | chr10:65727030–65727741 (−)//98.34//q25 | 26.7 | 122.1 | 2.2 | 66.2 | 153.2 | 2.2 | 50.6 | 112.8 | 1.2 |
| Chemokine (C-C motif) ligand 2 | *Ccl2* | NM_031530 | chr10:70269885–70271688 (+)//96.43//q26 | 73.1 | 262.6 | 1.8 | 146 | 308.3 | 1.8 | 111.6 | 249.8 | 1.2 |
| Schlafen 8 | *Slfn8* | BE107457 | chr10:71205849–71206602 (−)//47.07//q26 | 28.8 | 130.6 | 2.2 | 46.2 | 141.3 | 2.2 | 38.3 | 111.2 | 1.5 |
| Ribosomal protein S6 kinase, polypeptide 1 | *Rps6kb1* | AI236624 | chr10:76788330–76789841 (−)//86.43//q26 | 110.7 | 416.4 | 1.9 | 141.1 | 523.5 | 1.9 | 124 | 430 | 1.8 |

Microarray analyses in the DS and LEW kidneys identified 8 differentially expressed genes in the Ch10 QTL region. Transcribed loci (1)–(3) have not been precisely annotated. QTL, quantitative trait loci; LEW, Lewis rats; DS, Dahl salt-sensitive rats.

### Sequence Analyses of Rat *Ccl2*

Genomic DNAs were extracted from the livers of 10-week-old DS and LEW rats using proteinase K and phenol (*8*). The entire sequences of the 5′-flanking region (up to −2.3 kb) of *Ccl2* of DS and LEW were determined. The primers used for sequencing are shown in Table 1.

### Construction of Reporter Plasmids and Luciferase Assay

The promoter regions of DS and LEW rats (up to −2.3 kb) were amplified with PS4 and PA1 primers (Table 1) using KOD-Plus- DNA polymerase (TOYOBO, Osaka, Japan). The PCR products were gel-purified and subcloned into the XhoI and HindIII site of the pGL3-Basic vector (Promega, Madison, USA). RAW264.7 cells were cultured in 6-well plates and transiently cotransfected with 4 μg of reporter plasmid and 0.4 μg phRL-TK (Promega) per well using the Lipofectamine 2000 reagent (Invitrogen). After being incubated for 24 h, the cells were washed with 1× PBS and incubated in fresh medium for 24 h in the presence or absence of 10 μg/ml LPS. The luciferase assay was performed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

## Results

### QTL Analyses for BP and Albumin

A total of 418 polymorphic markers in all of the 107 F2 rats were genotyped, and some of them gave identical genotype data. Thus, the data on 383 effective genotypes were finally obtained, as described previously (*4*).

Marker regression analysis indicated that the most significant marker for each of the 4 BP values was D1Rat27, and this locus was added to the background to perform a second marker regression for 4 BP values (D-SBP, D-DBP, N-SBP, N-DBP), which indicated that the Ch10 *Pex12* region was the second QTL for each of the 4 BP values. Figure 1A provides LRS plots for the linkage to BP for Ch 10 controlled by D1Rat27. This Ch10 QTL around *Pex12* was a suggestive QTL for BP values, except D-SBP (Fig. 1A).

Marker regression analysis of UA levels did not identify a statistically significant QTL for UA levels. However, a Ch10 region near *Pex12* (around D10Rat116) reached a level (LRS=11.3) just below a suggestive level (suggestive LRS=12.2) (Fig. 1B).

