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Fli-1 controls transcription from the MCP-1 gene promoter, which may provide a novel mechanism for chemokine and cytokine activation

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Abstract

Regulation of proinflammatory cytokines and chemokines is a primary role of the innate immune response. MCP-1 is a chemokine that recruits immune cells to sites of inflammation. Expression of MCP-1 is reduced in primary kidney endothelial cells from mice with a heterozygous knockout of the Fli-1 transcription factor. Fli-1 is a member of the Ets family of transcription factors, which are evolutionarily conserved across several organisms including *Drosophilla*, *Xenopus*, mouse and human. Ets family members bind DNA through a consensus sequence GGAA/T, or Ets binding site (EBS). Fli-1 binds to EBSs within the endogenous MCP-1 promoter by ChIP assay. In this study, transient transfection assays indicate that the Fli-1 gene actively promotes transcription from the MCP-1 gene promoter in a dose-dependent manner. Mutation of the DNA binding domain of Fli-1 demonstrated that Fli-1 activates transcription of MCP-1 both directly, by binding to the promoter, and indirectly, likely through interactions with other transcription factors.

Another Ets transcription factor, Ets-1, was also tested, but failed to promote transcription. While Ets-1 failed to drive transcription independently, a weak synergistic activation of the MCP-1 promoter was observed between Ets-1 and Fli-1. In addition, Fli-1 and the NFκB family member p65 were found to interact synergistically to activate transcription from the MCP-1 promoter, while Sp1 and p50 inhibit this interaction. Deletion studies identified that EBSs in the distal and proximal MCP-1 promoter are critical for Fli-1 activation from the MCP-1 promoter. Together,

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these results demonstrate that Fli-1 is a novel regulator of the proinflammatory chemokine MCP-1, that interacts with other transcription factors to form a complex transcriptional mechanism for the activation of MCP-1 and mediation of the inflammatory response.

Keywords

Transcriptional factors; Gene Regulation; Chemokines; Inflammation; Fli-1; MCP-1

1. Introduction

Monocyte chemoattractant protein (MCP-1), also known as Chemokine (C-C motif) ligand 2 (CCL2), is a chemokine family member that plays a major role in the inflammatory process. It has also been shown to be involved in immune regulation, wound healing, and act as a modulator of tumor immunity (1–5). First characterized as the platelet-derived growth factor (PDGF) inducible gene *JE* (6), both the sequence and structure of murine MCP-1 was found to be homologous to several cytokines including macrophage colony stimulating factor, IL-2, IL-6, and interferon α (7). One of the primary roles of MCP-1 is the recruitment of monocytes, B and T lymphocytes, and natural killer cells to sites of inflammation and infection (8). A wide variety of proinflammatory cytokines and chemokines, including MCP-1, have been shown to be activated through toll-like receptor (TLR) mediated stimulation of NF κ B (9). Several transcription factor binding sites have been identified in both murine and human promoter regions of the MCP-1 gene. Binding sites for NF κ B have been mapped to distal and proximal regulatory regions within the MCP-1 promoter and an AP-1/GC box binding site that binds the transcription factor Sp1 is also located in the proximal regulatory region (10), although the proximal NF κ B site was shown to be non-functional in the human promoter (11). Transcriptional activation of both the human and murine MCP-1 promoter can be induced by TNF α (10, 11) and transcription of the human promoter has been shown to be induced by INF in astrocytes (12). Sp1 and the NF κ B transcription factor p65 produce a synergistic enhancement of the transactivation of the murine MCP-1 promoter in *Drosophila* cells and lack of the Sp1 transcription factor impairs activation and inhibits the *in vivo* assembly of the promoter/transcription factor complex (13). The regulation of MCP-1 across various cell types is complex and involves a variety of events including phosphorylation and translocation of NF κ B into the nucleus, the assembly of transcription factors, and responsiveness to stimulation from other cytokines including TNF α (10).

The Ets family of transcription factors play a role in the regulation of a variety of cellular functions including: angiogenesis, apoptosis, cell proliferation, differentiation, invasiveness, immune response, lymphoid cell development, and tumor progression (14–16). They can be both positive and negative regulators of transcription and bind DNA through a winged helix-turn-helix DNA binding domain recognizing the consensus sequence GGAA/T (14, 15, 17, 18). The region surrounding the core consensus motif and cell type specificity helps to distinguish which Ets family members will bind the DNA. Ets family members may bind the same site and this redundancy may be a key aspect of their transcriptional regulation (15). Most Ets transcription factors bind to the DNA as monomers, but interaction with other

transcription factors through the pointed domain (required for protein-protein interactions) can enhance their DNA binding ability resulting in synergistic activation or repression (19).

