Transcriptional Regulation of The Human Monocyte Chemoattractant Protein-1 Gene

COOPERATION OF TWO NF-κB SITES AND NF-κB/Rel SUBUNIT SPECIFICITY

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Human monocyte chemoattractant protein-1 (human MCP-1) mRNA accumulated in THP-1 cells 2 h after lipopolysaccharide (LPS) stimulation. DNase I footprinting revealed that LPS stimulation induced protein binding to the two closely located NF-κB sites, A1 and A2. By electrophoretic gel mobility shift assay and supershift assay, the binding of (p65)2, c-Rel/p65, p50/p65, and p50/κB to the A2 oligonucleotide probe was detected after LPS stimulation. In contrast, 12-o-tetradecanoylphorbol 13-acetate did not induce a significant amount of MCP-1 mRNA in THP-1 cells 2 h after stimulation, and only p50/p65 bound to the A2 probe. Activity of each NF-κB/Rel dimer was investigated by transfecting P19 cells with p65, p50, and/or κB expression vectors, and a luciferase construct containing the enhancer region of the human MCP-1 gene. Expression of recombinant p65 or p65 and c-Rel resulted in elevated luciferase activities, indicating that (p65)2 and c-Rel/p65 had trans-activity. The binding of (p65)2 and κB or c-Rel/p65 to the A2 probe was also detected from 12-o-tetradecanoylphorbol 13-acetate-stimulated HeLa, HOS, and A172 cells in which expression of MCP-1 mRNA was elevated. Finally, the role of the A1 site was investigated. Both (p65)2 and c-Rel/p65 bound to the A1 probe by electrophoretic mobility shift assay and a mutation in the A1 or A2 site resulted in a loss of the enhancer activity. These results suggest that the binding of (p65)2 and c-Rel/p65 to the A1 and A2 sites of this gene is important for the tissue- and stimulus-specific transcription of the human MCP-1 gene.

Blood monocytes infiltrate into the sites of inflammation and play major roles in host defense through their ability to present antigens and to produce various mediators. Although the mechanisms of monocyte infiltration have not been fully understood, locally produced monocyte chemotactants seem to be responsible for the recruitment of blood monocytes into the sites of inflammatory reactions.

Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC subfamily of the chemokine family and attracts blood monocytes both in vitro and in vivo (1–3). MCP-1 mRNA or protein was detected at high levels in the lesions of several diseases such as atherosclerosis (4, 5), arthritis (6), idiopathic pulmonary fibrosis (7, 8), and various tumors (9–11), strongly suggesting that MCP-1 plays a critical role in the recruitment of monocytes in these diseases. A wide variety of cells, including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells, produces MCP-1 in vitro in response to various stimuli such as lipopolysaccharide (LPS), interleukin-1 (IL-1), tumor necrosis factor-α (TNFa), platelet-derived growth factor (PDGF), IFN-γ, or 12-o-tetradecanoylphorbol 13-acetate (TPA) (1–3). However, the mechanisms of MCP-1 production remain unknown.

To understand the mechanisms involved in the expression of human MCP-1 mRNA in different types of cells at the molecular level, we previously investigated the transcription of human MCP-1 gene in TPA-, IL-1β, and TNFα-stimulated human malignant glioma cell line A172 cells (12). The basal level of human MCP-1 mRNA was detected in A172 cells without any stimulus, and the expression of human MCP-1 mRNA was strongly enhanced after stimulation with these stimuli. The binding of Sp1 to the proximal GC box located between bp −64 and −59 was critical for the maintenance of the basal transcription of this gene. Two NF-κB sites (A1 and A2 sites) were located 2.6 kilobases upstream of the transcription initiation site. Mutations in the A2 sequence resulted in a loss of enhancer activity, indicating that the A2 site was critical for the enhancer activity after stimulation. The role of the A1 site was not investigated in the previous study.