### Identification of Differentially Expressed Genes

There are 514 annotated genes between the markers of D10Rat32 and D10Rat93 (Table 2). Microarray analyses in the kidneys indicated that 8 genes in this Ch10 QTL region

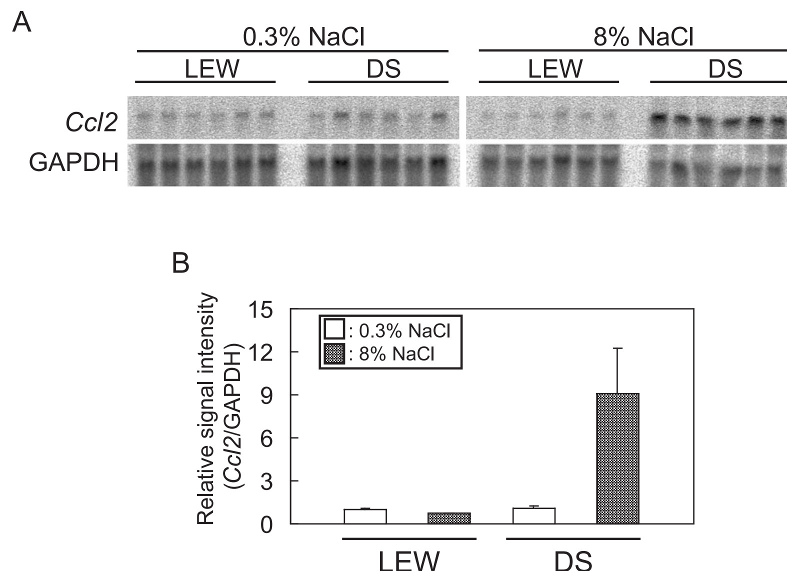


**Fig. 2.** *Expression levels of* Ccl2 *mRNA in the kidneys of DS and LEW rats. A: The transcript level of* Ccl2 *was assessed by Northern blot analysis. Total RNA samples (10 μg) obtained from DS or LEW kidneys fed an 8% or a 0.3% NaCl diet for 8 weeks were subjected to agarose gel electrophoresis. The separated samples were transferred to Zeta-Probe membranes, and mRNAs of* Ccl2 *and* Gapdh *were then detected by using specific probes. B: The expression level of* Ccl2 *mRNA was quantified by BAS-2500 using the* Gapdh *mRNA level as an internal control. The expression level of* Ccl2 *in LEW rats fed 0.3% NaCl was arbitrarily defined as 1.0. Vertical bars indicate SDs.*

might be differentially expressed (Table 3). However, subsequent RT-PCR and Northern blot analyses showed that *Ccl2* was the only gene that was differentially expressed between DS and LEW rats after salt loading. As described above, this Ch10 QTL for BP incidentally overlapped the possible QTL for UA. UA is a marker of renal injury, and thus it is likely that the gene that contributes to hypertension in DS in this Ch10 QTL may be related to kidney injury. Since MCP-1 (the product of *Ccl2*) is well known to be involved in various types of renal injury, *Ccl2* is the most likely candidate for hypertension, and we focused on this gene for further analysis in the present study.

## Confirmation of the Differential Expression of *Ccl2*

Northern blot analysis confirmed that the *Ccl2* mRNA levels were increased in DS rats compared to those in LEW rats after salt loading (Fig. 2A). While the expression levels of *Ccl2* mRNA were not different between the kidneys of DS and LEW rats fed a normal diet, those in DS were 10-fold higher than those in LEW under a high-salt diet (Fig. 2B).

Sequence analysis of *Ccl2* in DS and LEW rats identified seven polymorphisms in the promoter region (up to −2.3 kb) (Fig. 3A). However, a luciferase reporter assay in a mouse monocytic cell line (RAW264.7) indicated that the differential expression between DS and LEW rats was not due to these promoter polymorphisms (Fig. 3B). Luciferase activity under this 2.3 kb promoter region was upregulated by LPS stimulation, but the promoter activity in DS rats was not significantly different from that in LEW rats (Fig. 3B). The expression levels of *Ccl2* mRNA might be influenced by an enhancer sequence at a distance of several megabases, intronic sequences, or sequences in 3′ untranslated regions. Even if the differential expression might be due to sequence differences between DS and LEW rats, it would be difficult to pinpoint the responsible sequence differences.

To rule out the possibility that the differential expression of *Ccl2* between DS and LEW rats was due to the secondary influences of hypertension after salt loading, monocytes were isolated from peripheral blood and the production of immunoreactive MCP-1 was assessed in both strains. The production of MCP-1 was very low under unstimulated conditions, and was upregulated by LPS treatment (Fig. 4). The production of MCP-1 under LPS treatment in monocytic cells isolated from DS rats was 2.5 times higher than that in monocytic cells from LEW rats ($p<0.01$).