The friend leukemia insertion site 1 (Fli-1) gene is an Ets family transcription factor that is expressed in hematopoietic cells including B and T cells and vascular endothelial cells (20). Fli-1 has been implicated in the pathogenesis of both human and murine systemic lupus erythematosus (SLE) (21–24) of which glomerulonephritis, proteinuria, and autoantibody production are hallmarks (25, 26). Overexpression of Fli-1 in transgenic mice resulted in severe immune dysfunction due to an increase in infiltrating B and T lymphocytes and autoantibody production; these mice ultimately died due to tubulointerstitial nephritis and glomerulonephritis (27). Heterozygous expression of Fli-1 reduced autoantibody production, proteinuria, renal inflammation and necrosis, and prolonged survival (23, 24) in MRL/*lpr* mice and NZM2410 mice, murine models for lupus (28–30). Reduced proliferation of naïve B cells, independent of BCR and TLR expression in Fli-1 deficient mice has been observed (31). MCP-1 has also been implicated in the development of autoimmune disease. MRL/*lpr* mice deficient in MCP-1 showed a reduction in macrophage and T cell recruitment to the kidney and glomerulus as well as reduced proteinuria, a decrease in apoptotic cells, and prolonged survival (32). Similar results have also been observed in Fli-1^{+/-} NZM2410 mice, which exhibit significantly less infiltration of inflammatory cells and decreased expression of MCP-1 in kidneys (33). Based on our previous results showing decreased expression of MCP-1 in kidneys and primary endothelial cells, and demonstrating that Fli-1 binds to the MCP-1 promoter (33); it appears that there is a direct link between Fli-1 and MCP-1 gene expression.

The goal of this study was to establish that the Fli-1 transcription factor directly regulates expression of the MCP-1 gene through transcriptional activation of the promoter. Results show that very little Fli-1 is necessary to drive transcription at a high level from the MCP-1 promoter, corroborating our previous results. Mutation of the DNA binding domain of Fli-1 demonstrated that while some of the transcriptional activation of MCP-1 was due to Fli-1 binding directly to the promoter, most of the activation was due to indirect activation of the promoter. Therefore we decided to investigate the ability of Fli-1 to interact with other transcription factors. The Ets-1 transcription factor, surprisingly, failed to drive transcription from the MCP-1 promoter. Despite the inability of Ets-1 to activate transcription on its own, when co-transfected with Fli-1 there was a slight transcriptional enhancement observed. Fli-1 and NFκB family member, p65, were found to interact to activate transcription from the MCP-1 promoter, while Sp1 and p50 inhibit this interaction. While Fli-1 is able to bind at least three cis-regulatory regions in the promoter (33), it appears that similar to previous studies (10, 11, 13) binding sites in the distal and proximal regions are of greatest importance for transcriptional activation. Combined with our previous results, we have demonstrated that Fli-1 plays a critical role in the activation of the proinflammatory chemokine MCP-1. Thus, we have discovered that Fli-1 plays a novel role in the complex transcriptional mechanisms responsible for the activation of MCP-1 and the inflammatory response.