The mouse homologue of human MCP-1, termed JE, was cloned from PDGF-stimulated BALB/c 3T3 cells as one of early response genes (13, 14). The transcriptional mechanisms of the mouse MCP-1/JE gene was recently investigated. Ping et al. (15) investigated the regulatory site occupancy during the activation of mouse MCP-1 gene in TNFa-stimulated BALB/3T3 cells by using in vivo genomic footprinting. In response to TNFa, both distal promoter and proximal regulatory regions became occupied in vivo. The binding of (p65)2, p50/p65, and (p50)2 to the distal regulatory region of the mouse MCP-1 gene containing both NF-κB sites was detected by electrophoretic mobility shift assay (EMSA), but the trans-activity of each NF-κB/rel dimer remains unclear. A remote NF-κB site on the mouse MCP-1 gene was also reported to be important for PDGF-induced mouse MCP-1 mRNA expression in BALB/c 3T3 cells (16). These reports indicate the importance of the NF-κB...
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sites located in the distal region of both the human and mouse MCP-1 genes.

In our preliminary study, NF-κB (p50/p65) was activated in human B-lymphocytic IM9P3 cells and Raji cells without any stimulus. The binding of p50/p65 to the A2 site of the human MCP-1 gene was detected by EMSA. However, the expression of human MCP-1 mRNA was not detected in these cells.² It has recently become evident that certain NF-κB/Rel dimers such as (p65)₂ or c-Rel/p65 specifically regulate the transcription of certain genes.³ Thus, more detailed study on the interaction of the NF-κB sites and different NF-κB/Rel dimers is necessary to understand the mechanisms of the human MCP-1 gene expression. In the present study, we investigated the roles of two NF-κB sites, A1 and A2 sites, and different NF-κB/Rel dimers in the transcription of the human MCP-1 gene and have found that the binding of (p65)₂ and c-Rel/p65 to both A1 and A2 sites regulates the transcription of this gene.

EXPERIMENTAL PROCEDURES

Reagents—LPS (Escherichia coli 055:B5) was from Difco Laboratories (Detroit, MI). TPA and cycloheximide were from Sigma. The plasmid containing the 3.3-kb basic luciferase assay system was from Promega. The full-length murine p65 expression vector pCMV-LDp65, human p50 expression vector pCMV-399 (this vector coded residues 1–399 of p50), human c-Rel expression vector pCMV-NA, and rabbit polyclonal antibodies against p50, p52, p65, RelB, and c-Rel were kind gifts from Dr. Nigel Mackman (The Scripps Research Institute, La Jolla, CA). In the plasmid, four tandem copies of the double-stranded DNA were introduced from Promega.

RESULTS

MCP-1 mRNA expression in THP-1 cells—THP-1 cells were stimulated with LPS (10 μg/ml) or TPA (100 ng/ml) in the presence or absence of cycloheximide (10 μg/ml). 5 μg of total RNA were electrophoresed to a 1.2% agarose gel and blotted onto a nylon membrane as described under “Experimental Procedures.” The membrane was hybridized with α-³²P-labeled human MCP-1, IL-8, or β-actin cDNA probes.

Effects of LPS and TPA on the Expression of MCP-1 and IL-8 mRNA by THP-1 Cells—First, we investigated LPS- and TPA-induced MCP-1 mRNA expression in THP-1 cells by Northern analysis (Fig. 1). The expression of MCP-1 mRNA was first detected 2 h after LPS stimulation (lane 3). The expression

² A. Ueda, Y. Ishigato, T. Okubo, and T. Yoshimura, unpublished results.
level increased at 8 h and reached the peak at 16 h (lanes 5 and 6). The same level of MCP-1 mRNA expression was maintained until 24 h (lane 7). In contrast, MCP-1 mRNA was not detected until 8 h after TPA stimulation (lanes 11–14). Addition of cycloheximide did not affect the expression of MCP-1 mRNA by LPS- or TPA-stimulated THP-1 cells (lanes 8, 9, 17, and 18). The expression of IL-8 mRNA was also investigated (Fig. 1). A rapid induction of IL-8 mRNA was detected with both TPA and LPS stimulation (lanes 2 and 11). Addition of cycloheximide superinduced IL-8 mRNA expression in LPS-stimulated THP-1 cells (lanes 8 and 9).

