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The Fli-1 Transcription Factor Regulates the Expression of CCL5/RANTES

Mara L. Lennard Richard,* Shuzo Sato,*1 Eiji Suzuki,*1 Sarah Williams,† Tamara K. Nowling,*† and Xian K. Zhang*†

The friend leukemia insertion site 1 (Fli-1) transcription factor, an Ets family member, is implicated in the pathogenesis of systemic lupus erythematosus in human patients and murine models of lupus. Lupus-prone mice with reduced Fli-1 expression have significantly less nephritis, prolonged survival, and decreased infiltrating inflammatory cells into the kidney. Inflammatory chemokines, including CCL5, are critical for attracting inflammatory cells. In this study, decreased CCL5 mRNA expression was observed in kidneys of lupus-prone NZM2410 mice with reduced Fli-1 expression. CCL5 protein expression was significantly decreased in endothelial cells transfected with Fli-1–specific small interfering RNA compared with controls. Fli-1 binds to endogenous Ets binding sites in the distal region of the CCL5 promoter. Transient transfection assays demonstrate that Fli-1 drives transcription from the CCL5 promoter in a dose-dependent manner. Both Ets1, another Ets family member, and Fli-1 drive transcription from the CCL5 promoter, although Fli-1 transactivation was significantly stronger. Ets1 acts as a dominant-negative transcription factor for Fli-1, indicating that they may have at least one DNA binding site in common. Systematic deletion of DNA binding sites demonstrates the importance of the sites located within a 225-bp region of the promoter. Mutation of the Fli-1 DNA binding domain significantly reduces transactivation of the CCL5 promoter by Fli-1. We identified a novel regulator of transcription for CCL5. These results suggest that Fli-1 is a novel and critical regulator of proinflammatory chemokines and affects the pathogenesis of disease through the regulation of factors that recruit inflammatory cells to sites of inflammation. The Journal of Immunology, 2014, 193: 000–000.

Inflammatory cytokines and chemokines are important regulators of the immune system and actively recruit inflammatory cells to sites of inflammation. CCL5, also known as RANTES, is a member of the C–C chemokine family of inflammatory cytokines (1, 2), was originally thought to be T cell specific because it was cloned from a cDNA library enriched for T cell–specific sequences, and mRNA expression was found only in cytotoxic and Th cell lines (3). However, the expression of CCL5 has since been observed in a variety of cell types, including T cells (1, 3), endothelial cells (4), renal tubular epithelial cells (5), mesangial cells (6), and fibroblasts and macrophages (7, 8). The CCL5 gene consists of a 23-aa leader peptide followed by 68 residues, 4 of them cysteines, and it lacks a transmembrane domain (1, 3). The murine CCL5 gene was first isolated from renal tubular epithelial cells and is 90% homologous to the human gene (5). Analysis of the human CCL5 promoter region, ~1 kb in length, identified a wide variety of transcription factor binding sites, including NF-κB, AP-1, C/EBP, and at least one Ets-1 binding site. Deletion studies of the promoter demonstrated that different transcriptional mechanisms may control CCL5 in different tissue and cell types (1). CCL5 gene expression is stimulated by LPS, TNF-α, INF-γ, and IL-1β (4–11). Within the murine CCL5 promoter, one NF-κB and one IRF binding site are responsible for stimulation of the promoter by TNF-α and INF-γ, and activation is regulated by the p65 subunit of NF-κB and IRF1, respectively (8, 9). Studies aimed at understanding the transcription factors that regulate the CCL5 promoter have been performed, and many transcription factors have been identified. The Ets family member PU.1 was shown to bind to the CCL5 promoter and may be involved in the recruitment of other transcription factors (11, 12). Increased binding to the CCL5 promoter by NF-κB, AP-1, and C/EBP was observed in glomeruli after stimulation with LPS (10). LPS induction of the human CCL5 gene was found to be mediated by the transcription factors ATF and Jun, through a CRE/AP-1 binding site (11). Another transcription factor, KLF13, was shown to bind to the CCL5 promoter, and binding is a requirement for transactivation and synergistic activation with NF-κB proteins (13, 14).

CCL5 plays a role in the pathogenesis of a variety of inflammatory-mediated diseases, including asthma (15, 16), rheumatoid arthritis (7), and systemic lupus erythematosus (SLE) (17, 18), by actively recruiting leukocytes, macrophages, and eosinophils (15, 16, 19, 20) to sites of inflammation. A variety of renal diseases has been linked to the CCL5 gene (21), and its expression has been documented in the kidney cortex, glomerulus, and renal tubular epithelial cells (5, 6, 10, 20, 22–24). Analysis of microarray data from glomerular gene expression in MRL/lpr mice, a murine model of lupus, with active lupus nephritis demonstrated a profound upregulation of CCL5 mRNA (24). In MRL/lpr mice, CCL5 gene expression increased...

