Sp1 Binding Is Critical for Promoter Assembly and Activation of the MCP-1 Gene by Tumor Necrosis Factor*

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The monocyte chemoattractant protein-1 gene (MCP-1) is induced by the inflammatory cytokine tumor necrosis factor through the coordinate assembly of an NF-κB-dependent distal regulatory region and a proximal region that has been suggested to bind Sp1 as well as other factors. To provide a genetic correlation for Sp1 activity in this system, a cell line homozygous for a targeted truncation of the Sp1 gene was derived and examined. We found that the lack of Sp1 binding activity resulted in the inability of both the distal and proximal regions to assemble in vivo even though the binding of NF-κB to distal region DNA was unaffected in vitro. We also found that Sp1 and NF-κB were the minimal mammalian transcription factors required for efficient activity when transfected into Drosophila Schneider cells. Additionally, Sp3 was able to compensate for Sp1 in the Drosophila tissue cell system but not in the Sp1−/− cell line suggesting that Sp1 usage is site-specific and is likely to depend on the context of the binding site. Together, these data provide genetic and biochemical proof for Sp1 in regulating the MCP-1 gene.

TNF is a proinflammatory cytokine that has broad effects on immune responses. Although TNF has the ability to induce apoptosis in some cell systems and in some tumors, TNF carries out a majority of its function through the stimulation of gene expression. A large number of genes have been identified that are responsive to TNF (1, 2). Such genes include cytokines, transcription factors, adhesion molecules, and structural proteins. The mechanics of TNF-mediated gene induction has concentrated on the factor NF-κB (3). NF-κB represents a diverse family of homologous proteins that interact to form a variety of heterodimers, which appear to have distinct functions (4, 5). The major form is composed of the p50 and p65 subunits. NF-κB is held in an inactive state in the cytoplasm by the inhibitor IκB (reviewed in Ref. 5). Upon TNF signaling, IκB is phosphorylated by the IκB kinase, IκK (6), polyubiquitinated, and degraded (5, 7). The separation of IκB from the NF-κB complex reveals a nuclear localization sequence on NF-κB that allows its nuclear transport and gene activation. In most genes, NF-κB alone is not sufficient to activate gene expression, and numerous other factors appear to be involved in mediating expression. In some cases, a complex of proteins, which includes at least one NF-κB molecule, forms a compact regulatory unit that has been termed an enhanceosome (8, 9). However, in other genes, such as the monocyte chemoattractant protein-1 gene (MCP-1) (10) and the manganous superoxide dismutase gene (11), the regions controlling gene expression are separated by more than 2 kb of DNA. Such systems are likely to require more extensive and complex interactions for which little is known.

Monocyte chemoattractant proteins (MCP) are a family of proinflammatory C-C chemokines that recruit macrophages, monocytes, T cells, and basophils to regions of infection and disease (12–15). Five MCP genes have been identified in the mouse (reviewed in Ref. 15). The murine JE gene is highly homologous to the human MCP-1 gene and is regulated in a similar fashion. Thus, JE has been considered to be the ortholog of human MCP-1 for several years (15). Expression of MCP-1 is associated with a variety of disease states, including the pathogenesis of atherosclerosis (16), HIV replication (17, 18), glomerular nephritis (19), and allergic and chronic inflammatory diseases (15, 20, 21). MCP-1 expression is regulated by a host of cytokines that include tumor necrosis factor (TNF) (10), platelet-derived growth factor (PDGF) (22), and interferon-γ (IFN-γ) (23). Additionally, MCP-1 expression can be induced by agents and factors that stress cells (24–27). Other intercellular agents, such as retinoic acid, glucocorticoids, and estrogen, can inhibit the induction of the MCP-1 gene (28–31).