**DNase I Footprinting of the Enhancer Region of the Human MCP-1 Gene**—We previously identified two NF-κB binding sites, A1 and A2, in the enhancer region of the human MCP-1 gene (12). The enhancer region of the mouse MCP-1 gene was recently characterized by two independent groups (Table I). Freter et al. (16) reported four PDGF-responsive elements, elements I–IV, with PDGF-stimulated NIH 3T3 cells. In contrast, Ping et al. (15) reported that three regions (site A, x-B, and x-B-2) were occupied by proteins during the activation of the mouse MCP-1 gene in TNFα-activated NIH 3T3 cells. As shown in Fig. 2A, element I, element III/site A, x-B-1, and element IV/x-B-2 of the mouse MCP-1 gene are highly conserved in the human MCP-1 gene. However, element II of the mouse MCP-1 gene is not conserved in the human MCP-1 gene.

The binding of nuclear proteins to the enhancer region of the human MCP-1 gene was characterized by DNase I footprinting with the nuclear extracts of stimulated or non-stimulated THP-1 cells (Fig. 2B). Three regions were protected from DNase I digestion after LPS or TPA stimulation. Regions 2 and 3 corresponded to the previously reported NF-κB binding sites, A1 and A2 (12). Region 1 was occupied before LPS or TPA stimulation (lanes 3 and 4) and seemed to be identical to the element III/site A of the mouse MCP-1 gene (15, 16). Constitutive protein binding to the element III/site A of the mouse MCP-1 gene was previously reported (15). These results suggest that three cis-elements (regions 1, 2, and 3) are likely to be involved in the enhancer activity of the human MCP-1 gene. However, only LPS stimulation induces protein binding to A1 and A2 sites of the human MCP-1 gene.

**LPS Induces the Formation of Multiple Nuclear Protein-MCP-1 A2 Probe Complexes**—We performed EMSA to identify which NF-κB/Rel dimer bound to the A2 probe using the nuclear extracts of LPS- or TPA-stimulated THP-1 cells. As shown in Fig. 3, four different DNA-nuclear protein complexes (C1, C2, C3, and C4) were found 2 h after LPS stimulation (lanes 1 and 2). The formation of the complexes was inhibited with an excess amount of the unlabeled A2 probe (lane 3) but not with the MA2 probe carrying three nucleotide substitutions (lane 4), indicating that these complexes were specific. We also performed EMSA with the A2 probe and nuclear extracts of TPA-stimulated THP-1 cells. Although a significant amount of MCP-1 mRNA was not detected 2–3 h after TPA stimulation by Northern analysis as shown in Fig. 1 (lane 13), the formation of C3 was detected 2 h after stimulation (lanes 5–7). The formation of C4 was also detected 16 h after stimulation when high level MCP-1 mRNA was detected (lanes 8–10 and Fig. 1, lane 15). Neither C1 nor C2 was detected after TPA stimulation.

**Table I**

| Factors          | Species | Binding sites | Regions                  | References |
|------------------|---------|---------------|--------------------------|------------|
| NF-κB            | Human   | A1 and A2 NF-κB sites | Distal enhancer region    | 12         |
| Sp1              | Human   | GC box        | Proximal promoter region  | 12         |
| Unknown          | Human   | Region 1      | Distal enhancer region    | Present study |
| NF-κB            | Mouse   | x-B-1 and x-B-2 (element IV) | Distal enhancer region    | 15, 16, 50 |
| Sp1              | Mouse   | GC box        | Proximal promoter region  | 15         |
| AP-1             | Mouse   | Element IV    | Distal enhancer region    | 50         |
| NF-IL6           | Mouse   | 7-mer sequence | Not determined            | 52         |
| Unknown          | Mouse   | Element II    | Distal enhancer region    | 50         |
| Unknown          | Mouse   | Site A (element III) | Distal enhancer region    | 50         |