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; EBS, Ets-1 DNA-binding site; Fli-1, friend leukemia insertion site 1; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; WT, wild-type.

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with disease progression and promoted renal injury through the recruitment of macrophages and CD4+ and CD8+ T cells to the kidney, particularly the area surrounding the glomeruli (20). A mouse model that exhibits autoimmune nephrotic serum nephritis was shown to have increased CCL5 mRNA expression in diseased mice, whereas proteinuria was decreased in CCL5-deficient mice, and milder versions of glomerulonephritis were observed (22). Increased CCL5 expression has been detected in the serum of patients with SLE, although correlation with patients’ SLE Disease Activity Index scores appears to depend on further classification of the disease (17, 18). Additionally, it was found that, in patients with primary glomerulonephritis, concomitant urinary excretion of IL-1Ra, TNF-R1, TNF-R2, and CCL5 predicted a favorable response of patients to immunosuppressive therapy (25).

The Friend leukemia insertion site 1 (Flt-1) gene is a member of the Ets family of transcription factors that regulates a variety of cellular processes, including the immune response (26–28). Ets family members recognize the consensus DNA binding motif GGAA/T and bind the DNA through a winged helix turn helix domain (27, 29, 30). Ets transcription factors can be both positive and negative regulators of transcription, and interactions with other transcription factors can lead to increased DNA-binding ability and synergistic activation or repression (27, 31). Several studies demonstrated a role for Flt-1 in the pathogenesis of renal injury and the autoimmune disease SLE (32–37). Transgenic mice that overexpressed the Flt-1 gene exhibited an increase in infiltrating B and T lymphocytes and eventually died of tubulointerstitial nephritis and glomerulonephritis (38), whereas murine models of lupus with heterozygous expression of Flt-1 had a reduction in renal inflammation, necrosis, proteinuria, and autoantibody production, as well as prolonged survival (34, 35, 37). In addition, Flt-1 heterozygous lupus-prone mice displayed a significant decrease in the infiltration of inflammatory cells to the kidney, and the expression of another proinflammatory chemo- kine, CCL2/MCP-1, was dramatically reduced (39).

Because Ets DNA-binding domains have been identified in the CCL5 promoter (1, 11, 27), CCL5 plays a role in attracting inflammatory cells, and murine models of lupus with reduced expression of Flt-1 have significantly reduced nephritis with markedly decreased inflammatory cell infiltration, the current study was conceived to determine whether Flt-1 is involved in the regulation of the CCL5 gene expression. We discovered decreased CCL5 mRNA expression in kidneys of Flt-1 heterozygous NZM2410 mice prior to the onset of disease. CCL5 protein levels also were decreased in MS1 endothelial cells stimulated with LPS after knockdown of Flt-1 with small interfering RNA (siRNA). Chromatin immunoprecipitation (ChIP) assays demonstrated that Flt-1 binds to Ets binding sites in the distal region of the CCL5 promoter, and transient transfection assays showed that Flt-1 drives transcription from the promoter. Taken together, our results indicate that the Flt-1 transcription factor plays a previously undiscovered role in the transcriptional control of the CCL5 gene, and a reduction in the expression of Flt-1 leads to a decrease in CCL5 gene expression both in vitro and in vivo. Combined with our previous studies, it appears that Flt-1 plays a critical role in the transactivation of inflammatory chemokines, which affects the development of glomerulonephritis and the pathogenesis of kidney disease in SLE.

Materials and Methods

Mice

NZM2410 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The heterozygous (Flt-1+/−) NZM2410 mice and wild-type (WT) littermates (Flt-1+/+) NZM2410 mice were generated by backcrossing with Fli-1+/− B6 mice for 12 generations, as described previously (35). All mice were housed under pathogen-free conditions at the animal facility of the Ralph H. Johnson Veterans Affairs Medical Center, and all animal experiments were approved by the Institutional Animal Care and Use Committee.

Genotyping

PCR was used to detect fragments of the WT and Flt-1−/− alleles, as previously described (34). Briefly, the following primers were used in the PCR: Fli-1 exon IX/forward primer (positions 1156–1180), 5′-GACCA-ACGGGGGATTTCAAAATGACG-3′; Fli-1 exon IX/reverse primer (positions 1441–1465), 5′-GGAGGATTGOGTGAGAGCAGGCCAAAG-3′; and a Pol II/reverse primer. 5′-GGAAGTACGGTATTTAGTGGAGAGG-3′. The DNA was isolated from tail snips of 4-wk-old mice using the QIAamp Tissue kit (QIAGEN, Santa Clarita, CA). PCR analyses were performed under the following conditions: one cycle at 95˚C for 1 min, 60˚C for 1 min, and 72˚C for 1 min, followed by 72˚C for 7 min. A 309-bp fragment indicates the presence of the WT allele, and a 406-bp fragment is amplified from the mutated allele.

