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TNF Regulates the In Vivo Occupancy of Both Distal and Proximal Regulatory Regions of the *MCP-1/JE* Gene

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Summary

In vivo genomic footprinting (IVGF) was used to examine regulatory site occupancy during the activation of the murine inflammatory response gene *MCP-1/JE* by TNF. In response to TNF, both promoter distal and proximal regulatory regions became occupied in vivo. EMSA analysis showed that while some of the factors involved in expression, including NF-κB, were translocated to the nucleus following TNF treatment, others were already present and able to bind DNA in vitro. Protein kinase inhibitor studies showed that protein phosphorylation was required for TNF activation but not factor assembly. These studies provide evidence for a multistep model of TNF-mediated gene regulation involving chromatin accessibility, transcription factor complex assembly, and protein phosphorylation.

Introduction

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine that plays an important role in inflammation (Beutler and Grau, 1993), immunomodulation (Schattner, 1994), cachexia (Cerami, 1992), septic shock (Cerami, 1992; Tracey and Cerami, 1994), diabetes (Hotamisligil and Spiegelman, 1994), as well as other biological processes (Sidhu and Bollon, 1993; Tracey and Cerami, 1993; Birkedal-Hansen, 1993). The induction of such diverse functions by TNF is mediated through two distinct surface receptors (Sidhu and Bollon, 1993), TNF-R1 (55 kDa) and TNF-R2 (75 kDa). Both TNF-R1 and TNF-R2 are type II transmembrane proteins having homologous extracellular TNF-binding domains but different cytoplasmic domains. The binding of TNF to these receptors results in receptor oligomerization and activation of several signaling pathways that include the sphingomyelin (Kolesnick and Golde, 1994) and phospholipid pathways (Sidhu and Bollon, 1993). Separately or cooperatively, these signaling pathways induce a wide spectrum of cell type–specific genes responsible for the pleiotropism of TNF (Sidhu and Bollon, 1993; Gordon et al., 1992). Such genes include transcription factors (Kronke et al., 1992), cytokines (Kronke et al., 1992), cell surface receptors, and oxidative damage repair enzymes (Wong and Goeddel, 1988; Visner et al., 1990). Additionally, TNF modulates cellular immune responses by regulating genes involved in antigen processing, such as HLA class I and class II genes (Kronke et al., 1992).

The molecular mechanisms by which TNF activates expression of genes in a cell type–specific manner are unknown. One mechanism that is shared among many TNF-induced genes involves the activation and translocation to the nucleus of the transcription factor NF-κB. Once in the nucleus, NF-κB presumably binds its respective *cis*-acting regulatory element and activates expression (Schutze et al., 1992; Siebenlist et al., 1994; Mackman, 1995; Schindler and Baichwal, 1994; Ito et al., 1994; Collins et al., 1995; Krikos et al., 1992; Osborn et al., 1989; Mukaida et al., 1990). However, experiments involving the TNF induction of several genes indicate that NF-κB does not function alone but rather forms cooperative interactions with other factors, which are required for expression (Mackman, 1995; Collins et al., 1995). Some models propose that NF-κB-mediated gene expression involves the formation of a multiprotein complex termed an enhanceosome (Thanos and Maniatis, 1995b; Collins et al., 1995). Because these models are based on in vitro experimentation, the actual assembly of factors and their dependence on NF-κB has yet to be determined in vivo.

Human monocyte chemoattractant protein-1 (MCP-1) and its murine homolog, encoded by the *JE* gene, are small basic proteins that specifically attract and activate monocytes to sites of inflammation (Rollins, 1991). MCP-1/JE belongs to the CC chemokine family (Baggiolini and Dahinden, 1994) and functions in normal wound healing, the pathogenesis of atherosclerosis (Schwartz et al., 1991), the inflammatory process (Baggiolini and Dahinden, 1994), and modulation of tumor immunity (Mantovani et al., 1993). *MCP-1/JE* is transcriptionally induced immediately after exposure to TNF (Gordon et al., 1992; Hanazawa et al., 1993; Satriano et al., 1993). Transient transfection of the wild-type and mutant human *MCP-1/JE* reporter constructions have indicated that two promoter distal κB sites are important for TNF induction (Ueda et al., 1994). Other studies have suggested that TNF-mediated induction of the murine *MCP-1/JE* gene involves a promoter proximal AP-1 site (Hanazawa et al., 1993, 1994). A recent report identified an additional distal NF-κB binding site in the murine *MCP-1/JE* gene and showed that this site could bind NF-κB and activate expression of a heterologous promoter (Freter et al., 1995). Taken together, these studies suggest that the TNF regulation of *MCP-1/JE* may also require the formation of multiprotein complexes containing NF-κB.

Because models of TNF-regulated gene expression have been based on in vitro DNA binding protocols, the in vivo state of the regulatory regions of TNF-induced genes is not known. To examine the assembly of factors in response to TNF induction, in vivo genomic footprinting (IVGF) of the murine *MCP-1/JE* upstream regions that correlated with TNF induction was performed. IVGF provides a snapshot of the occupancy of the regulatory sites of a gene before and during exposure to TNF, and thus can potentially identify sites that are truly involved in expression. To correlate *MCP-1/JE* expression levels and transcription factor DNA binding activities with the IVGF analysis, Northern blot analysis and electrophoretic mobility shift assays (EMSAs) were performed. We found that in untreated cells, guanines in

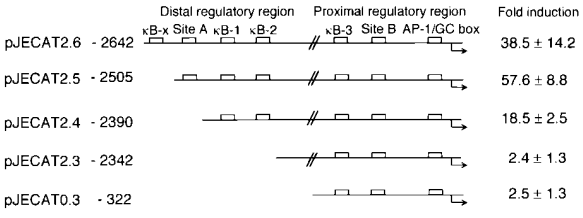


Figure 1. Induction of the *MCP-1/JE* Gene by TNF Requires the Distal Regulatory Region

Schematic maps of the *MCP-1/JE* upstream DNA sequence with the indicated *cis*-acting control elements and the *MCP-1/JE*–CAT reporter constructions are shown. Transient transfections of each plasmid were carried out as described in the materials and methods. CAT protein concentration is plotted as the fold induction for each construction of the TNF-treated sample divided by the untreated control sample. The standard deviation from the mean is presented. The results of Student's t test comparisons between samples were as follows: pJECAT2.6 and pJECAT2.5, $p = 0.0188$; pJECAT2.6 and pJECAT2.4, $p = 0.0068$; pJECAT2.5 and pJECAT2.4, $P < 0.0001$; and pJECAT2.3 and pJECAT0.3, $p = 0.8441$.

the majority of the regulatory regions were sensitive to dimethyl sulfate (DMS) methylation, indicating that these sites were unoccupied by DNA-binding factors. These methylation-sensitive regions included sites that bind factors that were readily detected in the nuclear extracts by EMSA. Upon TNF treatment, sites encoding the two distal κB sites, a proximal GC box, and two new sites became resistant to DMS methylation, and therefore represent specific regions that were occupied by DNA-binding proteins. Treatment of cells with cycloheximide (CHX) or pyrrolidinedithiocarbamate (PDTC, an inhibitor of NF-κB activation), revealed differential binding and separated the functions of the distal and proximal regulatory regions, suggesting that full induction may require interactions between these two regions. Inhibition of cellular protein kinase activity with H7 blocked TNF induction of *MCP-1/JE* but did not prevent the TNF-induced binding of transcription factors to any of the sites examined, suggesting that at least one of the regulatory factors requires phosphorylation for transcriptional activation but not binding. Together, these studies begin to outline the components of a complex regulatory system for the inflammatory gene *MCP-1/JE* and suggest a model of TNF-mediated gene induction that involves the global induction of transcription factor occupancy that may apply to other TNF-regulated genes. Thus, the ability of TNF to induce expression in a cell type–specific fashion may depend on the availability of regulatory sites, the profile of nuclear DNA-binding factors, and the ability to kinase one or more transcription factors.

Results

Identification of TNF-Response Elements in the Murine *MCP-1/JE* Gene

Sequence analysis and studies from other groups have revealed several DNA-binding consensus elements that could function in the TNF control of the murine *MCP-1/JE* gene (Figure 1) (Rollins et al., 1988; Timmers et al., 1990; Ueda et al., 1994; Freter et al., 1995). As with the human *MCP-1/JE* gene (Ueda et al., 1994), the region between −2390 and −2342 (the distal regulatory region) of the murine gene contains two κB sites, termed κB-1 and κB-2 (Figure 1). An additional κB site, (κB-x), which was identified by Freter et al. (1995), is located in the region between −2527 and −2502. The region between κB-x and κB-1 contains two *cis* elements, termed elements II and III (Figure 1, not shown), that function in the platelet-derived growth factor induction of *MCP-1/JE* (Freter et al., 1995). Site A (Figure 1) contains element III. The proximal regulatory region contains a third κB site, (κB-3), an AP-1 binding site, a GC box, and a TATA box (Figure 1).

To identify the TNF-response elements of the murine *MCP-1/JE* gene, a 2724 bp fragment (−2642 to +82 with respect to the *MCP-1/JE* start of transcription) was amplified by polymerase chain reaction (PCR) of BALB/3T3 mouse DNA and fused to the coding region of the chloramphenicol acetyltransferase (*CAT*) gene. The resulting reporter construct was termed pJECAT2.6. A 38-fold increase in the concentration of CAT protein was observed following the transient transfection of pJECAT2.6 into NIH 3T3 cells that were treated with TNF versus control transfectants (Figure 1). Therefore, this 2724 bp fragment contains TNF-response elements. To delineate further the region required for TNF inducibility, four 5′ deletion constructs were generated having 2505 bp (pJECAT2.5), 2390 bp (pJECAT2.4), 2342 bp (pJECAT2.3), and 322 bp (pJECAT0.3) of upstream sequence. Transient transfection experiments were again carried out (Figure 1) and showed that the region between −2505 and −2342 was required for TNF-mediated induction (Figure 1). The region between −322 and −1 (the proximal regulatory region) was only sufficient to maintain a low level of transcriptional activity in the presence of TNF. A negative element may reside between −2642 and −2505, as TNF-induced expression increased following its deletion; however, the overall effect of this site was less than 2-fold. Together, these data suggest a complex regulatory mechanism involving both distal and proximal regulatory regions. However, the above transient transfection experiments did not determine whether all of these sites function together or are actually used during induction by TNF.