**The Binding of Different NF-κB/Rel Proteins to the Human MCP-1 A1 Site, the hIL-8 NF-κB Site, and the Ig κ-Chain NF-κB Site**—The binding affinity of each NF-κB/Rel dimer to a NF-κB site depends on the sequence of each NF-κB site (25, 27, 28), and NF-κB binding sequences of different genes have been classified as the binding site for the heterodimer or homodimer of NF-κB/Rel proteins. As shown in Fig. 4, a 2-h LPS-stimulation induced the binding of (p65)2, c-Rel/p65, p50/p65, and p50/c-Rel, respectively. The complex formation after TPA stimulation was also characterized. C3, which was detected after a 2-h TPA stimulation (lane 9 and Fig. 3, lane 5), was supershifted by the addition of anti-p50 or anti-p65 (lanes 10 and 12). Normal rabbit IgG and antibodies against p52, RelB, and CEBP/β had no effect on the complex formation (lanes 11, 13–17). These results indicated that a 2-h TPA stimulation could induce the binding of p65/p50 to the A2 site of the human MCP-1 gene. C3 and C4 detected after a 16-h TPA stimulation (Fig. 3, lane 8) contained p65/p50 and p50/c-Rel, respectively (data not shown).

**The Binding of NF-κB/Rel Dimers to the Human MCP-1 Gene**—As shown in Fig. 5, the A1 probe as well as the A2 probe showed affinity to (p65)2, c-Rel/p65, p50/ p65, and p50/c-Rel to the A2 probe containing 5'GGGAAATTTCC-3'. Four different NF-κB/Rel dimers are capable of binding to the A2 site of the human MCP-1 gene. Since the sequences of the human MCP-1 A1 site and the human MCP-1 A2 site are similar, the binding affinity of each NF-κB/Rel dimer to a NF-κB site depends on the sequence of each NF-κB site (25, 27, 28). The human MCP-1 A1 site (5'-GGGAAACTTCC-3') and the human MCP-1 A2 site (5'-TGGGAAATTTCC-3') are different from that of the human MCP-1 A2 site, the binding affinity of NF-κB/Rel dimer to each NF-κB site could be different. As shown in Fig. 5, the A1 probe as well as the A2 probe showed affinity to (p65)2, c-Rel/p65, p50/ p65, and p50/c-Rel (lanes 1–5). In contrast, the IL-8 NF-κB probe showed affinity to (p65)2 and (p65/p50 or p50/c-Rel but not to (p65), or c-Rel/p65 (lanes 6–9). The Ig κ-chain NF-κB probe showed affinity to (p65)2 and p50/c-Rel but not to (p65), or c-Rel/p65 (lanes 10–13). Thus, human MCP-1 NF-κB sites bind to a broader range of NF-κB/Rel family proteins compared with IL-8 or the Ig κ-chain NF-κB site.
To investigate the trans-activity of each NF-κB/Rel dimer for the human MCP-1 gene transcription, P19 mouse embryonic carcinoma cells were cotransfected with pGLM-ENH containing the enhancer region of the human MCP-1 gene and different combinations of pCMV-LD (p65 expression vector), pCMV-339 (p50 expression vector), and pCMV-NA (c-Rel expression vector). In P19 cells, endogenous NF-κB/Rel proteins were undetectable (29), but Sp1, essential for the basal transcription of the human MCP-1 gene, was detectable (data not shown).

A basal level of luciferase activity was detected after transfection with 15 μg of pGLM-ENH (Fig. 6, A–C). Cotransfection with 15 μg of pGLM-ENH dose-dependently increased the luciferase activity up to 12-fold (Fig. 6A), but cotransfection with p50 or c-Rel expression vector did not increase the basal activity (Fig. 6, B and C). These results indicated that (p65)2 had a potent trans-activity for the human MCP-1 gene transcription, but (p50)2 and (c-Rel)2 did not activate the human MCP-1 gene transcription. To investigate the trans-activity of p50/p65 or c-Rel/p65 heterodimer, p50 or c-Rel expression vector was cotransfected with 600 ng of p65 expression vector into P19 cells along with the pGLM-ENH. As shown in Fig. 6D, the luciferase activity, which was elevated by (p65)2, was dose-dependently decreased by the cotransfection with the p50 expression vector, suggesting that p50/p65 is not a potent trans-activator for the human MCP-1 gene transcription. In contrast, cotransfection with p50 or c-Rel increased the luciferase activity up to 12-fold (Fig. 6B and C). These results suggested that the trans-activity of p50/p65 or c-Rel/p65 heterodimer was not as potent as that of (p65)2.
Nucleic acid extract from LPS-stimulated THP-1 cells was precipitated with appropriate antibody before addition of 32P-labeled probe. Arrowheads indicate specific nuclear protein-DNA complexes.