2. Materials and Methods

2.1 Reporter and Expression Constructs

The full length MCP-1 promoter region was PCR amplified from the pJECAT2.6 vector (a generous gift from Dr. Jeremy Boss, Emory University School of Medicine, Atlanta, GA) with a forward primer containing the *NheI* enzyme site (underlined) and a reverse primer containing the *BglIII* enzyme site (underlined). All primer sequences can be found in Table 1. The PCR program used to amplify the full length sequence was as follows: 94°C for 3min.; thirty cycles of 94°C for 30sec, 60°C for 30sec, 72°C for 1min; 72°C for 10min; held at 4°C until the program was stopped. The MCP-1 promoter region was then directionally cloned into the pGL3 basic vector upstream of the Luciferase gene (Promega, Madison, WI). Deleted portions of the MCP-1 were also PCR amplified from the pJECAT2.6 vector, using the same reverse primer as the full length promoter. The forward primers used to amplify them are described in Table 1. The PCR program used was primarily the same as the one described above, except that the annealing temperature for regions B and C was 58°C and for region D was 62°C. All of the promoter constructs were confirmed by DNA sequencing (Genewiz, South Plainfield, NJ). The mouse Fli-1 gene containing a 5' kozak sequence and Flag tag, was cloned into the pcDNA3.0 expression vector (Life Technologies, Grand Island, NY), which is under the control of a CMV promoter. The Ets1 cDNA was isolated from pGEM7ZEts1 through digestion with *BamHI* and *EcoRI*. The *BamHI* site was filled in and the isolated fragment was ligated into the *EcoRI* and *EcoRV* sites of the pcDNA3.0 vector. Both expression vectors have been described previously (34). A mouse Fli-1 construct in the pSG5 expression vector (Agilent Technologies, Santa Clara, CA) that contains a single amino acid mutation to prevent DNA binding was provided by Dr. Maria Trojanowska (Boston University, School of Medicine Arthritis Center, Boston, MA) and has been described previously (35) and for experiments with this construct, a mouse Fli-1 construct with an intact DNA binding domain in the same expression vector was used. Expression constructs (13) containing the NFκB family transcription factors p50 and p65, as well as an Sp1 construct were also generously donated from the Boss laboratory (Emory University School of Medicine, Atlanta, GA).

2.2 Cell line and DNA Transfection

The mouse embryonic fibroblast NIH3T3 cell line was grown in Dulbecco's Modified Eagle's Medium (Mediatech Inc., Manassas, VA) with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained at 37°C with 5% CO₂. Cells were seeded at 4×10⁵ cells per well in 6 well plates, one day prior to transfection. Transfections were performed using the Fugene 6 transfection reagent (Promega) following the manufacturer's instructions. For all of the transfection experiments 2μg of the reporter constructs pGL3/basic and pGL3/MCP-1 were used. Equimolar concentrations of the expression constructs were transfected into the cells. For the Fli-1 dose response study increasing amounts (0.05μg, 0.1μg, 0.2μg, 0.25μg, 0.5μg, and 1μg) of the Fli-1 expression construct were transfected into the cells. For the DNA binding mutant transfections 1μg of the expression constructs (pSG5, pSG5/Fli1, and pSG5/Fli1DNAbindMut) was transfected into the cells. For the initial transfection comparing the activation of Fli-1 to Ets-1, 1μg of all expression constructs (pcDNA3.0, pcDNA/Ets1, and pcDNA/Fli1) was transfected into the cells. For

the competition studies between Fli-1 and Ets-1, either 0.5µg of the Fli-1 expression construct was held constant and increasing amounts of the Ets-1 expression construct (0.25µg, 0.5µg, 1µg, and 2µg) were transfected into the cells or the Ets-1 expression construct was held constant at 0.5µg and increasing amounts of the Fli-1 expression construct (0.25µg, 0.5µg, 1µg, and 2µg) were transfected into the cells. For transfections with p50, p65 and Sp1 preliminary studies were performed to determine the best concentrations to use for each construct. The p50 and Sp1 constructs were unable to activate transcription from the MCP-1 promoter on their own at any concentration tested. The NFκB p65 construct, on the other hand, strongly activated transcription (data not shown). Based on the preliminary studies the following concentrations were used in the co-transfection experiments (0.1µg of pcDNA/Fli1 and NFκB p65; 1µg of NFκB p50 and Sp1). For the deletion studies, 2µg of the reporter constructs and 0.5µg of either the pcDNA/Fli1 or the pcDNA3.0 were transfected into the cells. Either the pcDNA3.0 or pSG5 construct was added to all transfection experiments when necessary to ensure that equimolar amounts of total DNA were transfected into the cells. In all experiments 200ng of a *Renilla* luciferase construct pRL/TK (Promega) was used as a transfection control. All of the transfected cells were harvested 48 hours after transfection.

2.3 Reporter Assays

Luciferase activity was measured using the Dual-luciferase reporter assay system (Promega) and the Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA). Transfection activity was normalized to the activity of the co-transfected *Renilla* luciferase construct. Total molar amount of DNA was kept constant with empty expression vectors as needed. Fold activation was calculated based on the activity over the pGL3/basic vector and the values are reported as mean ± standard error. Variances in the transfection data were calculated and statistical analysis was performed using a two tailed Student's *t*-test, which was applied based on the calculated variances.