Both the Proximal and Distal Regulatory Regions Become Occupied by Transcription Factors Following Treatment by TNF

To determine the in vivo role of each of these sites in the expression of *MCP-1/JE*, IVGF for both the proximal and distal regulatory regions of the *MCP-1/JE* gene was carried out in the presence and absence of TNF. To correlate the level of *MCP-1/JE* mRNA expression with the IVGF, RNA blot analysis was performed. In control untreated BALB/3T3 cells, the basal level of *MCP-1/JE* mRNA is barely detectable by RNA analysis (Figure 2A). In these control cells, IVGF showed that with the exception of site A (-2437 and −2417), which showed both DMS-resistant and DMS-hypersensitive sites, no differences between the in vitro and the in vivo methylation protection pattern could be discerned (Figures 2B and 2C; lanes V and 0). Thus, while site A appears to be

occupied prior to TNF treatment, the distal and proximal regulatory regions are unoccupied when *MCP-1/JE* expression is low. Interestingly, site A was found previously to be important in platelet-derived growth factor–regulated expression of *MCP-1/JE* (Freter et al., 1995).

As previously described, the addition of TNF to fibroblasts induces the expression of *MCP-1/JE* dramatically and immediately (Gordon et al., 1992). Maximal levels of *MCP-1/JE* mRNA were achieved at 4 hr following TNF addition and remained at this level through 24 hr of TNF treatment (Figure 2A). Corresponding directly to the TNF induction of *MCP-1/JE* mRNA, the binding of factors in vivo to both the distal and proximal regulatory regions of *MCP-1/JE* was observed (Figures 2B and 2C). After 30 min of TNF treatment, full occupancy of both κB-1 and κB-2 sites was observed. This occupancy is defined by both DMS-resistant and DMS-hypersensitive sites. The DMS protection pattern is consistent through 4 hr of TNF treatment; however, at 24 hr, there is a decrease in protection at some of the bases within the region (G^{-2378}, G^{-2377}, G^{-2376}, G^{-2352}, G^{-2351} of the coding strand, and G^{-2370}, G^{-2369}, G^{-2344}, G^{-2343} of the noncoding strand) (Figure 2B). The full protection of κB-1 and -2 sites induced by TNF suggests that TNF treatment of cells initiates the binding of transcription factors in all or most of the cells within the culture. The decrease in protection at some base pairs within the distal κB sites following extensive treatment with TNF may suggest dissociation of factors from the sites in some of the cells within the culture. This binding pattern correlates with the TNF-mediated activation profile of NF-κB for other genes in which the highest activity of in vitro NF-κB binding was observed after 30 min of TNF treatment, followed by a gradual reduction in binding activity during prolonged TNF treatment (Thompson et al., 1995). Thus, these data support the hypothesis that these sites bind NF-κB in vivo.

TNF treatment also results in the induction of DMS-hypersensitive sites in the distal regulatory region. The DMS-hypersensitive sites are located in the center of κB-2 (G^{-2350} of the coding strand), and between κB-1 and κB-2 (A^{-2357} and G^{-2356} of the coding strand, and T^{-2365}, G^{-2364}, A^{-2363}, G^{-2361}, and G^{-2360} of the noncoding strand; Figure 2B). Hypersensitivity of these positions to DMS implies that TNF-induced occupancy results in conformational changes in the DNA that may be due to DNA bending, the binding of additional factors, or both (Spana and Corces, 1990).

The proximal regulatory region was also occupied at several sites after 30 min of TNF treatment. Protection was observed at the GC box, the 5′ end of the κB-3 site, G^{-50} bp of the AP-1 site, and a novel site located at −74 to −72 bp (termed site B). These sites remained protected after 24 hr of TNF treatment (Figure 2C). Notably, the protection pattern at the κB-3 site was distinct from that of the distal κB-1 and κB-2 sites in that only a single base pair, G^{-154}, was protected at the κB-3 site. In contrast, the guanine residues at the 5′ and 3′ ends of both κB-1 and κB-2 sites were protected. Additionally, G^{-154} was fully protected after 24 hr of TNF treatment, whereas full protection at κB-1 and κB-2 sites was lost. Because the other protected base pair in the region of κB-3 is G^{-156}, which is located outside of the κB-3 site, it is likely that the κB-3 site binds a factor other than NF-κB. The TNF-mediated occupancy of both the distal and proximal regulatory regions suggests that prior to induction either the DNA-binding factors are not present in the nucleus, the DNA is inaccessible to the DNA-binding proteins, or that the assembly of factors requires the induction or modification of one or more factors. It is also possible that all of these mechanisms are involved in controlling factor occupancy.

TNF-Mediated Binding of Transcription Factors to the Distal Regulatory Region Is Partially Inhibited by CHX, Whereas the Binding of Transcription Factors to the Proximal Regulatory Region Is Induced by CHX

To determine whether the binding of factors to the TNF-response elements requires de novo protein synthesis, CHX was added to the cells 2 hr prior to TNF treatment. Incubation of cells with CHX alone increased the basal level of the *MCP-1/JE* mRNA by 36-fold. Subsequent TNF treatment increased the overall induction of *MCP-1/JE* an additional 2-fold over control cells (Figure 3A).

As determined by IVGF, treatment of cells with CHX alone did not result in the induction of factor binding to the distal regulatory region (Figure 3B, lanes V, 1, and 3). Treatment of cells with CHX prior to addition of TNF abolished TNF-induced factor binding to κB-1 and partially inhibited TNF-induced factor binding to κB-2. The intensity of DMS-hypersensitive sites in the distal regulatory region was also decreased (Figure 3B, lanes 2 and 4). Thus, the decrease in binding activity of the distal κB sites in cells treated with CHX and TNF correlates with the lowered fold-induction of mRNA, suggesting that full activity of the distal regulatory region may require the synthesis of a labile factor, whose activity is lost after 2 hr of CHX treatment. Additionally, the responses to CHX treatment were different between κB-1 and κB-2, implying that these sites may bind either different NF-κB family members or complexes. In contrast, CHX treatment did result in an IVGF pattern in the proximal regulatory region that was similar to that observed with TNF treatment (Figure 3C), suggesting that the proximal sites are likely to be responsible for the observed increase in basal expression.

Inhibition of Cellular Protein Kinase Activity Results in Loss of Transcription of *MCP-1/JE*, but Not in the Loss of Regulatory Region Occupancy

H7 is a general serine/threonine protein kinase inhibitor that blocks signal transduction through protein kinase C, protein kinase A, and other similar kinases. Previously, H7 has been shown to inhibit the induction of *MCP-1/JE* by TNF (Hanazawa et al., 1993). However, in other studies TNF-induced activation and nuclear translocation of NF-κB proteins was not influenced by H7 addition (Meichle et al., 1990). To determine whether transcription factor assembly on DNA was affected by the absence of protein kinase activity, IVGF was carried out in the presence of H7. As shown by RNA blot analysis in Figure 4A, H7 inhibited TNF-mediated induction of *MCP-1/JE* mRNA when added 1 hr prior to TNF treatment. This effect does not appear to be general, as the