# Discussion

We previously identified two QTL regions (Ch1 and Ch10) that influenced BP using an F2 cohort from DS and LEW, and reported that one of the candidate genes in the Ch1 QTL region is *Klk1* (*4*). In the present study, we further analyzed the second QTL region (Ch10). This Ch10 QTL for BP, in contrast to the Ch1 QTL region, appears to overlap a possible

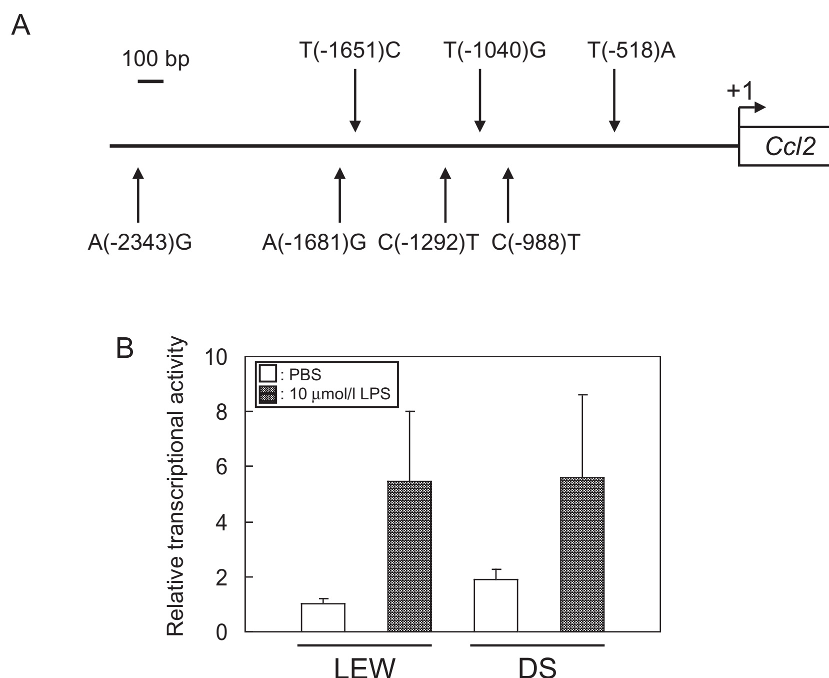


**Fig. 3.** *Functional analysis of the* Ccl2 *promoter. A: Sequence analysis of the* Ccl2 *gene in DS and LEW rats identified seven polymorphisms in the promoter region (up to −2.3 kb). The sites of the polymorphisms are indicated by arrows. +1 indicates the start site of translation. B: A luciferase reporter assay was performed in a mouse monocytic cell line (RAW264.7). The data are shown as the mean±SD. The luciferase activities of LEW in the absence of 10 µmol/l lipopolysaccharide (LPS) treatment are arbitrarily defined as 1.0. Two-way ANOVA indicated that significant differences were observed among the 4 groups (*$p<0.0001$*). Subsequent analysis (Turkey's HSD test) indicated that luciferase activity was significantly upregulated by LPS treatment (*$p<0.01$*), while no significant difference was observed between DS and LEW.*

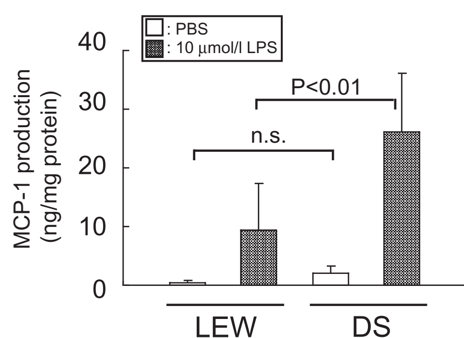


**Fig. 4.** *MCP-1 ELISA in monocytes from DS and LEW rats. The release of MCP-1 from monocytes in DS and LEW rats (*$n=5$* each) was measured by ELISA. Samples and standards were assayed in duplicate. The data are shown as the mean±SD. Two-way ANOVA indicated that significant differences were observed among the 4 groups (*$p<0.0001$*). Subsequent analysis (Turkey's HSD test) indicated that the MCP-1 release from DS monocytes was significantly higher than that from LEW after lipopolysaccharide (LPS) treatment (*$p<0.01$*).*

QTL for UA, a marker of renal injury. This coincidence may indicate that a gene that contributes to high BP in this region may mediate its influence through renal injury.