2.4 Western Blot Analysis

Cell lysates obtained during the luciferase assay were subsequently run on a 4–15% Criterion TGX Precast gel (Bio-Rad, Hercules, CA) for two hours at 120V. The proteins were then transferred to a PVDF membrane (Life Technologies) and probed with a Fli-1 polyclonal antibody described previously (33) and an antibody to β-actin (Cell Signaling, Beverly, MA). The results were analyzed on an Odyssey Imaging System (LI-COR, Lincoln, NE).

3. Results

3.1 Fli-1 Activates Transcription from the MCP-1 promoter

To investigate whether the Fli-1 transcription factor was able to directly regulate transcription of the murine MCP-1 promoter, a series of transient transfection studies were performed. The Fli-1 transcription factor was transfected into NIH 3T3 cells along with the full length MCP-1 promoter. A schematic diagram of the MCP-1 promoter illustrating the location of the NFκB binding sites, the AP-1/GC box that binds Sp1 and the known and putative EBSs can be found in Figure 1A. The Fli-1 transcription factor strongly induced

activation of the MCP-1 promoter alone in a statistically significant manner when compared to the activation of the reporter construct (Figure 1B). The results demonstrate that Fli-1 drives transcription from the MCP-1 promoter in a dose-dependent manner, with as little as 50ng of Fli-1 needed to significantly activate transcription. The level of activation begins to decrease at the highest concentrations (Figure 1B). Fli-1 protein expression corresponds with the increasing amounts of the plasmid transfected into the cells (Figure 1C).

3.2 Fli-1 has direct and indirect effects on MCP-1 gene transcription

A Fli-1 DNA binding mutant that contains a single amino acid mutation which prohibits Fli-1 from binding to the DNA was tested to determine if the transactivation of the MCP-1 gene promoter by Fli-1 was due to Fli-1 binding directly to the DNA. Activation by the Fli-1 DNA binding mutant was significantly decreased when compared to the intact Fli-1 construct; however, both constructs were able to drive transcription from the MCP-1 promoter over the reporter construct alone (Figure 2). Mutation of the Fli-1 DNA binding domain results in a nearly 27% loss of activity when compared to activation by Fli-1 with no mutation. These results suggest that activation of the MCP-1 promoter by Fli-1 is not only due to direct binding of the DNA, but through indirect means as well.

3.3 Ets-1 fails to drive transcription from the MCP-1 promoter

Since Ets-1 is in the same family of transcription factors as Fli-1, their expression patterns are very similar, and the cognate DNA binding sites are the same (GGAA/T) for both factors, we examined whether Ets-1 was able to activate transcription from the MCP-1 promoter. Surprisingly, the Ets-1 transcription factor failed to drive transcription from the MCP-1 promoter (Figure 3). Several different concentrations of the Ets-1 expression construct were tested and in all cases the Ets-1 transcription factor failed to drive transcription above the activation seen by the MCP-1 reporter construct alone (data not shown). Transcriptional activation from the MCP-1 promoter by Fli-1 was significantly higher compared to the activation observed by Ets-1 (Figure 3).

3.4 Weak synergistic activation of the MCP-1 promoter by Fli-1 and Ets-1

Given the finding that the Ets-1 transcription factor fails to enhance transcription from the MCP-1 promoter in NIH 3T3 cells and that both Ets-1 and Fli-1 bind to similar consensus sequences, we next investigated whether Ets-1 could compete with Fli-1. Transfections were carried out where the amount of Fli-1 remained constant and increasing amounts of the Ets-1 transcription factor were added. Interestingly, Ets-1 does not compete with Fli-1. In fact, a weak, but statistically significant effect was observed when adding an increasing amount of Ets-1. Ultimately, this effect disappears when a high concentration of Ets-1 is added (Figure 4A). Similar results were obtained when holding the amount of Ets-1 transfected into cells constant and increasing the amount of Fli-1 added (Figure 4B). The decrease in activation at higher concentrations is consistent with the results obtained above and may be due to saturation of the available binding sites.