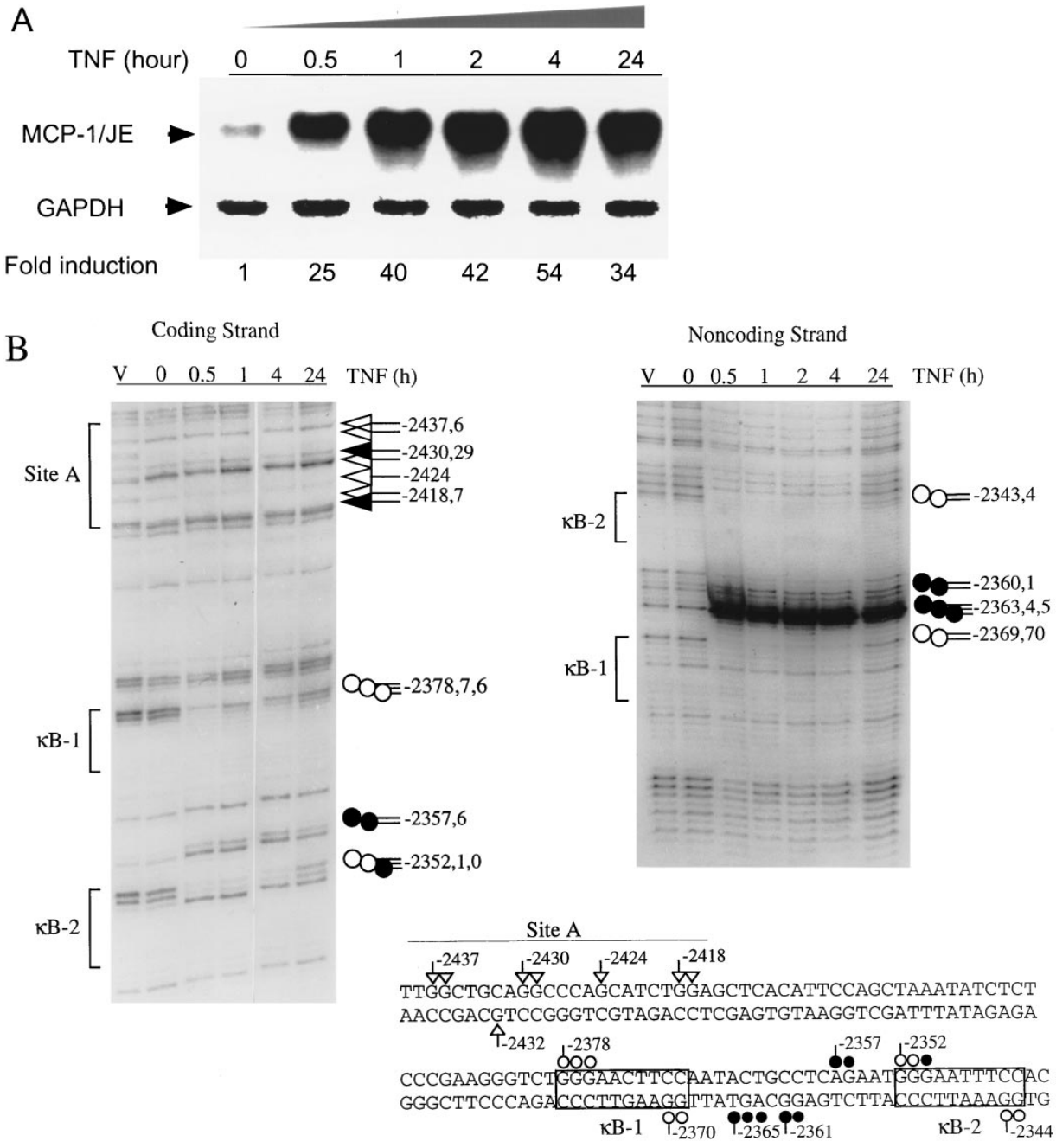
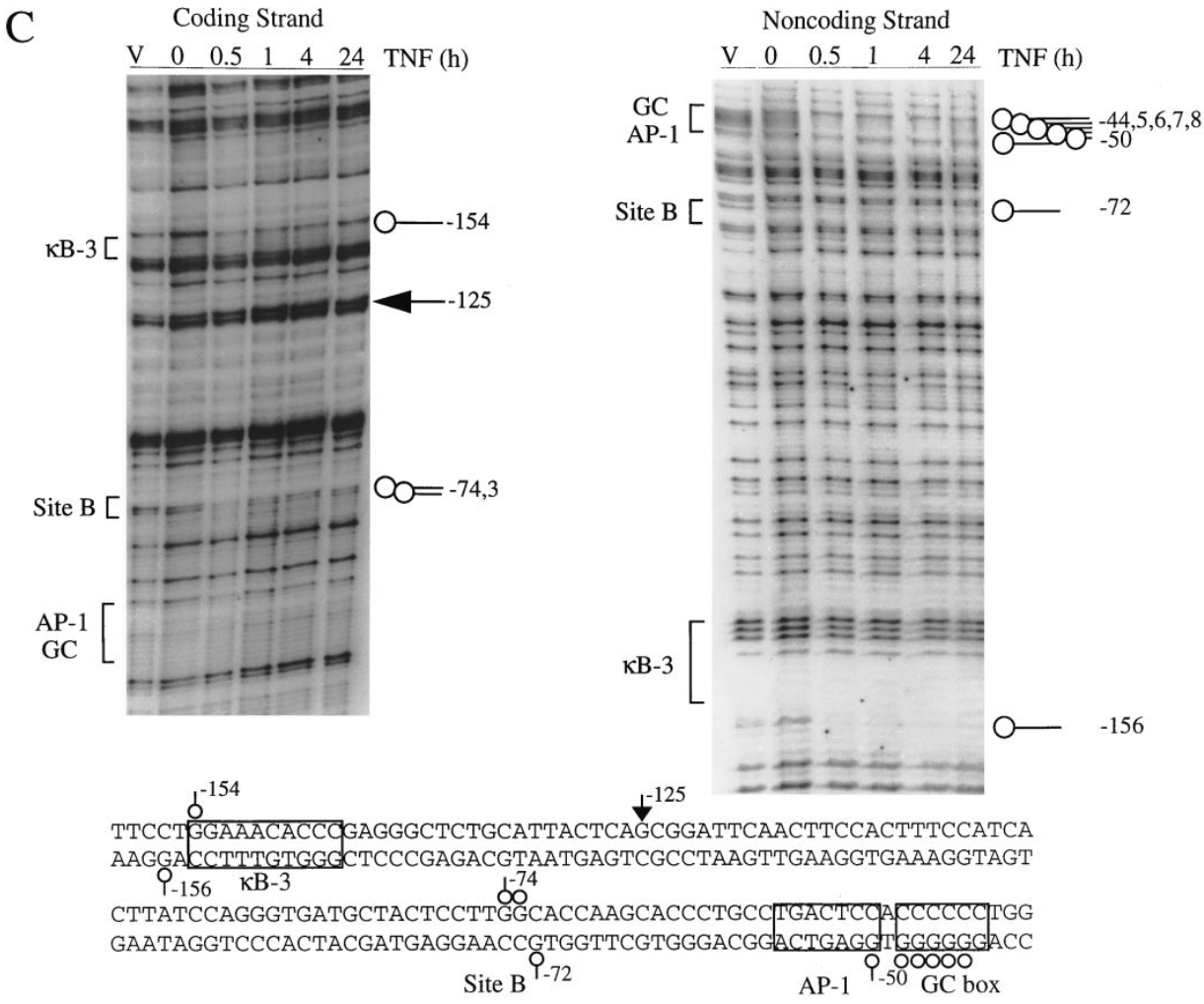


Figure 2. Induction of *MCP-1/JE* by TNF Results in the Immediate Binding of Transcription Factors to Both the Distal and Proximal Regulatory Regions

Northern blot analyses (A) of RNA prepared from BALB/3T3 fibroblasts after TNF treatment for the indicated time are shown with two probes, *MCP-1/JE* and *GAPDH* (control). IVGF carried out for the *MCP-1/JE* gene on cells treated with TNF for the indicated times are shown for the distal (B) and the proximal (C) regulatory regions. Lanes marked with V indicate in vitro methylated DNA and lanes marked by 0 represent the samples from control or untreated cells. Arrows indicate the constitutive changes from in vitro DNA that result in the DMS-resistant bases (open) and DMS-hypersensitive bases (closed). The circles indicate the TNF-induced binding of transcription factors that results in the DMS-resistant bases (open) and DMS-hypersensitive bases (closed). Brackets on the left side of the sequences delineate the binding sites as described in the text and Figure 1. A sequence summary labeled as above is presented.

(*Figure 2 continued top of next page*)



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RNA levels for the glyceraldehyde phosphate dehydrogenase gene (*GAPDH*) control was not affected by H7 treatment. Interestingly, IVGF analysis showed that H7 did not prevent the TNF-induced binding of transcriptional factors to any of the sites described above (Figures 4B and 4C). These data indicate that the simple binding of factors to their regulatory sites is not sufficient for activation of *MCP-1/JE* gene expression and suggest that one or more of the factors that interact with the *MCP-1/JE* regulatory regions require modification by protein kinases.

PDTC Inhibits the Occupancy of the Noncoding Strand of the Distal κB Sites

The role of NF-κB in TNF-mediated induction was examined by treatment of cells with PDTC. PDTC is an antioxidant that blocks the activation of NF-κB through the inhibition of IκB protein degradation (Thompson et al., 1995; Schreck et al., 1992; Henkel et al., 1993). Interestingly, in comparison to control cells, incubation of cells with PDTC alone resulted in a 5-fold increase in the level of *MCP-1/JE* mRNA. The combined treatment of cells with PDTC and TNF resulted in only an additional 3-fold induction over PDTC treatment alone (Figure 4A). While IVGF of cells treated with PDTC alone showed no protection at κB-1 and κB-2 sites, the DMS hypersensitivity at G^{-2350} of the coding strand and T^{-2365}, G^{-2364}, A^{-2363} of the noncoding strand was observed (Figure 4B). Following PDTC and TNF treatment, IVGF showed that the noncoding strand within the κB sites of the distal element remained sensitive to DMS, whereas the coding strand became partially resistant to DMS, suggesting some but not complete factor occupancy. In contrast, IVGF of cells treated with PDTC alone (Figure 4C) showed full protection at the proximal regulatory region (G^{-50} of AP-1 binding site, GC box, G bases upstream of κB-3 site, and site B). Further treatment with TNF did not result in alteration of the IVGF pattern. These data suggest that the proximal sites were utilized during PDTC induction of *MCP-1/JE*, and that further induction by TNF is aided by the distal elements.