In vivo genomic footprinting (IVGF) and mutational analyses of the upstream region of murine MCP-1 have identified two regulatory regions, proximal and distal, that are separated by ~2.4 kb (10, 32). The proximal regulatory region contains three sites that became occupied upon TNF induction of MCP-1: a site with partial homology to a κB-binding site, termed κB-3, site B, and a GC box. The κB-3 site does not bind NF-κB family members (10). However, in the human gene, κB-3 has homology to an IFN-γ response element and was shown to be required for IFN-γ induction (23), suggesting that this region might display IFN-γ responsiveness in the mouse. Mutation of site B and κB-3 did not affect TNF induction; however, mutation of the GC box proved to be critical for regulation by both TNF and PDGF (33). We have recently found that both Sp1 and Sp3 can bind to this site in vitro (33), but it is not clear which of these factors function in vivo. Sp1 was one of the first sequence-specific eukaryotic transcription factors purified and cloned (34, 35). Sp1-binding sites (GGGCGGGG) are found in numerous genes and in genes lacking TATA boxes, suggesting...
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that it may provide a link to the recruitment of TFIID to TATAless promoters (36, 37). Sp1 has also been found to interact with NF-κB in vitro and in vivo (38–40). However, despite the number of genes containing Sp1 sites, little information is available for genes that “require” Sp1 for expression. To address this issue, Marin et al. (41) constructed ES cells that contained a targeted disruption of the Sp1 gene. This mutation proved to be an embryonic lethal when homozygous in a mouse, demonstrating a critical role for Sp1 in development. The second Sp1 allele was knocked out as well, creating an ES cell line with both genes disrupted. The only major change in gene expression observed in early Sp1+/− embryos was a decrease in the McCP-2 gene. McCP-2 encodes a protein that functions in the maintenance of gene silencing at regions of methylated DNA. All other genes examined were close to their normal levels of expression, suggesting some compensating mechanism for Sp1. The role of Sp1 in cytokine induced expression was not examined.

The MCP-1 distal regulatory region contains four elements as follows: site A, B-B1, B-B2, and the HS site. Site A is constitutively occupied irrespective of MCP-1 gene expression and is required for maximal induction by TNF (10). The HS site is sensitive to hypermethylation by DMS in cells treated with TNF. The proteins that interact with sites A and HS are currently unknown. The two B-B sites are essential for TNF induction and in murine fibroblasts bind p50/p65, NF-κB heterodimer (32). With the exception of site A, the induction of MCP-1 by TNF leads to the occupancy and therefore assembly of both distal and proximal regulatory regions by factors already present in the nucleus and those that are transported into the nucleus, such as NF-κB (10). The coordinate occupancy of both the distal and proximal regions by TNF suggests that interactions between the proteins that bind to these sites may occur that promote factor assembly and/or chromatin remodeling.

To investigate factor assembly of both the proximal and distal regions, it is important to identify the factor(s) that could bind to the GC box in vivo and to determine their role in promoter/regulatory region assembly. In this report, the Sp1+/− ES cells were differentiated into a fibroblast-like cell line and used to study the mechanism of TNF induction on MCP-1 in the absence of functional Sp1. Additionally, a Drosophila cell line system, which lacks endogenous Sp1, was used to determine the minimal mammalian components that were necessary for MCP-1 expression. The results show that Sp1 is required for maximal MCP-1 expression. In the absence of Sp1, assembly of the distal regulatory NF-κB region was drastically inhibited, suggesting communication between these two regions. Moreover, the minimal mammalian components necessary for expression in Drosophila cells included Sp1 and the p65 NF-κB subunit. Sp3, a member of the Sp1 family of DNA transcription factors and found to be expressed at normal levels in Sp1+/− cells, could substitute for Sp1 in the Drosophila system. Together, these results provide a genetic proof of the involvement of Sp1 in MCP-1 expression and suggest interactions between Sp1 and NF-κB that span 2.5 kb of DNA.

MATERIALS AND METHODS

Cell Lines and Culture—NIH3T3 and BALB/3T3 murine fibroblasts were obtained from the ATCC. Both of these cell lines respond to TNF induction with similar kinetics (10, 32). Fibroblasts were cultured in Dulbecco’s modified Eagle’s media supplemented with 10% bovine serum (HyClone, Inc., Logan, UT), 1 mM glutamine, and antibiotics. Cells were harvested 48 h post-transfection. In these assays, 20 μg of the indicated reporter was used with 10 μg of each of the indicated expression vectors (p65, Sp1, or Sp3) and 1 μg of the alkaline phosphatase reporter control. NF-κB p50 expression vector was added in the indicated amount. Promoterless CAT reporter plasmid DNA (pCATBasic) was added to bring the total amount of DNA transfected to 50 μg/assay.