Real-time PCR

Measurement of CCL5 mRNA expression in kidneys was completed by isolating total RNA using TRIzol reagent (Life Technologies/Invitrogen, Grand Island, NY) from the kidneys of 18-wk-old Flt-1−/− NZM2410 mice and WT littermates. Two micrograms of RNA was used to synthesize cDNA with the SuperScript First-Strand Synthesis System (Life Technologies/Invitrogen). Real-time PCR was performed in duplicate using the Platinum SYBR Green PCR SuperMix UDG (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions, with three independent RNA preparations. The primers for the CCL5 gene were purchased from SABiosciences (QIAGEN), and the cycling conditions for all genes followed instructions from the company. PCR was carried out using the CFX connect Real-time PCR Detection System (Bio-Rad), and relative expression analysis was conducted using the program provided by SABiosciences (QIAGEN).

Chemokine measurement

Concentration of the CCL5 protein in the supernatants of MS1 endothelial cells was determined by ELISA using the DuoSet ELISA Development System for Mouse CCL5/RANTES from R&D Systems (Minneapolis, MN). The assays were performed following the manufacturer’s instructions.

ChIP assay

The ChIP assay was performed as described previously using an anti-Flt-1 rabbit polyclonal Ab (39–41). Briefly, total MS1 endothelial cells were cross-linked with formaldehyde; chromatin was isolated and immunoprecipitated with specific Fli-1 Abs or rabbit IgG control. The genomic fragments associated with immunoprecipitated DNA were amplified by RT-PCR. Putative Ets-1 DNA-binding sites (EBSs) were identified by examination of the CCL5 promoter sequence using the MatInspector software tool (Genomatix, Ann Arbor, MI). Based on these sites, the primer pairs for the ChIP assay were designed and are shown in Table 1. PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining.

