Histone Acetylation and Activation of cAMP-response Element-binding Protein Regulate Transcriptional Activation of MKP-M in Lipopolysaccharide-stimulated Macrophages*

Tipayarat Musikacharoen‡, Yasunobu Yoshikai§, and Tetsuya Matsuguchi‡

From the ‡Laboratory of Host Defense and Germfree Life, Research Institute for Disease Mechanism and Control, Nagoya University Graduate School of Medicine, Nagoya 466-8550 and §Division of Host Defense, Research Center of Prevention of Infectious Diseases, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

MKP-M is a dual specificity phosphatase that preferentially inactivates JNK. mkp-M gene expression is rapidly induced by lipopolysaccharide (LPS) stimulation in macrophages and is involved in the negative regulation of LPS-mediated JNK activation and tumor necrosis factor-a secretion. To reveal the transcriptional regulation of the mkp-M gene, we isolated the mouse mkp-M gene and mapped its transcriptional start site. Luciferase reporter plasmids containing 5′-upstream regions of the mkp-M gene were stably transfected into RAW264.7 cells. The assays using these cells revealed that the promoter region between −252 and −135 is required for mkp-M promoter activation. Sequencing analysis revealed E box and CREB-responsive elements in this region, and electromobility shift assays and mutagenesis confirmed that both of these elements are essential for LPS responsiveness of the mkp-M gene. We also utilized chromatin immunoprecipitation assay and found that LPS stimulation caused acetylation of histone H3 and H4 at mkp-M promoter in RAW264.7 cells. Consistent with this, a histone deacetylase inhibitor, trichostatin A, increased endogenous mkp-M gene transcription. Finally, DNase I hypersensitivity site mapping revealed the inducible hypersensitivity site after LPS stimulation around the location of the E box and CREB-responsive elements. Altogether, our data indicated that the activation of mkp-M gene transcription in macrophages by LPS is associated with histone acetylation and chromatin remodeling.

The dual specificity protein phosphatases (DSPs)¹ are an emerging subclass of the protein-tyrosine phosphatase gene superfamily which have been shown to inactivate mitogen-activated protein kinases (MAPKs) through dephosphorylation of both threonine and tyrosine residues within a signature sequence of TXY that are essential for the enzymatic activity (1). The members of DSPs share two common features as follows: a catalytic domain with significant amino acid sequence homology to a vaccinia virus dual specificity phosphatase, VH-1; and an N-terminal region homologous to the catalytic domain of the cdc25 phosphatase (rhodanese homology domain). Among its family members, some show highly selective substrate specificity, whereas others efficiently inactivate all three classes of MAPKs: extracellular signal-regulated kinase, c-Jun N-terminal kinase/stress-activated protein kinase, and p38/RK/CSBP (p38). Three distinct classes of MAPKs play important roles in various cellular events. Because the activation of MAPKs is a reversible process and gene expression of many DSPs is significantly induced following stimulation with growth factors, cytokines, or cell stresses, the induction of MKP-M is likely to provide the important mechanism for control of MAPK activity.

To date, 10 members of the mammalian DSPs gene family have been isolated and characterized. These members of DSPs have been defined recently into three subgroups by their basic structural prediction from genomic sequence (2): subgroup I, DUSP1 (hVH1/CL100/MKP-1/3CH134), DUSP2 (hPAC-1), DUSP4 (hVH2/TYP1), and DUSP5 (hVH3/B23/MKP-2); subgroup II, DUSP6 (PYST1/MK-3/rVH6), DUSP7 (PYST2/B59/MKP-X), DUSP9 (Mkp-4), and DUSP10 (Mkp-5); and subgroup III, DUSP 8 (hVH5/M3/6) and DUSP16 (Mkp-7/MKP-M).

MKP-M, a dual specificity isolated from macrophage, also referred to as MKP-7 (3, 4) is a member of DSPs family which is homologous to the human protein MKP-7 (2). MKP-M preferentially inactivates JNK (3, 5) when it is overexpressed in COS7 cells, and it also efficiently inactivates p38α and p38β in NIH3T3 cells (4). In any cell system, it does not show any significant phosphatase activity toward extracellular signal-regulated kinase. We have shown previously (5) that mkp-M gene expression is rapidly induced by LPS stimulation in mouse macrophage cell lines. Notably, it is not significantly increased by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-2 (IL-2), and IL-15. Expression of a phosphatase-inactive version of MKP-M enhanced and elongated both JNK activation and TNF-α secretion in macrophages, indicating that MKP-M may have a
mkp-M Gene Expression Associated with Histone Acetylation