In Vivo Genomic Footprinting (IVGF)—IVGF assays were done exactly as described (10, 32). For MCP-1, IVGF assays of the coding strand of the distal regulatory region and the noncoding strand for the proximal regulatory region are shown as these are the most informative strands. IVGF of the distal region and proximal regions were performed in BALB/3T3 cells using the JE promoter (32). Drosophila Schneider cells were transfected with 0.8 ml of DNA and 1 μg of pSV_AlkPhos, an alkaline phosphatase reporter vector (transfection efficiency control plasmid). Cultures were harvested at 48 h post-transfection after receiving TNF or media for the indicated time. BALB/3T3 cells were not used for transient transfections because of their poor transfection efficiency (10, 32, 42). Schneider cells were transfected by electroporation as above. Cultures were harvested 48 h post-transfection. In these assays, 20 μg of the MCP-1 reporter DNA and 1 μg of the coding strand of IP-10 gene were as follows: 5′-CAGCACTTTGGGTCTATGTTGC, 5′-GGATGTCTCTCAGCGGTG-3′, and 5′-AAGCGGTGATGACGGCGGTTG-3′, respectively.

Nuclear Extracts and EMSA—Nuclear extracts were prepared from BALB/3T3 or the Sp1−/− cell lines as described previously (10, 32, 33). In each case, 20, 10-cm plates were used. The standard DNA binding reaction contained 4 μg of nuclear extract, 0.6 μg of poly[dIC]poly[dIC], 250 ng of denatured sonicated salmon sperm DNA, 15 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.12 mM EDTA, 5 μg of bovine serum albumin, 12 mM dithiothreitol, 5 mM MgCl2, and the indicated amount of competitor DNA. Labeled probe was added after a 30-min incubation, and the reaction was then incubated for an additional 30 min on ice. The sequence of the coding strand of the MCP-1 GC box-specific probe used in the EMSAs was as follows: 5′-GACCACTTC-CTAGACTCCACCCCCTGCTGGTTAACA. Competitor DNA for consensus Sp1 and AP-1 sites were 5′-ATTACATGCGGGCGGCAG and 5′-GGCTTGATGCTGACTCGCCCGGA (Santa Cruz Biotechnology, Inc.; Valencia, CA), respectively, where the underlined bases represent the
consensus sites. The sequence for the α-b-binding site in the distal region used as a probe spanned α-2 and is 5’ACTGGCCCTCAAGATG-GGAATTTCCAGGCCTTATC. Non-specific DNA competitors encoded site A and site B of the MCP-1 gene are 5’AGAAGCTTGGTGGCA-GCCGATCTGAGGCCACTGTCGCA and 5’TGATGCTACCTGCATTG-CACCAAGCCCTTAC, respectively. NF-κB p65, p50, Sp1, and Sp3 antibody supershift assays were carried out by adding 1 μl of antibody (Santa Cruz Biotechnology, Inc.) to the reaction mixture 5 min prior to the addition of the labeled probe. Samples were separated by electrophoresis in a 5% polyacrylamide gel (49:1, acrylamide:bisacrylamide) at 4 °C with recirculating buffer and analyzed by autoradiography.

RESULTS

Creation of an Sp1-deficient Fibroblast Cell Line—Our previous work suggested a role for Sp1 in both TNF- and PDGF-induced MCP-1 expression (10, 32, 33). However, without direct genetic evidence, several important questions about the function of Sp1 in this system could not be answered. These included the following: is Sp1 essential for the expression of MCP-1; does Sp1 control assembly of the proximal regulatory region; and does Sp1 influence the assembly of the distal regulatory region? The creation of ES cells homozygous for an Sp1-targeted disruption provided an opportunity to obtain a genetic correlation between Sp1 and MCP-1 expression (41). Unfortunately, the Sp1+/− genotype resulted in an embryonic lethal. To obtain cell lines that could be used to link MCP-1 and Sp1, ES cells containing a homozygous disruption of the Sp1 gene were differentiated in culture. After supplementing the media with basic fibroblast growth factor and insulin, cells with a fibroblast-like appearance emerged and were passaged. These cells will be referred to as Sp1+/− cells.