Cooperation of the A1 and A2 Sites in Human MCP-1 Gene Transcription—To investigate the role of the A1 site and a possible cooperation with the A2 site, the sequences of the A1 and the A2 sites were mutated (Fig. 8), and then these constructs were transfected into p65-expressing P19 cells or TPA-stimulated HeLa cells. As shown in Fig. 8, 8-fold higher luciferase activity was detected with pGL-ENH (containing only the proximal region (GC box)) of the human MCP-1 gene. This enhancer activity was significantly reduced by the mutation in either the A1 or A2 sequence, indicating that the enhancer activity was caused by the binding of (p65)2 to both A1 and A2 sites. The luciferase activity in TPA-stimulated HeLa cells, in which (p65)2, c-Rel/p65, p50/p65, and p50/c-Rel were induced (Fig. 8B), was also 12-fold higher with the pGL-ENH compared with the basal activity obtained with the pGL-PRM. The mutation in either the A1 or A2 sequence resulted in a significant loss of the enhancer activity. These results suggest that the binding of (p65)2 and/or c-Rel/p65 to both the A1 and A2 sites is important for the human MCP-1 gene transcription.

DISCUSSION

We previously identified two closely located NF-xB binding sites, A1 and A2 sites, in the distal 5'-flanking region of the human MCP-1 gene (12). A2 site was found to be important for the transcription of the human MCP-1 gene in TPA-, IL-1-, and TNF-a-stimulated malignant glioma cell, A172. Although the A1 site was also included in the CAT construct we used for the assay, the role of A1 site was not investigated. Recently, two independent groups investigated the transcriptional regulation of the mouse MCP-1 gene (15, 16). Ping et al. (15) found that two NF-xB binding sites in the distal regulatory region of the gene were occupied by proteins upon stimulation of 3T3 fibroblast with TNF-a. The sequences in the distal regulatory region including two NF-xB sites are highly homologous between human and mouse MCP-1 genes, suggesting important roles of the two NF-xB sites. In the present study, we investigated the roles of two NF-xB sites in the transcription of the human MCP-1 gene by using human monocytic cell line, THP-1 cells. Although MCP-1 is a potent chemotaxtractant for monocytes, monocytes themselves produce MCP-1 in vitro, and infiltrating monocytes into the lesions of inflammatory diseases are often found positive for MCP-1 in vivo (1). Therefore, investigating the mechanisms of MCP-1 mRNA expression in monocytes is particularly interesting.

Binding of nuclear proteins to both A1 and A2 sites were detected 2 h after LPS or TPA stimulation of THP-1 cells by DNase I footprinting. 2-h LPS stimulation caused accumulation of MCP-1 mRNA and formation of four nuclear protein-A2 probe complexes, whereas a 2-h TPA stimulation did not induce accumulation of MCP-1 mRNA, and only one nuclear protein-A2 probe complex was formed. The formation of the same four complexes was detected with the A1 probe. Kunsch et al. (28) previously investigated the nucleotide sequence that was optimal for the binding of each NF-xB/Rel protein. According to the study, the A2 (5'-GGGAATTTCC-3') sequence has high affinity to both p65 and c-Rel. Although the A1 (5'-GGGACTTTCC-3') sequence does not match any sequence shown by Kunsch et al., this sequence could also be considered to have high affinity to both p65 and c-Rel because of its high similarity. In contrast, the NF-xB site of the Ig k-chain gene (5'-GGGACTTTCC-3') has high affinity to p50, and the hIL-8
The binding of (p65)_2 to the NF-κB sequence, 5′-GGGAATTCCC-3′, has high affinity to only p65. In the present study, we showed the binding of (p65)_2, c-Rel/p65, p50/p65, and p50/c-Rel to the A1 and the A2 probes after LPS stimulation of THP-1 cells, thus showing their affinity to both p65 and c-Rel. In contrast, the NF-κB site of the human IL-8 gene or the Ig κ-chain gene showed more selective affinity. These results indicate that the NF-κB sites of the human MCP-1 gene have a broader binding affinity to different NF-κB/Rel dimers in comparison with that of the human IL-8 gene or the Ig κ-chain gene and also suggest the role of different NF-κB/Rel dimers in the transcription of each gene.