After differentially expressed genes were screened by microarray, we determined that *Ccl2* is the only differentially expressed gene in this Ch10 QTL region. *Ccl2* is well known to be involved in various types of renal injury (*9–11*), and the down-regulation of *Ccl2* has been reported to ameliorate renal injuries in various pathological models (*12–14*). Thus, *Ccl2* appears to be the most likely candidate gene for hypertension in this Ch10 QTL.

The possible involvement of *Ccl2* in the pathogenesis of hypertension in DS has not been reported. However, tubulointerstitial injury with macrophage infiltration has been reported to be one of the features of DS kidneys (*15*). Moreover, tubulointerstitial inflammation is now recognized as one of the mechanisms for salt-sensitive hypertension (*16*). It is likely that a higher expression of *Ccl2* might aggravate macrophage infiltration, which in turn could aggravate tubulointerstitial injury, which finally could accelerate salt-sensitive hypertension. In support of this hypothesis, anti–MCP-1 gene therapy has been reported to ameliorate various types of renal injury, including ischemia-reperfusion injury (*17*), protein-overload renal injury (*18*), unilateral ureteral obstruction

nephropathy (*19*), and a nephritis of lupus model (*20*). Moreover, it has been reported that tubular injury in nephrotoxic serum nephritis is remarkably less common in *Ccl2* knockout mice than in wild-type mice (*21*).

The existence of QTLs for salt-sensitive hypertension in Ch10 has also been reported by Deng and Rapp (*22*). They further divided this QTL region into four distinct QTLs (QTL1–4) by establishing congenic strains (*23–25*). Our Ch10 QTL involves their QTL1, QTL3, and QTL4. *Ccl2* appears to be localized in the border of their QTL4. They have not yet identified the causative genes in these regions.

We tried to identify cells that expressed *Ccl2* mRNA in the kidney. Immunohistochemical analysis showed that tubular cells were stained with an anti–MCP-1 antibody, while *in situ* hybridization analysis showed that uncharacterized interstitial cells were stained with an antisense probe (data not shown). MCP-1 production has been confirmed in monocytic cells in the present study and has been reported in mesangial cells (*26*). Further study will be required to identify the sites of MCP-1 production in the kidney.

Even though this Ch10 QTL for high BP appears to overlap the QTL for UA, and *Ccl2* is a good candidate for salt-sensitive hypertension in DS, our hypothesis depends solely on indirect evidence. It is possible that the increased expression of *Ccl2* in DS rats after salt loading may be secondary to kidney injury, and may not play a primary role in the pathogenesis of hypertension. Some genes other than *Ccl2* in this chromosomal region might be responsible for hypertension. In this regard, it is worth noting that we recently investigated the expression profiles of microRNAs in the kidneys of DS and LEW, and found no significant differences between the two strains (*27*). Direct evidence that anti–MCP-1 therapy could ameliorate salt-sensitive hypertension in DS will be needed to support our hypothesis. However, it is technically difficult to suppress MCP-1 activity during the salt loading period. Transgenic DS rats with anti–MCP-1 RNAi might be useful for obtaining definitive support for our hypothesis.

The Rat Genome Project is almost complete, and precise maps of protein-coding genes are now available. However, a recent analysis of mRNA transcripts has clarified that more than 70% of chromosomal DNAs are transcribed, and more than 50% of mRNAs are non-coding (*28*, *29*). The concept of the genome is being revolutionized, and it now seems misleading to limit candidate genes to protein-coding genes. Understanding the non-coding RNA world may be necessary to advance our understanding of polygenic diseases, including hypertension.

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