To characterize the Sp1+/− cells, Sp1 expression was examined, and the ability of these cells to respond to TNF was examined by examining components of the TNF-signaling pathway. Both alleles of the Sp1 gene in the Sp1+/− cell line produce a truncated protein with a molecular mass of 65 kDa (41). The truncated Sp1 protein lacks the DNA binding and activation domains. A Western blot containing lysates prepared from wild-type NIH3T3 cells and Sp1+/− cells was stained with antisera to Sp1 (Fig. 1A). The results show the expected sizes, demonstrating that wild-type (90 kDa) Sp1 is not present in the Sp1+/− cell line.

During induction of gene expression by TNF, the NF-κB inhibitor IκB is phosphorylated, ubiquitinated, and degraded. This process allows NF-κB to translocate to the nucleus. To determine if this process is active in the Sp1+/− cells, the cells were treated with TNF, and a whole cell lysate was prepared and analyzed by Western blotting for the presence of IκB. After 10 min the presence of IκB is diminished, suggesting that the signaling pathway is intact (Fig. 1B). The rapid reappearance of IκB is also normal and is a result of TNF-induced expression of the IκB gene (43). Finally, the ability of NF-κB subunits to translocate to the nucleus following TNF treatment was examined. Nuclear extracts prepared from Sp1+/− cells showed that the nuclear concentration of NF-κB p50 and p65 increased following exposure to TNF (Fig. 1C). Moreover, the transcription factor Sp3, which is not known to be affected by TNF, showed no increase in nuclear concentration. Together, these data demonstrate that the TNF signaling pathway is intact and is functional in Sp1+/− cells.

MCP-1 Induction Is Severely Reduced in the Absence of Sp1—To determine if the loss of Sp1 affects the ability of MCP-1 to be induced by TNF, a Northern blot was carried out on wild-type BALB/S3T3 and Sp1+/− cells. As expected, in the wild type, MCP-1 was induced rapidly and maintained expression over the time course of the assay (Fig. 2). However, in the Sp1+/− cells, the levels of MCP-1 were severely compromised (Fig. 2). Thus, Sp1 is required for maximal expression of MCP-1. The low levels of expression suggest an Sp1-independent pathway or that another factor is substituting for Sp1. To determine if the loss of expression was due to Sp1, the Sp1 cDNA was cloned into a mammalian expression vector. This clone was transiently transfected into the Sp1+/− cells. After 36 h, the cells were treated with TNF. In comparison to wild-type NIH3T3 cells, MCP-1 induction by TNF was partially restored (data not shown), suggesting that the disruption of the Sp1 gene was the cause of the lack of MCP-1 induction by TNF.

Sp1 Is Required for Efficient Assembly of Both the Proximal and Distal Regulatory Regions—During TNF-induced expression, both the distal and proximal regulatory regions of the MCP-1 gene become occupied when examined by IVGF. In particular, the two distal κB sites show substantial changes in the footprint pattern, which are due to NF-κB binding (10, 32). In addition to the proximal GC box, site B at −72 is occupied as α-B-3 (10). To examine the ability of TNF to induce the assembly of both the proximal and distal regulatory regions of MCP-1, IVGF was performed on cells treated with TNF for 0.5 and 4 h (Fig. 3). Compared with the wild-type control lanes, a slight footprint was observed in the distal κB-binding sites at 0.5 h. A slight change in footprint was also observed at the proximal GC box, but no change was observed at site B. These results suggest that assembly of both the proximal and distal regulatory regions is impaired in the absence of Sp1. Moreover, the slight protection of the GC box suggests that another factor with GC box binding activity may substitute for Sp1 but not
Fig. 2. TNF induction of the MCP-1 requires Sp1. An autoradiograph of a Northern blot of RNA (12 μg/lane) isolated from Sp1−/− cells and BALB/3T3 cells probed with labeled MCP-1, and GAPDH cDNA is shown. Cells were treated with TNF for 0–4 h as indicated.