Previous studies have shown that the binding of NF-κB (p50/p65) to κB sites is asymmetric in that the p50 subunit preferentially interacts with the 5′ half site and

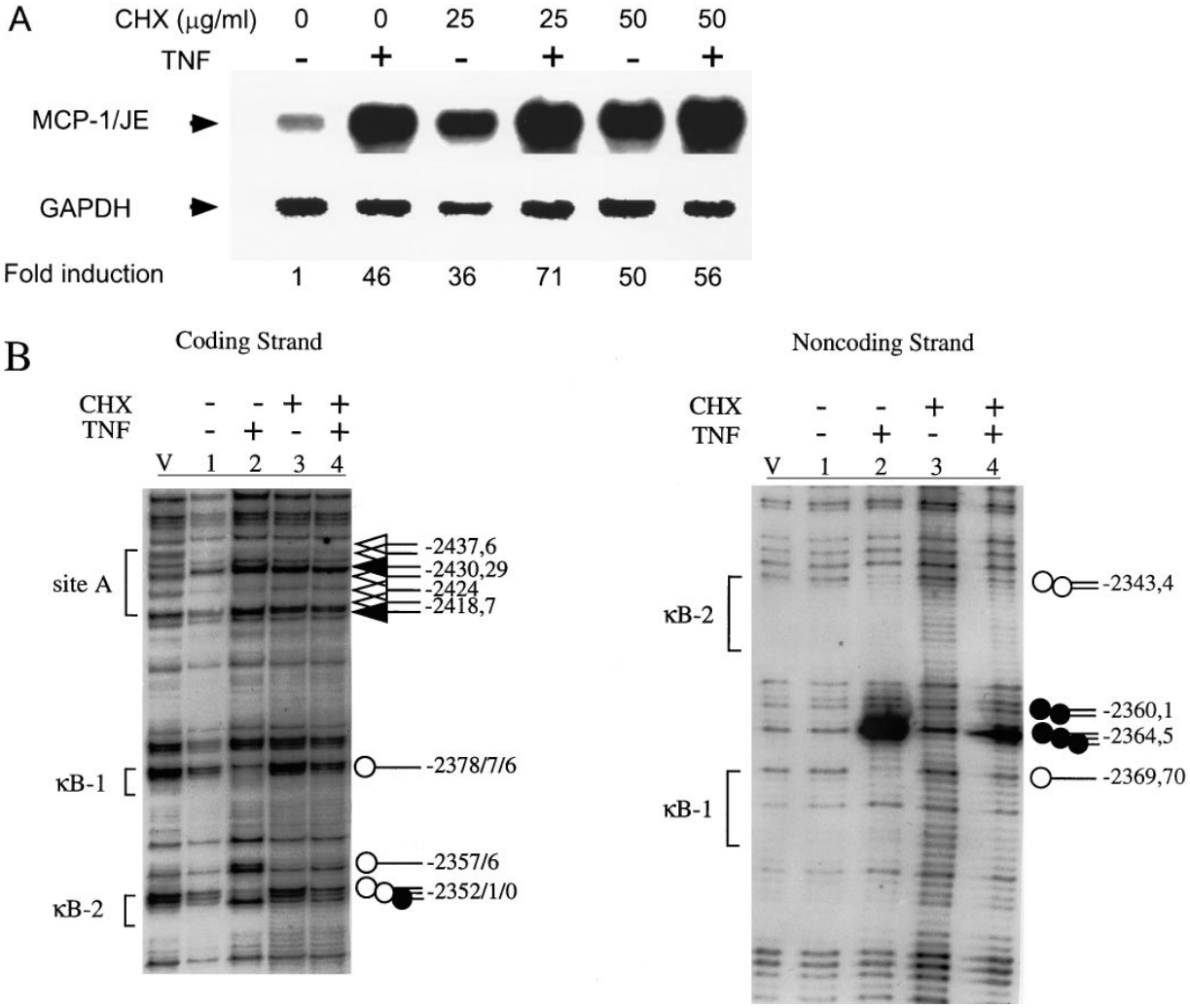


Figure 3. CHX Induces Basal Expression of *MCP-1/JE*, Induces Occupancy of the Proximal Regulatory Region, and Affects the Assembly of Factors at the Distal Regulatory Region

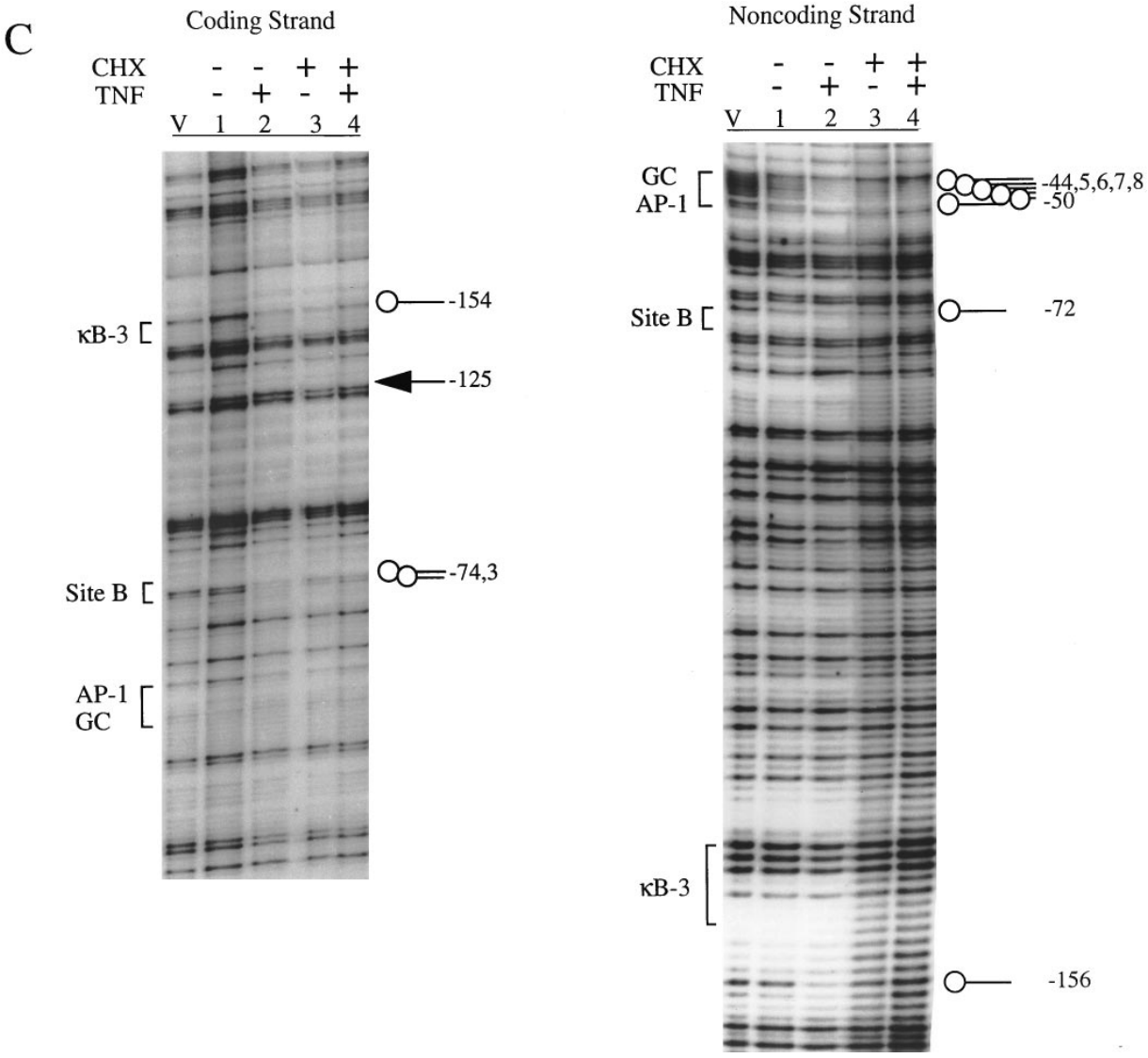
A Northern blot (A) of RNA prepared from cells treated with cycloheximide and TNF is shown. CHX was added to the cells 2 hr prior to a 2 hr TNF treatment. IVGF assays of the distal (B) and proximal (C) regulatory regions are shown. In vitro methylated genomic DNA (V), DNA from untreated cells (lane 1), and DNA from cells treated with CHX, TNF, or both, for 30 min are as indicated. Arrows and circles are as described in Figure 2.

(*Figure 3 continued on top of next page*)

p65 subunit preferentially interacts with the 3′ half site (Thanos and Maniatis, 1995a; Urban et al., 1991). If this is the case for the homologous κB-1 and κB-2 sites here, then p65 binding (noncoding strand) was inhibited by PDTC, whereas p50 binding (coding strand) was not. Alternatively, the absence of high affinity NF-κB proteins due to PDTC may either allow lower affinity family members that are not inhibited by PDTC or other non-NF-κB factors to bind to the distal κB sites.

TNF-Induced Formation of Protein–DNA Complexes in the Distal Regulatory Region Involves Both NF-κB p50 and p65

To investigate further the roles of individual TNF-response elements in the expression of *MCP-1/JE*, EMSAs were performed using oligonucleotide probes containing either the distal κB sites, the combined AP-1/GC box, the κB-3 site, or site B. EMSA analysis using a probe encoding both distal κB sites and nuclear extracts prepared from untreated and TNF-treated BALB/3T3 cells revealed three TNF-inducible protein–DNA complexes, complexes a, b, and c (Figure 5A, lanes 6 and 7). The TNF-inducible protein–DNA complexes were also detected in the nuclear extracts from cells that were treated with CHX or H7 prior to TNF treatment (Figure 5B, lanes 5 and 6) but were not detected in nuclear extracts from cells treated with PDTC and TNF (Figure 5B). All three complexes were specific for the distal κB probe, as specific but not nonspecific DNA competitors were able to compete with the probe for DNA binding activity. Antisera directed towards members of the c-Rel



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family of transcription factors (p50, p65, p52, c-Rel, and Rel-B) were used in EMSA supershift assays to determine whether the complexes were composed of known members of the c-Rel family. Complexes a and c were specifically supershifted by antisera to either p50 or p65, producing complexes d and e, respectively. This suggests that the κB sites within this region can bind p50/p50, p65/p65, or p50/p65 dimers (Figure 5C). Because the probe encodes two κB sites, it is possible that complex a represents binding to one of the two κB sites and complex c represents binding to both sites. Complex b is supershifted by p65 antibody only, suggesting that it contains p65/p65 homodimers (Figure 5C). Thus, the combined in vitro and in vivo data suggest strongly that NF-κB is involved in the TNF-mediated regulation of *MCP-1/JE*, and that binding to the distal κB sites occurs following the translocation of that NFκB to the nucleus upon TNF treatment.