Reporter and expression constructs

A portion of the murine CCL5 promoter (from −1236 to −52 bp upstream of the transcription start site) corresponding to the previously defined promoter region in humans (1) was PCR amplified from genomic DNA isolated from a B6 mouse by modifying previously published primers (9). The forward primer 5′-ATT AAG CTA GCT CCT GTG CCC ACC A-3′ contains the NheI enzyme site (underlined), and the reverse primer 5′-ATT ATCA TCC GAC GCA AGG GT G CTG CTG CA-3′ contains the XhoI enzyme site (underlined). The PCR program used to amplify the full-length sequence was as follows: 94˚C for 3 min; 30 cycles of 94˚C for 30 s, 62˚C for 30 s, and 72˚C for 1 min; 72˚C for 10 min; and held at 4˚C until the program was stopped. The CCL5 promoter region was directionally cloned into the pGL3 basic vector upstream of the Luciferase gene (Promega, Madison, WI). Region A of the CCL5 promoter was amplified from the full-length clone using the same PCR program described above. The forward primer, located −520 bp upstream from the transcription start site, 5′-ATT AAG CTA GGG TGG TCT GTT CTT GAT ATG G-3′ contains the NheI enzyme site (underlined), and the reverse primer is identical to the one described above, but it contained a HindIII site instead of the XhoI site. Region A of the CCL5 promoter was directionally cloned into the pGL3 basic vector. The Region B forward primer is located −746 bp upstream of the transcription start site and
contains an EcoRI site (underlined) 5'-ATT AGA ATT CGA CAG AAG ATT CCG ATT CAA AGA-3'. The reverse primer was designed to the multi-cloning of the pGL3 basic vector and contains an EcoRI site (underlined) for easy ligation: 5'-TAA GTC ATG CCA CAC CGG TAA GA-3'. The linearized plasmid and the reverse primer were added to a PCR re-action, and the amplification began as follows: 95˚C for 3 min; three cycles of 95˚C for 30 s, 55˚C for 30 s, 68˚C for 6 min; and held at 80˚C, at which point 1 μl the forward primer was added to the reaction. The PCR reaction proceeded as follows: 18 cycles of 95˚C for 30 s, 55˚C for 30 s, 68˚C for 6 min; 68˚C for 10 min, and held at 4˚C until the program was stopped. A DpnI digestion was carried out overnight at 37˚C to remove the parent template. The PCR product was then digested with EcoRI and ligated together with T4 DNA ligase. All enzymes were obtained from New England Biolabs (Ipswich, MA). All of the promoter constructs were confirmed by DNA sequencing (GENEWIZ, South Plainfield, NJ). The mouse Fli-1 expression vector was obtained from Dr. Dennis Watson (Medical University of South Carolina) and confirmed by DNA sequencing. Briefly, the Fli-1 gene, containing a 5’ kozak sequence and Flag tag, was cloned into the pcDNA3.0 expression vector (Life Technologies), which is under the control of a CMV promoter. The mouse Ets-1 cDNA was isolated from pGEM7Zf(+) (from Dr. Dennis Watson) by digestion with BamHI and EcoRI. The junction sites of the expression constructs (pcDNA3.0, pcDNA/Ets1, and pcDNA/Fli1) were transfected into the cells. The Fli-1 expression level of Fli-1 in these cells is not detectable. NIH3T3 cells were seeded at 4000 cells/well in six-well plates 1 d prior to transfection. Transfections were performed using the Fugene 6 transfection reagent (Promega), following the manufacturer’s instructions. For all of the transfection experiments, 2 μg reporter constructs pGL3/basic, pGL3/ CCL5, pGL3/CCL5Region A, and pGL3/CCL5Region B were used. Equimolar concentrations of the expression constructs (pcDNA3.0, pcDNA/Ets1, and pcDNA/Fli1) were transfected into the cells. The Fli-1 dose-response study used increasing amounts (0.025, 0.05, 0.1, 0.25, 0.5, and 1 μg) of the Fli-1 expression construct transfected into the cells. In the Fli-1 and Ets1 transfection experiment, 0.5 μg of all expression constructs was transfected into the cells. For the competition studies, 0.25 μg of the Fli-1 expression construct was held constant, and increasing amounts of the Ets1 expression construct (0.25, 0.5, 1, and 2 μg) were transfected into the cells. For the deletion studies, 0.5 μg of the Fli-1 expression construct was used. The pcDNA3.0 construct was added to these studies so that equimolar amounts of total DNA were transfected into the cells. For the DNA-binding domain mutant studies, 1 μg the Fli-1 and Fli-1 DNA-binding mutant constructs was transfected into the cells, and the pG5 vector construct was used so that equimolar amounts of total DNA were transfected into the cells. In all experiments, 200 ng of Renilla luciferase construct pRL/TK (Promega) was used as a transfection control. All of the transfected cells were harvested 48 h after transfection. The murine endothelial MS1 cell line was purchased from the American Type Culture Collection and maintained with DMEM with 5% FBS. The cells were maintained at 37˚C with 5% CO2. The MS1 endothelial cell line was used for the siRNA transfection experiments and the ChIP assay because Fli-1 expression was shown to be detectable only in endothelial cells within the glomerulus (39). Specific Fli-1 siRNA and negative-control siRNA were purchased from Invitrogen (Life Technologies) and transfected into MS1 cells using Lipofectamine provided by Invitrogen (Life Technologies), following the manufacturer’s instructions. Transfected MS1 cells were cultured for 24 h with DMEM plus 5% FBS. One microgram of LPS (Sigma-Aldrich, St. Louis, MO) was added to each well and incubated for up to 24 h. The supernatants were collected at 0, 2, 6, or 24 h after stimulation with LPS, and CCL5 chemokine production was analyzed by ELISA.

**Statistics**

Variability was assessed using one-way or two-tailed ANOVA (Graphpad Instat; GraphPad Software, San Diego, CA). When a significant main effect was found, a Student-Newman-Keuls test was used for post hoc comparisons. When a significant interaction was found, a Student-Newman-Keuls test was performed within each level of the significant factor. All data are expressed as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 with respect to control.

**Results**

**Expression of the inflammatory chemokine CCL5 is significantly reduced in kidneys of Fli-1−/− NZM2410 mice**

To determine whether decreased Fli-1 plays a role in the expression of CCL5 mRNA in vivo, we examined the expression of CCL5 in kidneys of Fli-1−/− NZM2410 mice compared with WT littermates. The kidneys were removed from mice at the age of 18 wk, prior to the onset of disease, to exclude the influence of disease-related inflammatory cell infiltration. CCL5 mRNA expression was measured by RT-PCR. A >50% significant reduction (p < 0.05) in CCL5 mRNA expression was observed in Fli-1−/− NZM2410 mice (n = 6 in each group) compared with WT littermate controls (Fig. 1).