3.5 Fli-1 interacts with p65 to synergistically enhance transcriptional activation of MCP-1

Since Fli-1 activation of the MCP-1 promoter is through both direct and indirect means, the ability of Fli-1 to interact with other transcription factors involved in MCP-1 gene activation was explored. Given the recognized role that Sp1, NF κ B p50 and p65 play in binding the promoter, transcription factor complex assembly, and transactivation (10, 13), Fli-1 was tested in transient transfection assays with these transcription factors. Sp1 and p50 failed to increase transcription from the MCP-1 promoter when compared to activation by the reporter construct alone (Figure 5A and B, data not shown). Transcription from the MCP-1 promoter was greatly enhanced by p65, with as little as 0.1 μ g of expression construct needed to drive transcription in a statistically significant manner (Figure 5A). No statistical difference was observed in co-transfections of Fli-1 with p50 or Sp1 when compared to the activation by Fli-1 alone, although overall activation decreased slightly when Fli-1 and Sp1 were transfected together. A substantial and statistically significant increase was seen when Fli-1 and p65 were co-transfected into the cells (Figure 5A). Next, transfections with multiple transcription factor combinations were performed. Surprisingly, co-transfection of p65 and Sp1 yielded a statistically significant decrease in transcriptional activation when compared to p65 alone. Transfection of p50, p65 and Sp1 together showed no change in activation when compared to p65 alone (Figure 5B). The strong enhancement observed when Fli-1 and p65 were transfected together diminished significantly when Sp1 is transfected with them, although, the level of activation is still significantly higher than either Fli-1 or p65 alone (Figure 5B). Activation was further reduced when all four transcription factors were transfected together. The observed activation by the combination of all four factors was not significantly different than the enhancement observed by p65 alone, but was still significantly increased over the activation of Fli-1 alone (Figure 5B). Figure 5C illustrates that cotransfection of Fli-1 and p65 led to a super-additive enhancement of transcription from the MCP-1 promoter when compared to the results of adding the activation of each transcription factor alone together (additive results).

3.6 Ets binding sites in the distal and proximal promoter region are responsible for most of the transcriptional activation by Fli-1

In order to determine which EBSs were responsible for the majority of the transcriptional activation by Fli-1 a series of deletion constructs were made. The deletion constructs were designed such that a systematic removal of the regions known to bind to Fli-1 (33) or that contain putative EBSs was accomplished (Figure 6A). A significant decrease in transcriptional activation by the Fli-1 transcription factor was evident upon the removal of the most distal region of the promoter (Region A), containing a known Fli-1 DNA binding site. The second largest loss of activity occurred when a region containing five putative EBSs was removed (Figure 6B, Region F). This region is in the proximal promoter region from around -187bp to -243bp upstream of the transcription start site. Removal of the more distal region (Region A, with two EBS sites at -1992bp and -2050bp) resulted in the loss of 60% of the activity when compared to the transcriptional activation seen with the full length promoter (Figure 6C). Removal of the proximal region described above (Region F) caused a decrease in activity of 27% from the previous deletion construct and 96% from the full length promoter (Figure 6C). While removal of the other regions (B-E) did produce some

moderate effects, including some potential negative regulatory elements, these deletions were not nearly as potent as the removal of the other two regions.

4. Discussion

The data presented in this paper provide the first molecular evidence that the Fli-1 transcription factor directly regulates expression of the chemokine MCP-1. Transient transfection assays demonstrated that Fli-1 drives transcription from the MCP-1 promoter and validated our previous finding that Fli-1 plays a role in the pathogenesis of glomerulonephritis through regulation of MCP-1 (33). The regulation of MCP-1 by Fli-1 is through both direct and indirect means and our findings provide evidence that Fli-1 interacts with at least two other known transcriptional regulators of MCP-1, Ets1 and p65, to enhance transcriptional activation.

In this study we have shown that Fli-1 activates transcription of MCP-1 in a dose-dependent fashion, although activation appears to tail off at the higher concentrations (Figure 1). This may be due to the occupation of the available DNA binding sites within the MCP-1 promoter or maximal recruitment and interaction with other transcription factors. The western results indicate that the tailing off of activation is not due to protein concentration (Figure 1C). Mutation of the Fli-1 DNA binding domain led to a 27% reduction in activation from the MCP-1 promoter (Figure 2), demonstrating that activation by Fli-1 is not only through directly binding the DNA, but also through indirect means, such as interactions with or the recruitment of other transcription factors to the promoter complex. Interaction with other proteins to improve DNA binding and/or enhance transcriptional control is a hallmark of Ets transcription factor family members and has been well documented (19).