The Transcription Factors that Occupy the Combined AP-1/GC Box Site, κB-3 Site, and Site B Are Present in the Nucleus Prior to TNF Treatment

Using the combined AP-1/GC box site as a probe, two specific protein–DNA complexes, complexes a and b, were detected using nuclear extracts prepared from either control, untreated cells, or TNF-treated cells (Figure 6A, lanes 1 and 6, respectively). The same complexes were detected using nuclear extracts from cells that were treated with CHX, H7, or PDTC (Figure 6B). Therefore, the transcription factors that bind to the combined AP-1/GC box site were present in the nucleus prior to TNF treatment. By using antisera specific for c-Fos, c-Jun, SP-1, and ATF-4, complex b was shown to contain SP-1 protein (Figure 6C, lane 4). However, neither supershift nor the loss of the complex a or b was observed when antisera specific for c-Fos or c-Jun was

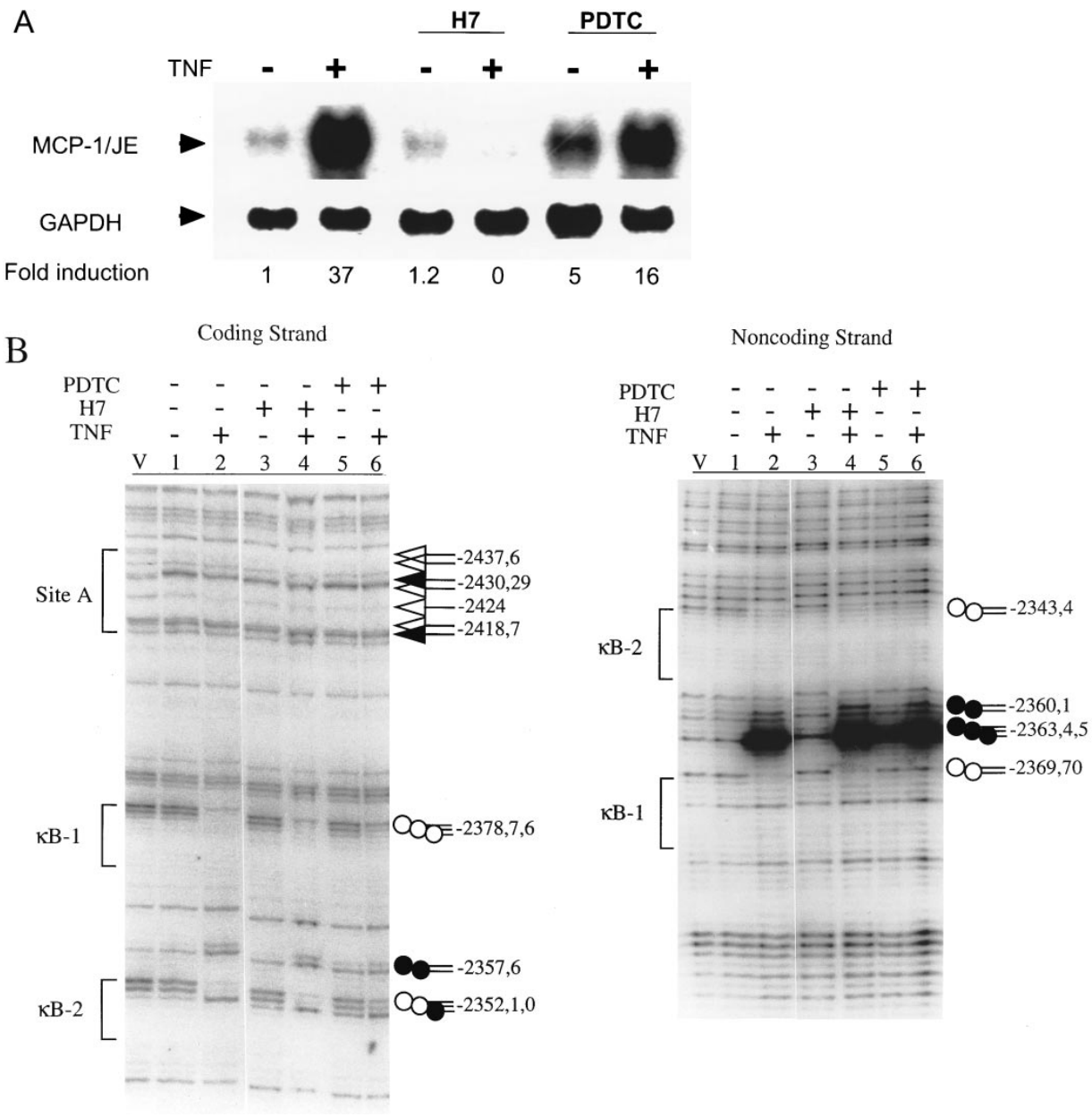


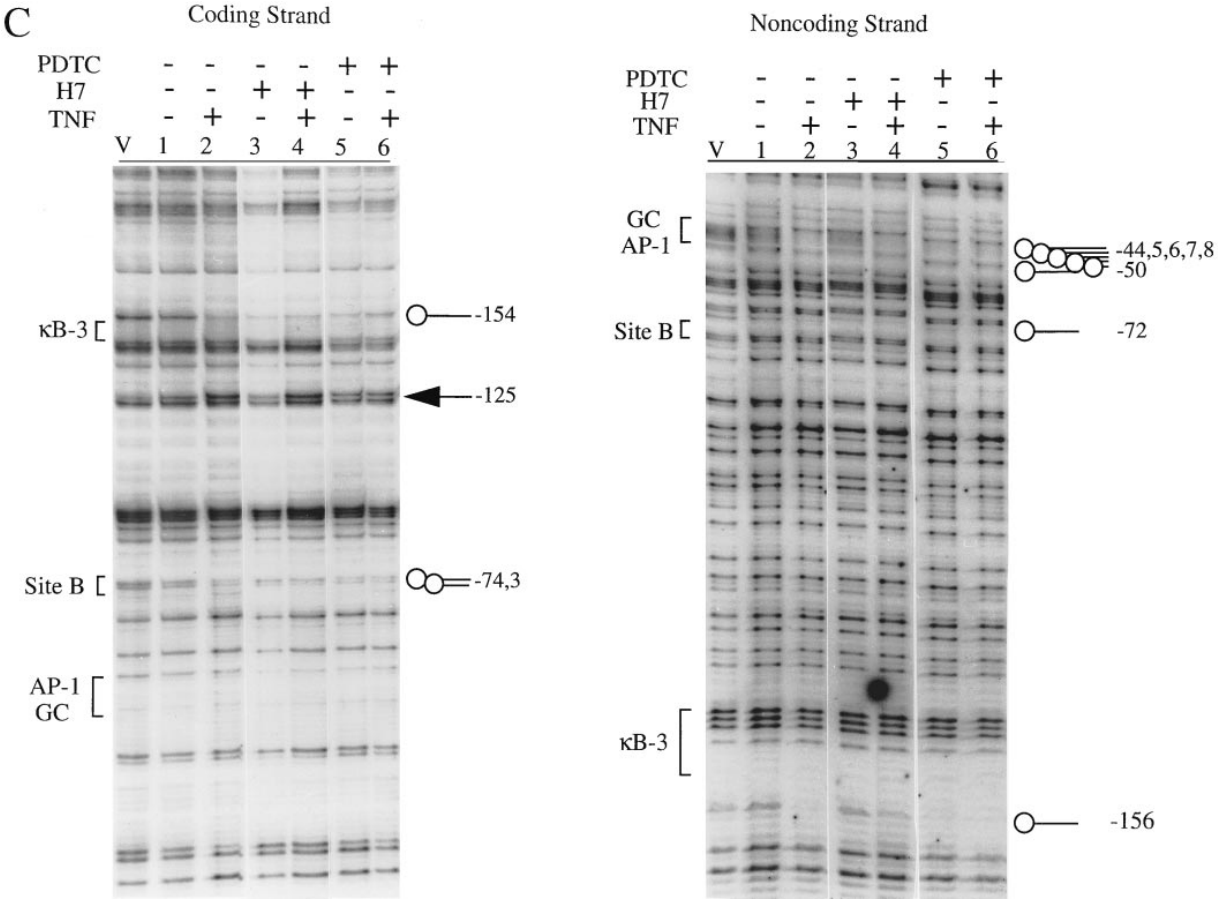
Figure 4. H7 and PDTC Affect the TNF Induction of *MCP-1/JE* but Have Different Consequences on the Occupancy of the Regulatory Regions

Northern blot analysis (A) of TNF-induced *MCP-1/JE* mRNA level in cells incubated in the presence and absence of 0.1 mM H7 or 0.1 mM PDTC for 60 and 90 min, respectively, prior to the addition of TNF. TNF treatment was for 2 hr. IVGF assays of the distal (B) and proximal (C) regulatory regions are shown. In vitro methylated genomic DNA (V), DNA from untreated cells (lane 1), and DNA from cells treated with H7, PDTC, or TNF for 30 min are as indicated. Arrows and circles are as described in Figure 2.

(*Figure 4 continued on top of next page*)

added. Also, no competition was observed when an authentic AP-1 oligonucleotide was added as a specific competitor in these EMSAs (Figures 6A and 6B). These data suggest that the combined AP-1/GC box site functions as a SP-1 site and not an AP-1 site.

Regardless of whether the nuclear extracts were prepared from control, untreated cells, or TNF-treated cells, a protein–DNA complex was detected in EMSAs using probes containing κB-3 or site B (Figures 7A and 7B). The same protein–DNA complex was also observed using nuclear extracts from cells that were treated with CHX, H7, or PDTC (Figures 7A and 7B). These experiments indicate that the transcription factors that bind to κB-3 and site B are already present in the nucleus prior to TNF treatment. To determine whether κB-3 binds NF-κB, DNA encoding κB-1 and κB-2 was used as competitor DNA in the EMSA. No competition was observed (data not shown). Additionally, antisera specific for



(*Figure 4 continued*)

members of the c-Rel family of transcription factors (p50, p65, p52, c-Rel, and Rel-B), the c-Fos, c-Jun, SP-1, CREB, and others were used in EMSA supershift assays but had no effect on the EMSA pattern (data not shown). Thus, the κB-3-binding factor remains to be identified.