**Inhibition of Fli-1 results in decreased production of CCL5 protein in endothelial cells**

Fli-1 expression within kidneys is primarily localized to endothelial cells within the glomerulus, and coexpression with CCL2/MCP-1, another proinflammatory chemokine, was observed (39). CCL5 expression in the glomerulus also was documented previously (6, 10, 22–24). Thus, we chose to investigate whether a disruption in Fli-1 expression affects CCL5 production in vitro by performing transfection studies using Fli-1–specific siRNA and negative-control siRNA in murine endothelial MS1 cells. The expression of Fli-1 protein was inhibited after transfection with specific siRNA (data not shown). Twenty-four hours after transfection with siRNA, CCL5 protein concentrations were measured in the supernatants of MS1 endothelial cells after stimulation with LPS (1 μg/ml) for 0, 2, 6, or 24 h. As shown in Fig. 2, endothelial cells transfected with Fli-1–specific siRNA had significantly lower CCL5 concentrations compared with the endothelial cells transfected with control siRNA at 0, 2, or 6 h after stimulation. Lower concentrations of CCL5 also were detected at 24 h after LPS stimulation, but the difference was not statistically significant.

**FIGURE 1.** Reduced CCL5 mRNA expression in kidneys from Fli-1−/− NZM2410 mice compared with WT littermates. Total RNA was prepared from kidneys at the age of 18 wk (n = 6, each group). Total RNA was converted to cDNA with the Superscript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed in triplicate with the appropriate primers. *p < 0.05.
Fli-1 binds to the CCL5 promoter in endothelial cells

A ChIP assay was carried out to determine whether Fli-1 directly or indirectly regulates the expression of CCL5 in murine endothelial cells. Twelve putative EBSs were identified in the murine promoter region of the CCL5 gene, and nine primer pairs were designed to cover these sites (Table I). After immunoprecipitation in endothelial cells with a Fli-1-specific Ab, five pairs of primers (ChIP1, ChIP2, ChIP3, ChIP6, and ChIP9) had PCR products, and one of these sites (ChIP3) was significantly enriched with specific Fli-1 Abs (Fig. 3A, 3B). Binding to ChIP1 was also increased, although the results were not statistically significant. These results clearly indicate that Fli-1 binds to the CCL5 promoter in endothelial cells and regulates the expression of CCL5.

Fli-1 drives transcription from the CCL5 promoter in a dose-dependent manner

Transient transfection assays were performed to demonstrate that Fli-1 regulates the expression of the CCL5 gene. The CCL5 murine promoter was amplified using PCR from genomic DNA and cloned into the pGL3 basic reporter construct. The ability of Fli-1 to drive transcription from the CCL5 promoter was assessed by increasing the concentration of Fli-1 (2 μg). As the concentration of Fli-1 used increased activation (Fig. 4B). Linear regression analysis of the data was performed. The results depict a decreasing trend as the amount of Fli-1 is increased and indicate that a 1-μg addition of Fli-1 causes a 37-fold increase in Fli-1-related activation (Fig. 6B), confirming that Fli-1 acts as a dominant-negative transcription factor for the activation of the CCL5 promoter by Fli-1.

Activation of the CCL5 promoter by Fli-1 occurs primarily between −746 and −520 bp

Deletion constructs were designed in an effort to determine where along the CCL5 promoter Fli-1 binds. The location of the EBSs and the deletion constructs are depicted in Fig. 7A. The Region B construct removes the most distal EBSs that cover the primers tested for in ChIP1, whereas the Region A construct removes the EBSs covered by the ChIP2 and ChIP3 primers (Table I). Removal of the most distal EBSs (Region B) does not result in loss of activation by Fli-1; rather, a statistically significant increase in activation was observed (Fig. 7B). Conversely, upon deletion of the ChIP2 and ChIP3 putative EBSs, activation of the CCL5 promoter by Fli-1 is drastically reduced (Fig. 7C). Compared with the activation of the full-length promoter by Fli-1, 65% of the activity is lost upon removal of the EBSs between −746 and −520 bp.