The fact that another Ets family member, Ets-1, was unable to drive transcription from the MCP-1 promoter (Figure 3) was particularly surprising given that Ets-1 has been previously identified as a critical regulator of MCP-1 in vascular smooth muscle cells in response to angiotensin 2 (36). The difference in the ability of Ets-1 to activate transcription may have to do with a requirement for angiotensin 2 to stimulate transactivation or the difference in cell type and potential availability of co-activators. Within the human MCP-1 promoter, of several putative EBSs, only two were found to bind Ets-1, one site in the distal promoter and one site more proximal to the transcription start site (36). Based on the location of the distal Ets-1 binding site in the human promoter, it is possible that Ets-1 may bind the murine promoter at a site close to the known Fli-1 site (33, 36). Given that Ets-1 failed to drive transcription from the MCP-1 promoter, the proximity and similarity of potential DNA binding sites, and that Ets-1 has been shown to act as a competitor to Fli-1 in the context of the CCL5 promoter (unpublished results), similar results were anticipated for the MCP-1 promoter. On the contrary, our results imply that Ets-1 and Fli-1 do not share a distal DNA binding site, but rather interact, perhaps physically, given the close proximity of the two EBS (around two to four hundred base pairs apart) to enhance transcription from the MCP-1 promoter (Figure 4). Previously, it has been shown that Fli-1 and Ets-1 in fibroblasts have opposing effects on the collagen type I promoter (35) and our own data suggest that Ets-1 limits the activation of the CCL5 promoter by Fli-1 (unpublished results). Thus our findings imply that both differences in cell type and the factors involved in the transcriptional

regulatory network are important elements that influence the interaction between these two transcription factors.

A strong, synergistic activation of the MCP-1 promoter was observed when Fli-1 was co-transfected along with the NF κ B family member p65 (Figure 5), suggesting another mechanism by which Fli-1 may indirectly affect the regulation of the chemokine. In previous studies, Ets transcription factors have been shown to interact with proteins that bind to AP-1 and NF κ B sites to control transcription of a variety of cytokine genes including GM-CSF, IL-2, and IL-3 (19, 37–39). Ets-1 was shown to cooperate with both NF κ B and AP-1 (c-Fos and c-Jun) factors to synergistically enhance transcription from the GM-CSF promoter; activation was strongest when all three transcription factors were transfected together. Additionally, Ets-1 bound to the GMCSF promoter along with an unknown second Ets family member that could be Fli-1 (37). The AP-1 transcription factor and another unknown Ets family member were shown to be vital regulators of the murine anaphylatoxin C3a receptor (40). Therefore, while protein-protein interactions between Fli-1 and NF κ B p65 to our knowledge has not been previously described, this novel interaction between these two factors is not completely without precedent.

Some of our results with the NF κ B proteins and Sp1 differ from those reported previously. The failure of Sp1 to activate transcription from the MCP-1 promoter alone (Figure 5A and B) is somewhat surprising, given that previous results show weak activation by Sp1 alone and indicate that Sp1 is required for MCP-1 promoter activation and assembly by tumor necrosis factor (TNF)(13), although perhaps most surprising are the differences between the interactions of the transcription factors when they are combined. In the 2000 study by Ping et al, Sp1 and NF κ B p65 combined to enhance activation from the MCP-1 promoter, while increasing amounts of NF κ B p50 inhibited activation (13). Here, a statistically significant decrease was observed upon co-transfection of p65 and Sp1. The addition of p50 to p65 and Sp1 showed no change from the level of activation seen by p65 alone (Figure 5B). The difference in these results may be due to the difference in cell types. In the study by Ping, these experiments were performed in the *Drosophila* Schneider cell line (13), whereas in this study the NIH3T3 cell line was used. Ets transcription factors are highly conserved (14), including the identification of eight orthologs to Ets family members in *Drosophila* (41), one which is most closely related to Fli-1 (42). Thus, it is possible that the Fli-1 ortholog is expressed in the *Drosophila* Schneider cell line and caused the differences observed between the two studies.