Fig. 3. Sp1−/− cells treated with TNF show reduced occupancy of both the proximal Sp1 region and the distal NF-κB region in vivo. DNA isolated from Sp1−/− cells or BALB/3T3 control cells treated with or without TNF for the times indicated were analyzed by IVGF. IVGF patterns of the most informative strands are shown as follows: for the proximal region the lower strand, and for the distal region, the upper strand. The DNA sequence with the wild-type pattern of IVGF protection in response to TNF is shown (10, 32). Open and solid circles show the sequences that became significantly occupied or became DMS-hypersensitive after TNF treatment in BALB/3T3 cells, respectively.

with high efficiency. The observed occupancy by IVGF provides an explanation for the low levels of MCP-1 mRNA that accumulate following TNF treatment in Sp1−/− cells.

The Lack of Sp1 Does Not Affect NF-κB Binding Activity or Sp3 Binding Activity—One explanation for the lack of activity might be that cells lacking Sp1 may have either lower levels of NF-κB or somehow the lack of Sp1 leads to decreased NF-κB binding activity. To determine if this may be the case, nuclear extracts prepared from BALB/3T3 cells and Sp1−/− cells treated with TNF were compared. Extracts from Sp1−/− cells treated with media instead of TNF were also prepared. EMSAs were performed on the nuclear extracts using the MCP-1 κB-2 sequence as a probe (Fig. 4A). This sequence has a high affinity for NF-κB (10, 32). Whereas many specific protein-DNA complexes form with this probe, complexes a and b appear only in extracts from cells treated with TNF. Complexes a and b appear only in extracts from cells treated with TNF.

Fig. 4. NF-κB and Sp3 bind DNA in Sp1−/− cells. Nuclear extracts were prepared from the indicated cell types following media control or TNF treatment (30 min). Four μg of extract was used in binding assay. A, TNF-activated NF-κB proteins bind the MCP-1 κB-2 site probe in EMSAs. Specific (SC) and nonspecific (NC) DNA competitors, κB-2 DNA or site B DNA, respectively, were added prior to the addition of the probe. Specific DNA protein complexes represented by bands a and b induced in TNF-treated cells were supershifted by NF-κB p50 and p65 antibodies, respectively, to bands c and d. Sp1 antibody did not supershift any of the complexes. B, Sp3 binds to the proximal AP-1/GC box oligonucleotide in EMSAs. Three bands, a–c, were detected in each of the extracts and were competed by specific but not nonspecific competitor, MCP-1 GC box DNA and site A DNA, respectively. Band b has similar mobilities in both Sp1−/− and NIH3T3 extracts and was supershifted by Sp3 antibody in each of the extracts. Band c has different mobilities in Sp1−/− cell extracts and NIH3T3 cell extracts. Band c is supershifted by antibody to Sp1 in NIH3T3 cells but not in Sp1−/− cells. A control antibody to c-Jun did not supershift any of the complexes.

To compare the binding activity of Sp3 in Sp1 wild-type and mutant cells, EMSAs were performed using the above extracts with the MCP-1 GC box probe (Fig. 4B). Three specific protein DNA complexes (bands a, b, and c) formed on the MCP-1 GC box. Two distinct mobilities were discerned for complex c, depending on whether the extracts were prepared from Sp1 wild-type or mutant cells. By using Sp1-specific antisera, antibody supershifted protein-DNA complexes were generated in wild-type but not Sp1 mutant cells (band d). The protein-DNA complex represented in band b is found in all cells and is
Supershifted by antisera to Sp3. These data demonstrate that Sp1 binding activity is not present in the Sp1/−/− cell type. Moreover, Sp3 binding activity is clearly present in both mutant and wild-type cells, suggesting that Sp3 may substitute for Sp1 in the mutant cell types.