Table I. Primers used in the ChIP assay of the CCL5 promoter

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Position from TSS (bp)</th>
<th>Amplicon Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIP1</td>
<td>5'-CAC CAT TCA CAC ACA TG-3'</td>
<td>5'-GTC AAG GGA TCT GAT AGG-3'</td>
<td>−983 to −765</td>
<td>218</td>
</tr>
<tr>
<td>ChIP2</td>
<td>5'-CCT ATC AGA TCC CCT GAC-3'</td>
<td>5'-GTT ACT GTG TGG CAT GTG-3'</td>
<td>−782 to −629</td>
<td>153</td>
</tr>
<tr>
<td>ChIP3</td>
<td>5'-CAC ATG TCA CAC ACT AGG-3'</td>
<td>5'-CCT ACT TAT CAG AAA CAG ACC AC-3'</td>
<td>−646 to −499</td>
<td>147</td>
</tr>
<tr>
<td>ChIP4</td>
<td>5'-GTT TGT GTT TGT TTA GAT ATT-3'</td>
<td>5'-GTT AAG GTC AAG CCA TGG G-3'</td>
<td>−518 to −392</td>
<td>126</td>
</tr>
<tr>
<td>ChIP5</td>
<td>5'-GTT AAG AAC AAT GGC TGG-3'</td>
<td>5'-CCT TGG CCA AAC AAT GGA GAT-3'</td>
<td>−417 to −271</td>
<td>146</td>
</tr>
<tr>
<td>ChIP6</td>
<td>5'-GTT ACT GGA TTA TGG GCA CAG-3'</td>
<td>5'-GCA AAG AAC ACT TGG TGC TGG C-3'</td>
<td>−288 to −156</td>
<td>132</td>
</tr>
<tr>
<td>ChIP7</td>
<td>5'-CCT ACT GGA TTA TGG GCA CAG-3'</td>
<td>5'-GAC TCT GCC TCT AAC TG-3'</td>
<td>−386 to −228</td>
<td>158</td>
</tr>
<tr>
<td>ChIP8</td>
<td>5'-CAG TTA GAG GCA GAG TCC-3'</td>
<td>5'-CAG GAC TGG GGG AGT-3'</td>
<td>−244 to −127</td>
<td>117</td>
</tr>
<tr>
<td>ChIP9</td>
<td>5'-ACT CCC CAC GCA GTC CTG-3'</td>
<td>5'-GAT GCA TGT GTC GTG TCA G-3'</td>
<td>−141 to −24</td>
<td>117</td>
</tr>
</tbody>
</table>

Primers are listed based on their distance from the transcription start site (TSS). The ChIP7 primers cover the two EBSs included in the ChIP5 and ChIP6 primers.
Mutation of the DNA-binding domain of Fli-1 impairs transcriptional activation of the CCL5 promoter

To establish whether Fli-1 drives transcription from the CCL5 promoter through direct binding of the promoter, transient transfection assays were performed using a Fli-1 construct in which a single amino acid change (tryptophan 321 to arginine) prevents DNA binding (43). Mutation of the Fli-1 DNA-binding domain resulted in significantly diminished activation from the CCL5 promoter. Compared with promoter activation with an intact DNA-binding domain, activity was reduced by 78% (Fig. 8).

Some ability to regulate transcription remains because the Fli-1 DNA-binding domain mutant was still able to activate transcription compared with the reporter construct alone.

Discussion

This study identified Fli-1 as a previously undiscovered transcriptional regulator of the inflammatory chemokine CCL5. CCL5 mRNA expression was significantly reduced in response to heterozygous expression of the Fli-1 gene in kidneys of NZM2410 mice prior to the onset of disease. Upon complete knockdown of the Fli-1 gene using siRNA, CCL5 protein expression in LPS-stimulated endothelial cells also was significantly affected. ChIP assay demonstrated that Fli-1 binds to the murine CCL5 promoter, and transient transfection studies further confirmed that Fli-1 directly regulates the expression of the CCL5 gene.

Chemokines, such as CCL5, play a critical role in the inflammatory response, particularly in the recruitment of inflammatory cells to areas of disease or injury (2, 15, 16, 19, 20). CCL5 has been implicated in the pathogenesis of several inflammatory-mediated diseases, including asthma, rheumatoid arthritis, and SLE (7, 15–18). Previously, we demonstrated a significant role for the Fli-1 transcription factor in the development of renal disease, glomerulonephritis, and the pathogenesis of SLE (34–39). Fli-1+/− NZM2410 mice had significantly reduced infiltration of inflammatory cells into kidneys, with a 50% decrease in CD3+ and CD11b+ cells and a 70% decrease in CD19+ cells compared with WT littermate controls (39). Additionally, we found that Fli-1 directly regulates expression of CCL2/MCP-1 (39). Given the profound effects on inflammatory cell infiltration, we hypothesized that Fli-1 regulates chemokines other than CCL2/MCP1. Based on reports that CCL5 plays a role in recruitment of inflammatory cells and the development of glomerulonephritis, we sought to determine whether Fli-1 regulates the expression of CCL5. To test this relationship in vivo, kidneys were harvested from Fli-1+/− NZM2410 mice before they displayed symptoms of murine lupus nephritis, thus removing the influence of migrating inflammatory cells related to the disease. CCL5 mRNA

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expression was reduced in vivo by >50% in kidneys of mice with heterozygous expression of Fli-1 (Fig. 1).