The combined EMSA analysis demonstrates that the proximal regulatory region DNA-binding proteins are present in the nucleus prior to TNF treatment and are able to bind in vitro, but not in vivo. This also implies that protein modification is not required for factor binding and site occupancy.

Discussion

The above analyses demonstrate the involvement of many sites and factors controlling the regulation of the *MCP-1/JE* gene by TNF. IVGF, which provides a snapshot of the sites utilized in vivo, revealed that TNF induces the binding of both induced factors, such as NF-κB, as well as factors already present in the nucleus. Only site A was occupied prior to TNF treatment. The induced occupancy of both proximal and distal sites suggests that at least one or possibly two multiprotein complexes or enhanceosomes are assembled in response to TNF to regulate the *MCP-1/JE* gene. The composition and roles of each of the regulatory regions are discussed below.

The Proximal Regulatory Region Controls the Basal Transcriptional Activity of Murine *MCP-1/JE* Gene

Three potential regulatory sites were identified in the proximal regulatory region of murine *MCP-1/JE* gene: the combined AP-1/GC box site, site B, and κB-3. Factors binding to site B and κB-3 are currently uncharacterized and their roles in the expression of *MCP-1/JE* are under investigation. The *MCP-1/JE* AP-1 site (TGA CTCC), which is conserved in the human (Ueda et al., 1994) and rat (Timmers et al., 1990) *MCP-1/JE* genes, differs from the consensus AP-1 sequence (TGACTCA) by 1 bp (Timmers et al., 1990). A role for the AP-1 site in the TNF and transforming growth factor β regulation of murine *MCP-1/JE* expression has been suggested by studies that showed decreases in *MCP-1/JE* expression when either antisense DNAs targeting c-Jun or c-Fos (Hanazawa et al., 1993; Takeshita et al., 1995) or inhibitors of c-Jun or c-Fos activities were used (Hanazawa et al., 1994). Other studies have suggested that the adjacent GC box of the human *MCP-1/JE* gene is responsible for the maintenance of basal transcriptional activity of *MCP-1/JE* (Ueda et al., 1994). The combined IVGF and EMSA data for the GC box are consistent with a role for SP-1 in TNF-mediated expression. The lack of occupancy at this site in untreated cells is consistent with the low level of expression observed by Northern blot

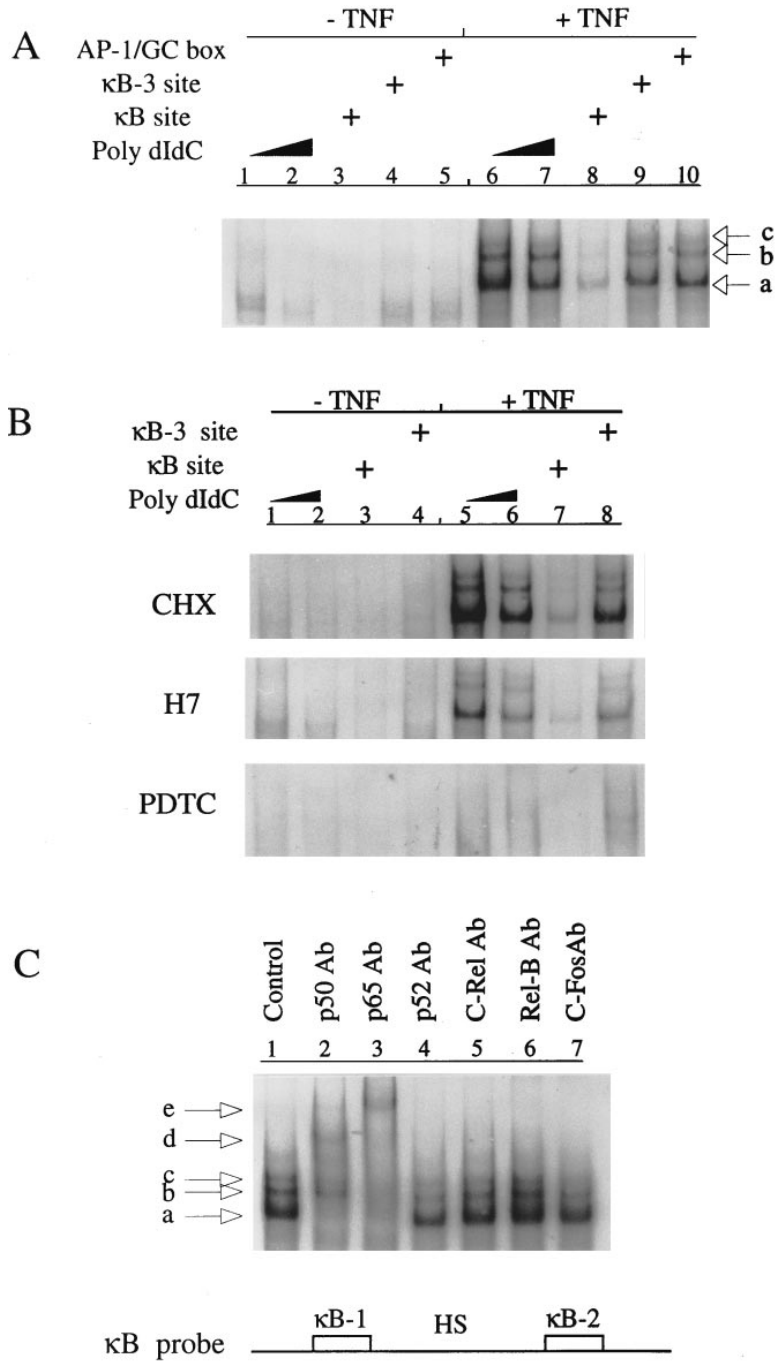


Figure 5. TNF-Activated NF-κB p50 and p65 Bind to the Distal κB Sites in EMSAs

EMSAs were carried out on nuclear extracts prepared from control cells (−TNF) and cells treated with TNF for 30 min (+TNF). The probe, which contains both κB-1 and κB-2 sites and was generated by PCR (−2331 to −2390), is schematically shown at the bottom of the figure.

(A) Three protein–DNA complexes, a, b, and c, are indicated. DNA competitors (35 ng each), unlabeled κB DNA, κB-3 DNA, and the AP-1/GC box DNA were added 5 min prior to the addition of probe. Poly (dIdC):poly (dIdC) (0.625 μg) was included in all reactions except lanes 2 and 7, which contained 1.3 μg.

(B) EMSAs were performed on nuclear extracts prepared from cells that were pretreated with CHX (25 mg/ml), H7 (0.1 mM), PDTC (0.1 mM), or TNF as above.

(C) EMSAs of nuclear extracts prepared from TNF-treated (30 min) and untreated cells were carried out in the presence of the indicated κB-reactive antisera. Complexes a, b, and c are the same as for (A), whereas d and e indicate complexes that are supershifted by p50- and p65-specific antisera, respectively.

analysis, as factor occupancy in a small percentage of the cells in culture would not be detected by IVGF. A recent study has shown interactions between NF-κB and SP-1 elements (Pazin et al., 1996). If such interactions occurred in the *MCP-1/JE* gene, this would serve to link the distal and proximal regulatory regions.

The role of the AP-1 site is not as clear. Although additional base pairs within the AP-1 site were occasionally protected following TNF treatment, this observation was not consistent. Furthermore, although control EMSAs showed that AP-1 was present in the cells (data not shown), EMSA analysis and antibody supershift assays using the combined AP-1/GC box as a probe showed no clear binding of AP-1. Interestingly, a similar IVGF analysis performed on a C3H-derived fibroblast cell line, C3HA, showed full protection of both the AP-1 and SP-1 sites (data not shown). This result may suggest cell line differences in the role of AP-1 in regulating *MCP-1/JE*.

Although EMSAs showed that factors that bind to the proximal sites were present in the nucleus of untreated cells, the level of *MCP-1/JE* mRNA in untreated cells was low. However, after cells were treated with CHX or PDTC, a substantial increase in *MCP-1/JE* mRNA level was detected without formation of new or enhanced

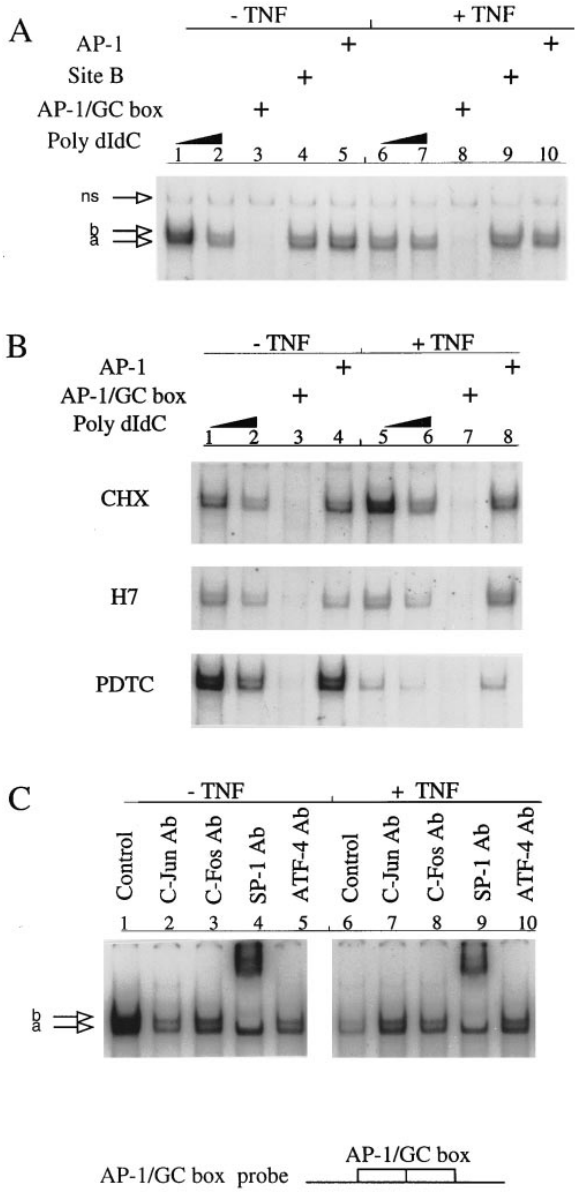


Figure 6. The AP-1/GC Box Site Binds the Transcription Factor SP-1, Which Is Present in the Nucleus Prior to TNF Treatment

Oligonucleotides encoding the AP-1/GC box (base pairs −66 to −33) were used in EMSAs prepared from cells as described in Figure 5.