The IP-10 Gene Is Induced by TNF in Sp1/−/− Cells—To determine if genes that do not contain an Sp1-binding site could be regulated by TNF in the Sp1/−/− cell line, the IP-10 gene which does not contain a GC box was examined after induction for RNA levels and occupancy of its xB-binding site(s). IP-10 is a chemokine that is regulated by both TNF and IFN-γ and is therefore a good candidate for this analysis. RT-PCR was carried out on RNA prepared from control and TNF-treated wild-type and Sp1/−/− cells (Fig. 5A). The results show only a small difference between Sp1 wild-type and mutant cells. To examine further the ability of NF-xB to function in this system, IVGF was carryed out on the coding strand of the IP-10 promoter (Fig. 5B). The IP-10 IVGF pattern has three points of reference. An AP-1 site at −81 is partially protected in both untreated and TNF-treated cells. The xB1 site is unoccupied in untreated cells and shows a sharp hypersensitive band at −114 and a slight decrease in the intensities of the G residues at −113 through −111 upon induction by TNF. At the xB2 site, the G at position −169 showed marginal protection after 30 min of TNF treatment. A similar pattern was observed in control and TNF-treated Sp1/−/− cells, further supporting the supposition that the Sp1/−/− cell line is responsive to TNF and that defects in NF-xB assembly at the MCP-1 gene are due to a lack of Sp1.

Sp1 or Sp3 Can Function with NF-xB to Activate Transcription of MCP-1—The accumulated data on MCP-1 gene regulation suggest that a minimal set of gene-specific transcription factors for the expression of the MCP-1 gene could consist of NF-xB and Sp1 (10, 32, 33, 44–46). To test this hypothesis, the Drosophila Schneider cell line was used as a model to demonstrate a role for Sp1 and NF-xB activity on a regulatory sequence (9, 39, 40, 48). Thus, to determine if these factors could be sufficient, a transcription reporter plasmid, driven by 2.6 kb of MCP-1 5′-flanking DNA fused to the CAT gene, was cotransfected into Schneider cells with plasmids expressing the p65 subunit of NF-xB and Sp1 (Fig. 6A). The wild-type MCP-1 vector contains both the proximal and distal regulatory regions. Whereas plasmids expressing Sp1 and p65 provided low levels of expression over background when introduced alone, a synergistic increase in expression was observed when both expression plasmids were introduced together. The synergistic effect was dependent on the presence of both xB-binding sites and the GC box, as mutations that scramble the sequence of these cis-acting elements had dramatic effects on transcription (Fig. 6C). Mutation of the HS sequence had no effect on expression of the reporter plasmid. This is in contrast to TNF-treated cells, where mutation of the HS element resulted in reduced expression (32), suggesting that the HS-DNA-binding protein is not present in Drosophila cells. Thus, this experiment suggests that Sp1 and p65 are the sufficient mammalian factors required for expression of MCP-1. Interestingly, the addition of an NF-xB p50 expression vector with p65 and the full MCP-1 reporter resulted in lower levels of transcription than when p50 was omitted (Fig. 6B). This is similar to previous observations in transfections in NIH3T3 cells (32), suggesting that the overexpression of p50 in cells may prevent activation of the MCP-1 gene. It is possible that the overexpression of p50 competes for binding to the MCP-1 xB sites and prevents access by p65.

To test the hypothesis that Sp3 could substitute for Sp1 in the regulation of MCP-1, an Sp3 expression vector was cotransfected into Schneider cells with the wild-type MCP-1 reporter (Fig. 7). Similar to Sp1 transfections, Sp3 was able to stimulate expression of the reporter gene in a synergistic manner with
NF-kB p65 (Fig. 7A). Also as above, mutations that destroyed the distal kB sites or the GC box resulted in reduced activity from the reporter (Fig. 7B), and a mutation in the HS element had no effect. NF-kB p50 also showed similar activity in repressing expression with p65 and Sp3. Thus, these data suggest that Sp3 can also synergize with NF-kB p65 to activate transcription of the MCP-1 gene. Moreover, these data support the hypothesis that Sp3 substitutes for Sp1 in the Sp1-/- cells in providing low levels of expression from the MCP-1 gene.