The localization of endothelial cells allows them to play a key role in the inflammatory response by homing immune cells through the production of chemokines and cytokines. CCL5 was shown to be present, and its expression was regulated by Th2-type cytokines (4). In the MS1 endothelial cell line, stimulation of CCL5 protein concentration by LPS increased over a 24-h period (Fig. 2). A rapid upregulation of CCL5 in response to induction by LPS was observed previously (11). In this study, CCL5 protein concentrations were significantly decreased at 0, 2, and 6 h after stimulation when Fli-1 was knocked down by siRNA (Fig. 2). Although CCL5 protein concentrations were decreased after 24 h, the results were no longer significant. This may be due to the fact that transient CCL5 stimulation by LPS occurs primarily between 3 and 12 h, after which induction subsides (11); this may impact the ability of Fli-1 to bind to the CCL5 promoter. These results are consistent with previous findings that glomerular CCL5 mRNA expression is induced by LPS 3–6 h after stimulation (10, 23), and it was shown to effect the binding of NF-κB, C/EBP, and AP-1 transcription factors to the CCL5 promoter (10). Our results suggest that a reduction in Fli-1 leads to a reduction in LPS-induced CCL5 expression, which may ultimately affect the ability of other transcriptional regulators of CCL5 to bind to their

FIGURE 6. Ets1 acts as a dominant-negative transcription factor for Fli-1 in the context of the CCL5 promoter. (A) Results of transfection experiments to determine whether Ets1 acts as a dominant-negative transcription factor over Fli-1. The table below the graph shows the amount of each expression vector (µg) used in the transfection. The amount of Fli-1 added was held constant at 0.25 µg, whereas increasing amounts of Ets1 were transfected. Values shown are fold activation over the empty vector control (mean + SE for three replicate experiments; n = 9). (B) Linear regression calculated from the transfection results when Fli-1 and Ets1 were cotransfected. The amount of each expression construct (µg) is depicted in the table below the graph, and the linear regression equation is located in the upper right corner of the graph. **p < 0.01, two-tailed Student t test with unequal variance.

FIGURE 7. Activation of the CCL5 promoter by Fli-1 occurs between −746 and −520 bp. (A) Schematic diagram of the murine CCL5 promoter region, depicting the location of two deletion constructs (and their size in bp) designed to determine the region responsible for Fli-1 activation. (B) Results of transfection experiments using Region B of the CCL5 promoter, which removes the three most distal EBSs. (C) Results of transfection experiments using Region A of the CCL5 promoter, which removes seven EBSs (including the ChIP2 and ChIP3 regions). Values shown are fold activation over the empty vector control (mean + SE for three replicate experiments; n = 9). **p < 0.01, two-tailed Student t test with unequal variance.

FIGURE 8. Fli-1 regulates CCL5 through direct binding of the promoter. Results of the luciferase assay using the Fli-1 DNA-binding domain mutant (Fli-1BindMut) on the full-length CCL5 promoter. Values shown are fold activation over the empty vector control (mean + SE for three replicate experiments; n = 9). **p < 0.01, two-tailed Student t test with unequal variance.
cognate DNA-binding sites. Previous studies determined that \( \geq 40\% \) of the LPS-induced promoter activation in CCL5 is mediated through the CRE/AP-1–binding motif, which is located near an EBS that binds another Ets family member, PU.1 (11). This highlights the complex transcriptional mechanisms involved in the chemokine-mediated inflammatory response and may suggest a role for Ets family members in the recruitment of other transcription factors and in the coordinated assembly of the regulatory complex.

ChiP assay demonstrated that Fli-1 binds to the murine CCL5 promoter (Fig. 3). It appears that Fli-1 binds primarily to the distal region of the CCL5 promoter. The region that showed the strongest Fli-1–binding affinity contains three potential EBSs within a 22-bp stretch of DNA (Fig. 4A). To further demonstrate that Fli-1 regulates the transcription of the CCL5 gene, transient transfection assays were performed. The results clearly establish that Fli-1 drives transcription from the CCL5 promoter, with as little as 25 ng of Fli-1 needed to activate transcription in a statistically significant manner (Fig. 4B), and prove that Fli-1 is a direct regulator of the CCL5 gene. Ets1 also was able to drive transcription from the CCL5 promoter but at a significantly lower level than Fli-1 (Fig. 5). Therefore, we investigated whether Ets1 and Fli-1 were in direct competition with one another. The results confirm that Ets1 acts as a dominant-negative transcription factor for Fli-1 (Fig. 6). Linear regression analysis reveals that increasing the amount of Ets1 decreases activation of the CCL5 promoter achieved by Fli-1 (Fig. 6B). These results suggest that Fli-1 and Ets1 are in competition for binding sites on the CCL5 promoter and imply another level of transcriptional regulation. In fibroblasts, Ets1 and Fli-1 were shown to possess reciprocal function in the regulation of the human \( \alpha 2(I) \) collagen promoter (43), and maintaining balance in the expression of these two factors is likely key to achieving homeostasis within the extracellular matrix (29). The binding of multiple Ets family members to the same EBS as a means of gene regulation also was reported previously (45). Thus, it seems likely that a complex mechanism of transcriptional control is responsible for the regulation of the inflammatory chemokine CCL5.