Previous studies have established that both the distal and proximal regulatory regions are required for *in vivo* promoter assembly, and TNF α induced transcriptional activation (10, 13). Ets-1 was also shown to bind to regions in both the distal and proximal promoter (36). Previous chromatin immunoprecipitation results demonstrated that Fli-1 binds to the MCP-1 promoter at a distal site as well as two more central sites (33). However, the deletion studies clearly indicate that both the most distal binding site and a more proximal region are critical for Fli-1 driven transcriptional activation (Figure 6). These results suggest that Fli-1 may be binding to the more proximal region containing five putative EBSs. Determining the specific site(s) within the MCP-1 promoter that bind Fli-1 may be a difficult task. Within the regions described above, a minimum of 14 putative EBS sites have been identified through sequence

analysis (Figure 1A). Further complicating matters is the possibility that Fli-1 may be indirectly binding the DNA through other transcription factors, such as NFκB p65 or Sp1. Overall, our results are consistent with previous findings (10) and it is apparent that the distal and proximal regions of the MCP-1 promoter are the most critical regions for transcriptional control of MCP-1 gene expression. Sp1 binds to the proximal region of the MCP-1 promoter, while NFκB p65 binds 2.5kb away in the distal region of the promoter and yet Sp1 binding affects activation of the MCP-1 promoter and in vivo binding of the DNA by NFκB (13). In order for p65 and Sp1 to physically interact, the chromatin would need to be rearranged, looping the DNA and bringing both regulatory regions closer. Sp1 is known to be involved in chromatin looping (43) and recently was shown to regulate chromatin rearrangement between an intronic enhancer and the distal promoter of heme oxygenase-1 in renal epithelial cells (44). Perhaps, in the context of the MCP-1 promoter, Fli-1 and Sp1 do not interact to enhance transcription (Figure 5A and B), but rather Fli-1 interacts with Sp1 to facilitate the looping of the DNA, allowing Sp1 to promote the assembly of the overall transcription factor complex. Protein-protein interactions between Fli-1 and Sp1 have been observed when bound to the collagen type I promoter and it was shown that Sp1 may facilitate binding of Fli-1 to the promoter (35).

In conclusion, the Fli-1 transcription factor drives transcription from the MCP-1 promoter in a dose-dependent manner and affects the regulation of the proinflammatory chemokine through direct binding to the DNA and indirectly, by interacting with other transcription factors. While Ets-1 alone fails to drive transcription from the MCP-1 promoter, an interaction between Fli-1 and Ets-1 leads to increased transcriptional activity. Fli-1 also synergizes with the NFκB family member p65 to enhance transcription from the MCP-1 promoter. Consistent with previous studies, EBSs in the distal and proximal promoter are the regions critical for the transcriptional activation by Fli-1. Together, this data suggests that Fli-1 is a key regulator of transcription for the chemokine MCP-1 and may be actively involved in the complex transcriptional mechanism responsible for MCP-1 gene activation.

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Highlights

Fli-1 activates transcription from the MCP-1 promoter in a dose-dependent manner.

Fli-1 activates transcription directly and indirectly.

Ets-1 alone failed to drive transcription, but with Fli-1 activates transcription.

Fli-1 and NF κ B p65 interact to activate transcription, while Sp1 and p50 inhibit.

EBSs in the distal and proximal promoter are critical for Fli-1 activation.

Figure 1. Fli-1 drives transcription from the MCP-1 promoter

(A.) Schematic diagram of the murine MCP-1 promoter construct cloned into the pGL3 expression vector. Known NFκB binding sites (black triangles) and the AP-1/GC box (grey square) are depicted. Ets binding sites (black circles) are also shown; the number of sites in a region is noted by number above and Ets sites that have been shown to bind Fli-1 are labeled below. (B.) Transcriptional activation of the MCP-1/pGL3 reporter construct is shown as fold activation over an empty pGL3 basic vector alone. All transfections were carried out at equimolar concentrations as described in the Materials and Methods section 2.2. Increasing

amounts of the Fli-1 expression plasmid (0.05, 0.1, 0.2, 0.25, 0.5, to 1 μ g) were transfected into NIH3T3 cells. Values shown are the mean + standard error for three replicate experiments (n=9). A two tailed student's t-test with unequal variance was used to calculate significance, $p < 0.01$. (C.) Representative western blot results demonstrating Fli-1 protein expression (upper arrow) compared to β -actin (lower arrow) upon transfection of increasing plasmid amounts into NIH 3T3 cells. The amounts of Fli-1 in the cell lysates are indicated. Cell lysates were used for both the luciferase assay and western analysis.