(A and B) Two specific protein–DNA complexes, a and b, identified in the nuclear extracts from both TNF-treated and untreated cells are indicated. The protein–DNA complex designated as ns is nonspecific. DNA competitors included DNA containing a consensus AP-1 site (TGACTCA), site B DNA, and unlabeled probe DNA as indicated.

(C) EMSAs of nuclear extracts prepared from TNF-treated (30 min) and untreated cells were carried out in the presence of the indicated antisera show that complex B reacts with SP-1 antisera.

protein–DNA complexes as determined in vitro by EMSA. This apparent inconsistency was resolved by IVGF analysis. While control cells showed no protein occupancy in the proximal regulatory region, treatment with CHX or PDTC resulted in full occupancy of the proximal regulatory region that was similar to the occupancy observed upon TNF treatment. Thus, treatment of cells with CHX, PDTC, or TNF either initiates post-translational modification processes that activate the transcription factors, alter the chromatin structure of the *MCP-1/JE* gene from an inactive ground state to a derepressed state (Paranjape et al., 1994), or both, to initiate regulatory site occupancy and transcription.

TNF Induction of the Murine *MCP-1/JE* Gene Involves the Assembly of a Multicomponent Complex in the Distal Regulatory Region

Compared with the identified κB sequences (Miyamoto and Verma, 1995), the κB-2 site is identical to the κB sites found in the IκBα gene, and κB-1 has a one base mismatch with the consensus sequence (GGGRNNY-YCC). Since TNF induces the binding of transcription factors to both sites with kinetics similar to the activation profile of NF-κB proteins by TNF (Thompson et al., 1995), it is likely that both sites are functional NF-κB binding sites in vivo. Using the κB sites as a probe in EMSAs, the TNF-inducible protein–DNA complexes were shown to contain p50 and p65. However, in the experiments reported here it was not possible to determine which NF-κB complex (e.g., p65/p65 or p65/p50) was bound at each of the sites.

IVGF of cells treated with TNF showed that TNF induces not only the protection of the κB-1 and κB-2 sites but also the appearance of DMS-hypersensitive base pairs between the two κB sites. DMS-hypersensitive sites indicate changes in the conformation of the DNA that could be due to DNA bending. The presence of both protected and DMS-hypersensitive bases over this region suggests the formation of a multiprotein complex similar to that described for the interferon β (IFNβ) (Thanos et al., 1993) and endothelial cell adhesion molecule (Collins et al., 1995) genes. The distal regulatory complex involves the NF-κB (or κB-like) proteins that bind to both κB sites and an additional unknown transcription factor(s). One possible additional factor may be the HMG-I/Y. HMG-I/Y is a minor groove DNA-binding protein that bends DNA upon binding (Grosschedl et al., 1994). HMG-I/Y has been found to stabilize the binding and assembly of multicomponent protein–DNA complexes that include NF-κB and ATF-2 (Thanos et al., 1993; John et al., 1995; Lewis et al., 1994). The κB-2 site contains an A–T-rich region in its center that is similar to the κB/HMG-I/Y motif found in the IFNβ enhancer (Thanos and Maniatis, 1992). It is likely that NF-κB p50 and p65 are not involved directly in the formation of the hypersensitive site as treatment with PDTC and TNF, which prevented NF-κB nuclear translocation in this cell line, did not prevent formation of the hypersensitive site. Moreover, the partial protection of κB-2 following PDTC and TNF treatment may suggest that other c-rel family members that are not affected by PDTC are activated in response to TNF and function in controlling *MCP-1/JE*. Alternatively, the absence of the NF-κB factors following PDTC treatment may allow the binding of secondary factors.

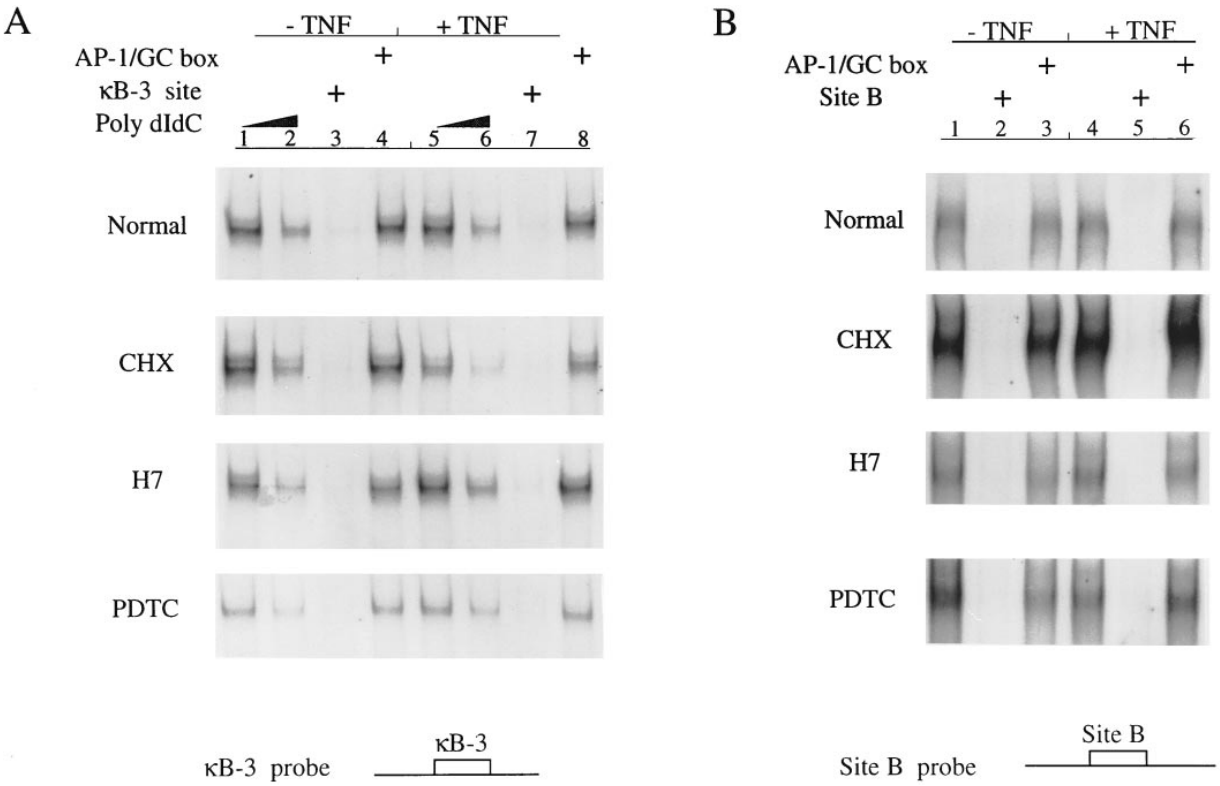


Figure 7. The κB-3 Site and Site B Bind to Factors that Are Present in the Nucleus Prior to TNF Treatment

EMSAs were carried out as in Figure 5. The probe containing (A) κB-3 site (−135 to −169) or (B) site B (−59 to −88) was generated from annealing complimentary oligos as described in Experimental Procedures.

Protein Synthesis and Posttranslational Processes Are Required for Full TNF-Mediated Induction of *MCP-1/JE*

Treatment of cells with CHX and the protein kinase inhibitor H7 provided important clues to the mechanisms of *MCP-1/JE* regulation. Although CHX treatment alone had been reported to activate NF-κB proteins (Henkel et al., 1993), this was not observed in either the in vitro EMSA or in vivo IVGF assays. CHX did not prevent the TNF-mediated activation of p50 or p65 as determined by in vitro EMSA; however, IVGF analyses of cells treated with both CHX and TNF showed that κB-1 was not protected, whereas κB-2 showed only slight protection when cells were pretreated with CHX for 2 hr prior to TNF treatment. The strong hypersensitive site is reduced considerably under these conditions as well. Thus, it appears as though CHX treatment results in the loss of a factor required for complete occupancy of the distal κB sites. The fact that TNF still superinduces the expression of *MCP-1/JE* in the presence of CHX suggests that this induction may either occur at the proximal regulatory region, which is fully occupied, or that the partially protected distal region is sufficient to allow the 2-fold increase in expression. Another contributing factor is the possibility that CHX treatment extends the half-life of the *MCP-1/JE* mRNA, which under normal circumstances is extremely short (unpublished data), thereby increasing steady-state mRNA levels without increasing the transcription rate.

H7, a serine/threonine protein kinase inhibitor, has been shown to inhibit the TNF induction of *MCP-1/JE* and other genes (Hanazawa et al., 1993; Mattila et al., 1992; Cao et al., 1992; Montgomery et al., 1991). In BALB/3T3 cells, H7 inhibits the TNF induction of *MCP-1/JE* in a dose-dependent manner with no inhibition at 5 mM and 100% inhibition at 60 mM (data not shown). H7 (100 mM) was used in these experiments to ensure that the footprinting data fully reflect the effects of H7. However, even at 100 mM H7, the TNF-induced binding of transcription factors to both the distal and the proximal regulatory regions was not inhibited. Therefore, TNF-initiated signal transduction pathways that lead to the binding of transcription factors to their regulatory sites in the *MCP-1/JE* gene do not require a phosphorylation step. Despite the fact that occupancy of both regulatory regions was complete, transcription was not activated. These data suggest that at least one of the factors required for activation of the *MCP-1/JE* gene by TNF requires phosphorylation.