So-called NF-κB, the p65/p50 heterodimer, was originally identified as a nuclear factor that bound to the κB enhancer motif of the Igκ-chain gene and was reported to be involved in the expression of various genes in different types of cells (34). Since then, different NF-κB/Rel dimers have been identified. The binding of (p65)_2 to the NF-κB site was first presented by Urban et al. (35) by an *in vitro* experiment in which purified p65 was used. However, Fujita et al. (25) reported that (p50)_2 could bind to the NF-κB sequence with an affinity about 10-fold higher than that of (p65)_2, and an equimolar mixture of p50 and p65 formed almost exclusively p50/p65. The findings by Fujita et al. led to a suggestion that (p65)_2 might not naturally exist *in vivo*. Presence of (p65)_2 was later shown by detecting the binding of (p65)_2 to the NF-κB sites (5′-GGGAATTCCC-3′) of the MHC class I and IL-2 receptor genes with the cytoplasmic extracts of deoxycholate-treated HeLa cells (36) and the nuclear extracts of TPA-stimulated Jurkat cells (37). The binding of c-Rel/p65 to the NF-κB sites of the uroskinase gene (5′-GGGAATTC-3′) was reported with the nuclear extracts of TPA-stimulated HeLa and HT1080 cells (38).

Recent studies on the tissue factor (TF) and vascular cell adhesion molecule-1 (VCAM-1) genes indicate the roles of different NF-κB/Rel dimers in the transcription of different genes (17, 18). TF is specifically induced in the cells such as monocyte or vascular endothelial cells, whereas VCAM-1 is induced in endothelial cells. Oeth et al. (18) characterized a nuclear protein complex from LPS-stimulated THP-1 cells that bound to the NF-κB-like site, 5′-CCGAGATTCG-3′, in the 5′-flanking region of the human TF gene and found that c-Rel/p65, but not p65/p50, bound to the NF-κB-like site and mediated the expression of the TF gene. In the promoter region of the VCAM-1 gene, two NF-κB sites are closely located, and both NF-κB sites are necessary for the transcription of this gene. Ahmad et al. (17) investigated the role of (p65)_2, p50/p65, and (p50)_2 in the transcription of this gene by using recombinant proteins. Although all dimers were capable of binding to the NF-κB sites, (p65)_2, not p65/p50 or (p50)_2, activated the transcription of the VCAM-1 gene. Since both MCP-1 and VCAM-1 are involved in the emigration of blood monocytes, induction of both proteins by (p65)_2 may be an effective way to promote the infiltration of monocytes.

Transcription of the human MCP-1 gene requires synergism between two NF-κB/Rel dimers and Sp1 that is essential for the basal transcription of this gene. A similar synergism between NF-κB/Rel and Sp1 is important for the activation of the human immunodeficiency virus-long terminal repeat promoter (39–41). In this promoter region, two NF-κB sites and three Sp1 sites are closely located. An interaction between p65 and Sp1 that binds to the Sp1 site closest to the NF-κB site mediates the inducible HIV-1 gene expression (41).