The results of the systematic deletion of the distal EBSs suggest that Fli-1 binds to the CCL5 promoter between \(-746 \) and \(-520\) bp (Fig. 7). These results are consistent with the results of the ChiP assay that indicated that Fli-1 had the strongest binding affinity to the ChIP3 region. That Fli-1 may activate transcription from the region between \(-746 \) and \(-520\) bp of the promoter is particularly interesting given the fact that most of the known active transcription factor binding sites have been mapped to the proximal promoter. It was originally shown that only the promoter region from \(-421\) bp to the transcription start site was required for activation in most cell types (1); a more recent review paper detailing the role of the regulation of CCL5 in renal disease maps a majority of the transcriptional mechanisms involved in CCL5 gene expression to the region from \(-195 \) bp to the transcription start site (21). The IRF1 and INF-\( \gamma \)-response element was mapped from \(-147 \) to \(-143\) bp within the murine CCL5 promoter (8). The IRF1 binding site and the NF-\( \kappa B \) binding site between \(-87 \) and \(-79\) bp work synergistically together and are responsible for stimulation by INF-\( \gamma \) and TNF-\( \alpha \) (9). These two transcription factors also interact with Ets family member PU.1, which binds to the murine promoter immediately upstream of the NF-\( \kappa B \) binding site (12). Within the rat CCL5 promoter, C/EBP binds at \(-486\) bp, and NF-\( \kappa B \) binding sites at \(-100\) bp, although distal promoter sites further upstream from the defined murine and human promoter regions also demonstrated transcription factor binding to the AP-1 site at \(-1149\) bp and an NF-\( \kappa B \) site at \(-1593\) bp (10). ATF and Jun bind to the CRE/AP-1 site at \(-197\) bp, PU.1 binds to the EBS at \(-205\) bp of the human promoter (11), and the KLF13 transcription factor binds between \(-71 \) and \(-53\) bp (14, 21). Thus, for the most part, transcription factor binding and activation primarily have been mapped to proximal regions of the CCL5 promoter. The results of this study indicate that Fli-1 transcription factors and, to a lesser extent, Ets1 transcription factors bind to the distal region of the murine CCL5 promoter and regulate transcription differently from other previously described transcription factors.

Deletion of the most distal region of the CCL5 promoter enhances transcriptional activation by Fli-1 (Fig. 7B), suggesting that there may be elements of this region that repress Fli-1 activation, further highlighting the complexity involved in the transcriptional control of CCL5. Unlike the modest results obtained with the human \( \alpha 2(I) \) collagen promoter (43), mutation of the Fli-1 DNA-binding domain reduced Fli-1–mediated activation of the CCL5 promoter by 78\% (Fig. 8). These results strongly suggest that Fli-1 is activating transcription by binding directly to the CCL5 promoter. Some transcriptional activity remains after Fli-1 DNA-binding ability has been removed, suggesting that some indirect protein–protein interactions also may contribute to the regulation of this gene.

Both Fli-1 and CCL5 are involved in the progression and pathogenesis of SLE. In this study, we demonstrated that a reduction in Fli-1 gene expression significantly impacts the expression of CCL5 mRNA in kidneys and CCL5 protein expression in MS1 endothelial cells. This is due to the fact that Fli-1 binds to the distal region of the CCL5 promoter and directly regulates gene transcription. Combined with our previous finding that Fli-1 regulates the expression of another inflammatory chemokine, CCL2/MCP-1 (39), it is evident that one of the ways in which Fli-1 impacts the development of SLE is through the regulation of proinflammatory chemokines that recruit infiltrating inflammatory cells to the kidney. Thus, further investigation into the precise transcriptional regulatory mechanisms, of which Fli-1 is a key player, will lead to a better understanding of the pathogenesis of disease and may lead to the development of novel therapies in the prevention of SLE.

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References