Figure 2. Mutation of the Fli-1 DNA binding domain partially inhibits transcriptional activation from the MCP-1 promoter

Graph depicting the change in transcriptional activation when the Fli-1 DNA binding domain is mutated (Fli-1BindMut). Transcriptional activation of the MCP-1/pGL3 reporter construct is shown as fold activation over an empty pGL3 basic vector alone. Values shown are the mean + standard error for three replicate experiments (n=9). A two tailed student's t-test with unequal variance was used to calculate significance, $p < 0.01$.

Figure 3. Ets-1 fails to activate transcription from the MCP-1 promoter

Transient transfection assay results demonstrating that Ets-1 does not activate transcription from the MCP-1 promoter. Empty vector controls are also included to show that negligible activation of the empty vector control (pGL3) is occurring by Ets-1 or Fli-1. Values shown are the mean + standard error for three replicate experiments (n=9). A two tailed student's t-test with unequal variance was used to calculate significance, $p < 0.01$.

Figure 4. Fli-1 interacts with the Ets-1 transcription factor to drive transcription from the MCP-1 promoter

Luciferase assay results illustrate fold activation over the empty vector control. In (A.) the amount of Fli-1 was held at 0.5 μ g and Ets-1 was increased from 0.25, 0.5, 1, to 2 μ g, n=12. In (B.) the amount of Ets-1 was held at 0.5 μ g and Fli-1 was increased from 0.25, 0.5, 1, to 2 μ g, n=6. Error bars represent the standard error from the mean. A two tailed student's t-test with unequal variances was used to determine p values, * = p<0.05, ** = p<0.001.

Figure 5. Fli-1 and NFκB p65 enhance transcription from the MCP-1 promoter, while NFκB p50 and Sp1 suppress

Transfection assays performed to determine the interaction between Fli-1 and p50, p65, and Sp1. (A.) The upper panel is a schematic diagram of the MCP-1 promoter noting the EBS (black circles), NFκB binding sites (black triangles) and AP-1/GCbox that binds Sp1 (grey square). The lower panel contains the luciferase assay results showing the interaction of Fli-1 with each factor through co-transfection, n=9. (B.) Results of the luciferase assay illustrating the combinations of multiple transcription factor combinations, n=9. (C.) Luciferase assay results demonstrating that the effect of Fli-1 and p65 co-transfection is synergistic when compared to the activation results of each factor added together (or the additive effect), n=18. Error bars represent the standard error from the mean. A two tailed student's t-test was applied based on the calculated variances, ** = p<0.001.

Figure 6. Ets-1 binding sites located in the distal and proximal promoter region are important for Fli-1 transcriptional activation

(A.) Diagram illustrating the deletion constructs made. The six deletion fragments were chosen to determine the effect of known and previously unknown Fli-1 binding sites. Luciferase assay results illustrate fold activation over the empty vector control and error bars represent the standard error from the mean (B.) and the loss of activity from the full length promoter of the deletion constructs (C.). Numbers indicate the % activity remaining compared to the full length promoter n=9.

Table I

Primers Used to Amplify the MCP-1 Promoter and Deletion Constructs

Primer Name	Primer	Expected Amplicon Size
Full Length Forward	5' TAA TTG <u>CTA GCA</u> CGT TCC ATT GTC CAT CTG GGT 3'	2736bp
MCP-1 Reverse	5' TTA ATTT <u>AGA TCT</u> GGT GGT GGA GGA AGA GAG AG 3'	2736bp
Region A Forward	5' TTA AAT <u>GCT AGC</u> CAA CTA CTG CCT CCA ATC 3'	1942bp
Region B Forward	5' ATT TAT <u>GCT AGC</u> CCT TTA CAG TCA TTG TCT C 3'	1340bp
Region C Forward	5' TTA TAT <u>GCT AGC</u> CAA GAG TCT GCT GAA ATG 3'	1169bp
Region D Forward	5' AAT TTT <u>GCT AGC</u> CTG CAC CAT GAG CTC ATG 3'	687bp
Region E Forward	5' ATA TAT <u>GCT AGC</u> CTC CAT TCA CAC TTG TG 3'	390bp
Region F Forward	5' TTT AAA <u>TGC TAG CGA</u> TGC TAC TCC TTG GCA C 3'	168bp

* The NheI and BglII enzyme sites in the forward and reverse primers are underlined and in bold font.