**DISCUSSION**

The data presented in this paper provide a genetic correlation between the expression and activity of Sp1 and MCP-1 gene regulation by the inflammatory cytokine TNF. This was demonstrated in two systems. The first utilized a fibroblast-like cell line that was derived from ES cells that contained disruptions of both of its Sp1 alleles. The second system employed the *Drosophila* tissue culture system that lacks Sp1 and NF-kB proteins. Each of these systems showed a dependence on Sp1 for activity. The data showed that Sp3 was able to substitute for Sp1 in the *Drosophila* cell lines and suggest that Sp3 may partially substitute for the lack of Sp1 in the Sp1-/- ES cells.

The finding that the lack of Sp1 resulted in a considerable loss of expression during TNF induction of MCP-1 was surprising considering that many genes thought to require Sp1 were not affected in the ES cells or in early Sp1-/- embryos (41). Even more surprising was the observation that the lack of Sp1 affected the in vivo footprint at the distal regulatory region, which is located ~2.4 kb upstream. This suggests that assembly of sequence-specific transcription factors on DNA regulating MCP-1 is coordinate. Previous studies examining the assembly of the distal and proximal regions in the presence and absence of the p50 and p65 NF-kB subunits showed that whereas the absence of p50 did not affect assembly in either region, p65 was required for assembly of the distal region. IVGF experiments conducted on the proximal GC box region in p65/-/- cells showed a moderate decrease in protection.2 Together these results suggest that the p65 and Sp1 may interact over the 2.5 kb of DNA separating their binding sites. NF-kB and Sp1 have been shown to interact in other systems, which include the HIV promoter (38–40) and VCAM-1 (48). But in each of these systems, the sites are in close proximity to each other. Close proximity could allow direct protein-protein interactions to occur between the DNA bound factors.

In the MCP-1 gene, Sp1 and NF-kB could also interact in a direct manner. The removal of the DNA between the distal and proximal regulatory regions had no effect on the overall level of activation in a transient transfection system (32), suggesting that there is no need to have the sites far apart. Interactions between the distal and proximal regions could occur through the looping of chromatin DNA. Another possibility is that Sp1 and NF-kB interact with a cofactor/coactivator that remodels chromatin, such as CBP/p300 or members of the SWI-SNF complex. NF-kB p65 has been shown by several groups to interact with CBP/p300 (49, 50). Because CBP/p300 have histone acetylase activity, this suggests that the recruitment of these factors could aid in the remodeling of chromatin encoding MCP-1. Nucleosome repositioning occurs in the HIV promoter upon activation and involves both NF-kB and Sp1 (51). It is possible that such repositioning by an Sp1-mediated event could translate upstream to the kB sites and allow binding and gene activation. Recently, several multiprotein coactivator complexes have been described that appear to link either Sp1 or NF-kB to the general transcription machinery have been described and termed CRSP and ARC (52–55). If active in the cells studied here, it is possible that these complexes may provide a physical connection between the proximal and distal regulatory regions of the MCP-1 gene.

The ability of Sp3 to activate or repress gene expression at GC boxes is not well resolved and appears to depend on the gene being assayed (56–58). Our data support a role for Sp3 in the activation of transcription. The ability of Sp3 to synergize with NF-kB to activate expression in the *Drosophila* system was indistinguishable from that of Sp1. However, Sp3 could not compensate completely for Sp1 in the regulation of MCP-1 by TNF in the Sp1-/- cells. Thus, differences in promoter composition are likely to play a large role in whether Sp1 or Sp3 is recruited or is able to activate expression.

Our data suggest that Sp1 may be required for the expression of other TNF-regulated genes. As stated above, genes that are activated by TNF use NF-kB as the primary activator of transcription. Many of these genes also have Sp1-binding sites located close to the start of transcription (59). Thus, it is reasonable to consider that in such genes NF-kB may require Sp1 to activate transcription or to aid in the repositioning/remodeling of chromatin. Such dependence of one activator on another may explain the lethality of Sp1-/- embryos, where the presence of one critical factor is missing. It is also possible that the observed effects of Sp1 on MCP-1 expression are unique to the distance between the Sp1 site and the kB sites. If this is the case, then we expect that analysis of other genes, such as the manganous superoxide dismutase gene, where the regulatory sites are also separated by several kB will have a similar dependence on Sp1, whereas genes that have compact kB/GC regulatory regions will not.

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