In the promoter region of the E-selectin gene, two NF-κB sites are closely located, and a cooperation of two NF-κB sites was necessary for the enhanced transcription of this gene (42, 43). This cooperation was mediated by high mobility group protein I (Y). HMG-I (Y). HMG-I (Y) was originally purified from CV-1 cells as a DNA-binding protein to α-satellite DNA of African green monkey (44). HMG-I (Y) specifically binds to AT-rich sequences (45) and changes the chromatin configuration and hence the accessibility to other DNA-binding proteins. In the case of the E-selectin gene, HMG-I (Y) recognizes the AT-rich region of the NF-κB sequence, 5′-GGGAAATTCG-3′, stimulates the binding of NF-κB to the NF-κB binding site, and enhances the assembly of NF-κB and other
NF-κB/HMG-I(Y) plays a role in enhancing the assembly of the two nuclear protein-DNA complexes. This sequence is completely identical to the A2 sequence of the human MCP-1 gene transcription. Schematic representation of human MCP-1 reporter constructs. The proximal promoter region and distal enhancer region of the human MCP-1 gene are indicated by open and closed boxes, respectively. A1 and/or A2 sites of the enhancer region were mutated (indicated by X) in some of the constructs. These constructs were transfected into P19 or HeLa cells, and luciferase activities were compared. P19 cells were cotransfected with pCMV-LDp65. HeLa cells were stimulated with 100 ng/ml TPA. 

Fig. 7. Correlation between human MCP-1 mRNA expression (A) and the binding of NF-κB/Rel subunits to the A2 probe (B) in different tumor cell lines. A, Northern blot analysis. RNAs were extracted after 3 h of incubation in the absence (lanes 1, 4, 8, 6, and 10) or presence (lanes 3, 5, 7, 9, and 11) of TPA (100 ng/ml). B, supershift assay. Nuclear extracts were prepared after 2 h of incubation in the absence (lane 1) or presence (lanes 2-5) of TPA and preincubated with antisera against p50 (lane 3), p65 (lane 4), or c-Rel (lane 5) before addition of 32P-labeled probe. Arrowheads indicate specific nuclear protein-DNA complexes.

trans-activators (42, 43). HMG-I(Y) also binds to the IFN-β NF-κB site, 5'-GGAATTTCC-3' (46), and the melanocyte growth-stimulating activity NF-κB site, 5'-GGAATTTCC-3' (47). This sequence is completely identical to the A2 sequence of the human MCP-1 gene. Since two NF-κB complexes cooperate for the human MCP-1 gene transcription, it is possible that HMG-I(Y) plays a role in enhancing the assembly of the two NF-κB/Rel complexes that bind to the enhancer region of the MCP-1 gene (48). Further investigation is necessary to determine whether HMG-I(Y) is involved in the human MCP-1 gene regulation.

However, there seem to be other mechanisms involved in the human MCP-1 gene expression. An elevated level of human MCP-1 mRNA was detected in TPA-stimulated THP-1 cells 16 h after stimulation, but the binding of neither (p65)2 nor c-Rel/p65 to the A2 probe was detected by EMSA. Since the stability of MCP-1 mRNA was prolonged in IL-1-stimulated U373 malignant glioma cells (49) and TPA- or LPS-stimulated A172 cells, an elevated level of MCP-1 mRNA expression in TPA-stimulated THP-1 cells might be due to the prolonged stability of MCP-1 mRNA. Freter et al. (50) recently reported that the binding of 90-kDa phosphoprotein coactivator to the mouse MCP-1 NF-κB site was required for PDGF-induced mouse MCP-1 expression in NIH 3T3 cells. The 90-kDa coactivator was obviously different from HMG-I(Y) because of the much higher molecular mass. Ping et al. (15) also suggested that one or more of the factors that interacted with the mouse MCP-1 regulatory region required modification by protein kinases (15). Thus, transcription of human MCP-1 gene may also require phosphorylation of factors that bind to the regulatory region of the gene. Constitutive binding of unknown protein to the element II (also called site B by Freter et al.) located near upstream of the mouse MCP-1 NF-κB sites was also reported (15, 16). Constitutive binding of a nuclear protein to the same region of the human MCP-1 gene was shown in the present study. The 7-mer sequence 5'-TTTTGTA-3' located in the 3' untranslated region of the mouse MCP-1 gene was reported to be involved in the PDGF-induced mouse MCP-1 gene transcription in NIH 3T3 cells (51). AP-1 (52) and CEBP/β (53) were also reported to be involved in the mouse MCP-1 gene regulation. Further investigation is necessary to completely understand the mechanisms of elevated levels of human MCP-1 mRNA expression in different types of cells.

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