These studies begin to define a bipartite regulatory system for *MCP-1/JE* with the distal regulatory region required for TNF-induced expression and the proximal regulatory region required for all aspects of expression, including the response to cellular stress (Figure 8). The above analysis also suggests several levels in which cell-type specific responses to TNF may be controlled. These levels include the accessibility of the regulatory regions to factors required for expression, the release

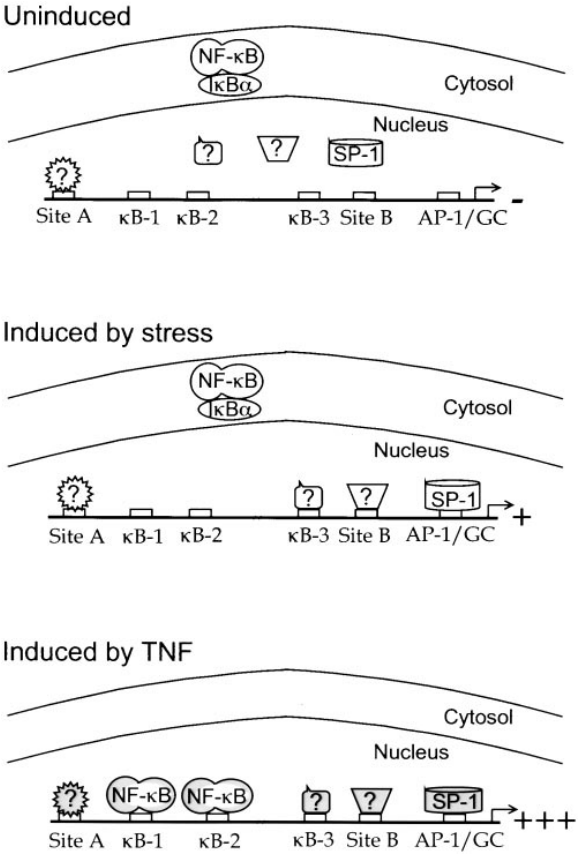


Figure 8. Model of TNF-Induced Regulation of the Murine *MCP-1/JE* Gene

In the uninduced state, factors at site A are bound to the DNA and the other factors are either in the nucleus or cytoplasm but are not bound to DNA. Expression during stress, such as when protein synthesis is inhibited, involves the assembly of nuclear factors and activation through the proximal control region, potentially with factors at site A. Induction by TNF involves the translocation to the nucleus of NF-κB, the assembly of all the factors on the DNA, and the activation of one or more of the factors by phosphorylation as indicated by the shading.

and translocation to the nucleus of transcription factors held in the cytoplasm, the assembly of both induced and "resident" nuclear transcription factors into a multiprotein complex, and finally the activation of some of the factors by a kinase. Cells unable to comply with any of these steps would therefore be unable to activate that gene in response to TNF.

Experimental Procedures

Cells and Cell Culture

BALB/3T3 clone A31 (American Type Culture Collection, CCL-163) and NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's media supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% calf serum (Intergen, Incorporated). Human recombinant TNF (Creasey et al., 1987; Wang et al., 1985) was provided by Chiron Corporation. Cells were grown to 90%–95% confluence and then treated with 500 U/ml TNF for the indicated time period. CHX, PDTC, and H7 were purchased from Sigma, Incorporated, and added 2 hr, 1.5 hr, and 1 hr, respectively, prior to TNF.

DNA Constructions and Transient Transfection Assays

The DNA fragments for the six *MCP-1/JE* promoter fusion reporter constructions shown in Figure 1 were generated from BALB/3T3 genomic DNA by PCR amplification using Pful polymerase and the following primer sets: pJECAT2.6, 5′-ACGTTCCATTGTCCATCTG GGT-3′ and 5′-GGTGGTGGAGGAAGAGAGAG-3′; pJECAT2.5, 5′-CA AGCCAGAGCTCAGACTAGGCCT-3′ and 5′-GGTGGTGGAGGAAGA GAGAG-3′; pJECAT2.4, 5′-CCGAAGGGTCTGGGAACTT-3′ and 5′-GGTGGTGGAGGAAGAGAGAG-3′; pJECAT2.3, 5′-ACGCTCTTATC CTACTCTGCCTC-3′ and 5′-GGTGGTGGAGGAAGAGAGAG-3′; pJECAT0.3, 5′-CAGTGAAAGCAGAGCCACTCCATTCAC-3′ and 5′-GGTGGTGGAGGAAGAGAGAG-3′. Amplified DNA fragments were subcloned into pCATBasic vector DNA that was linearized with XbaI and filled in with the Klenow fragment of DNA polymerase. Transient transfections were carried out by electroporation of 20 μg of DNA as previously described (Riley and Boss, 1993) using approximately 5×10^6 cells/assay. TNF (500 U) was added at 36 hr posttransfection, and extracts were prepared from cells 12 hr later as described previously (Riley and Boss, 1993). CAT protein expression was assayed using the CAT–ELISA kit supplied by Boerhinger Mannheim, Incorporated. All constructions were assayed at least three times. The data from these assays were averaged and analyzed for statistical significance using the Student's t test.

IVGF

In vivo methylation of cellular DNA and DNA preparation were performed as described in Mueller et al. (1994). The ligation-mediated PCR for footprinting was carried out according to the procedure of Mueller et al. (1994) with minor modification. Primer fill-in and amplification reactions were carried out using Vent Polymerase (New England Biolabs, Incorporated) with 0.4 mM each of dATP, dCTP, dGTP, and dTTP in the buffer recommended by the vendor. Ligation of the common linker (Mueller et al., 1994) was carried out overnight at 16°C using 3 U of T4 DNA ligase (GIBCO BRL) in the buffer provided by the manufacturer. The distal regulatory region and the proximal regulatory region were each analyzed by one set of upper-strand and one set of lower-strand primers. The sequences of the coding-strand primers for the distal regulatory region are as follows: JENFκB3-1, 5′-TCACCATTGCAAAGTGAATTGGC-3′; JENFκB3-2, 5′-AGTTAGCACAGGAGGCAGCGCAA-3′; and JENFκB3-3, 5′-CACAGGAGGCAGCGCAAATGTGAATCA-3′. The sequences of the noncoding-strand primers for the distal regulatory region are as follows: JENFκB5-1, 5′-CAAGCCAGAGCTCAGACTAG GCCT-3′; JENFκB5-2, 5′-TCAGATTCTCCGGCCCATGAGAGA-3′; and JENFκB5-3, 5′-CTCCGGCCCATGAGAGAACTGCTTGG-3′. The sequences of the coding-strand primers for the proximal regulatory region are as follows: JE3-1, 5′-AACTGTGAACAGCAGGCCCAGAA-3′; JE3-2, 5′-AGAAGCATGACAGGGACCTGCATGGT-3′; and JE3-3, 5′-GACAGGGACCTGCATGGTGGTGGAGGA-3′. The sequences of the noncoding-strand primers for the proximal regulatory region are as follows: JE5-7, 5′-CCCCCTACTCCCTGCGCA-3′; JE5-8, 5′-TCATTTGCTCCCAGGAGTGGCTAG-3′; and JE5-9, 5′-GCTCCCAG GAGTGGCTAGAAAAATACCA-3′.

Nuclear Extracts and EMSAs

Nuclear extracts were prepared from ~10^8 cells according to the procedures of Shapiro et al. (1988) and as we have previously described (Hasegawa et al., 1991, 1993). DNA binding assays were carried out on ice and the probes were incubated with ~2 μg nuclear extract in the appropriate reaction buffer for 30 min prior to loading on a 5% polyacrylamide gel. Electrophoresis was carried out at 4°C with recirculating buffer (Hasegawa et al., 1991). The reaction buffer contained 15 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.12 mM EDTA, 5 μg BSA, 1 mM dithiothreitol (DTT), and 5 mM $MgCl_2$. To assay for NF-κB binding activity, NP-40 (0.05%), and GTP (3 mM) were added to the above reaction buffer. The final concentration of DTT in reactions that assayed for AP-1/GC box binding activities was 12 mM. Antibody supershift assays were carried out by adding 1 μl of antibody (Santa Cruz Biotechnology, Incorporated) to the reaction mixture 5 min prior to the addition of the labeled probe.

The sequences of the coding strand of DNAs used in EMSAs were as follows: κB, 5′-CCCGAAGGGTCTGGGAACTTCCAATACTGCCT CAGAATGGGAATTTCCACGCTCTTATCC; AP-1/GC box, 5′-GCACC

CTGCCTGACTCCACCCCCCTGGCTTACAA; κB-3, 5′-TCTCTTCCA CTTCCTGGAAACACCCGAGGCTCTG; site B, 5′-TGATGCTACTC CTTGGCACCAAGCACCCTG; and AP-1, 5′-CTAGTGATGAGTCAGC CGGAT.

RNA Analysis

RNA was prepared from one 10 cm plate of cells grown to near confluence using the NP-40 lysis method as described (Sambrook et al., 1989; Gordon et al., 1992). RNA blot analysis was carried out under standard conditions using formaldehyde gels (Gordon et al., 1992). Approximately 5 μg of RNA was loaded in each lane. cDNA probes were labelled by a random primed DNA labeling kit (Boehringer Mannheim, Incorporated), and hybridization was carried out at 68°C in QuikHyb (Stratagene, Incorporated) for 1 hr. Filters were washed three times for 20 min each at 68°C in 2× SSC, 1× SSC, and 0.5× SSC, together with 0.1% SDS (1× SSC contained 0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.0]). Northern blots were exposed to phosphorimaging screens and the data were analyzed on a Molecular Dynamics PhosphorImager. *GAPDH* was used as an internal standard to normalize total mRNA in each lane.

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