role in preventing excessive inflammatory responses mediated by activated macrophages. To understand the transcriptional regulation of mkp-M gene in macrophages, we isolated mkp-M genomic DNA clones, identified the transcriptional initiation sites, and analyzed its 5’-upstream region. We show here that mkp-M gene expression is regulated by acetylation of histones H3 and H4 on the mkp-M promoter region containing the functional E box and CRE consensus motifs.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-acetylhistone H3 and anti-acetylhistone H4 antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). LPS from *Escherichia coli* serotype B5:055, trichostatin A, RPMI 1640 medium, and Dulbecco’s modified Eagle’s medium were obtained from Sigma. SB208530, a specific inhibitor of p38 kinase, 2 mM spermidine, 150 ng of actinomycin D, 5 \( \mu \)M RNA polymerase from *Escherichia coli* (Invitrogen) into annealed primer/RNA and incubated at 42°C for 2 h.

Mapping the Transcriptional Start Site of mMKP-M—Primarily extension analysis was applied to map the initiation start site of the mkp-M gene. Total RNA was prepared from LPS-stimulated RAW264.7 cells. Contaminating DNA was removed from RNA by treatment with 10 \( \mu \)g/ml DNase I (Sigma) for 2 h at 37°C. Following LPS stimulation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 1°C.

Enzymatic Analysis—To map the initiation start site of the mkp-M gene, were used to generate a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Many of the transcriptional regulators of the mouse are known to be regulated by acetylation of histones H3 and H4. To examine whether the expression of mkp-M gene is regulated by histone acetylation, we generated a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Mapping the Transcriptional Start Site of mMKP-M—Primarily extension analysis was applied to map the initiation start site of the mkp-M gene. Total RNA was prepared from LPS-stimulated RAW264.7 cells. Contaminating DNA was removed from RNA by treatment with 10 \( \mu \)g/ml DNase I (Sigma) for 2 h at 37°C. Following LPS stimulation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 1°C.

Enzymatic Analysis—To map the initiation start site of the mkp-M gene, were used to generate a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Many of the transcriptional regulators of the mouse are known to be regulated by acetylation of histones H3 and H4. To examine whether the expression of mkp-M gene is regulated by histone acetylation, we generated a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Mapping the Transcriptional Start Site of mMKP-M—Primarily extension analysis was applied to map the initiation start site of the mkp-M gene. Total RNA was prepared from LPS-stimulated RAW264.7 cells. Contaminating DNA was removed from RNA by treatment with 10 \( \mu \)g/ml DNase I (Sigma) for 2 h at 37°C. Following LPS stimulation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 1°C.

Enzymatic Analysis—To map the initiation start site of the mkp-M gene, were used to generate a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Many of the transcriptional regulators of the mouse are known to be regulated by acetylation of histones H3 and H4. To examine whether the expression of mkp-M gene is regulated by histone acetylation, we generated a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Mapping the Transcriptional Start Site of mMKP-M—Primarily extension analysis was applied to map the initiation start site of the mkp-M gene. Total RNA was prepared from LPS-stimulated RAW264.7 cells. Contaminating DNA was removed from RNA by treatment with 10 \( \mu \)g/ml DNase I (Sigma) for 2 h at 37°C. Following LPS stimulation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 1°C.

Enzymatic Analysis—To map the initiation start site of the mkp-M gene, were used to generate a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Many of the transcriptional regulators of the mouse are known to be regulated by acetylation of histones H3 and H4. To examine whether the expression of mkp-M gene is regulated by histone acetylation, we generated a series of 5’-deletion DNA fragments. An oligonucleotide, 5’TCTCTCTGCTAGTCAGCTGCT, was end-labeled with \( \alpha^{32}P \) RNA polymerase and subjected to poly(A)+ purification using mRNA purification kit (Amersham Biosciences). An oligonucleotide sequences upstream to the transcriptional start site were searched for the potential binding sites for transcription factors (7).

Mapping the Transcriptional Start Site of mMKP-M—Primarily extension analysis was applied to map the initiation start site of the mkp-M gene. Total RNA was prepared from LPS-stimulated RAW264.7 cells. Contaminating DNA was removed from RNA by treatment with 10 \( \mu \)g/ml DNase I (Sigma) for 2 h at 37°C. Following LPS stimulation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 1°C.
ice-cold PBS, harvested by scraping into PBS, and pelleted at 1500 rpm. The pellet was lysed in 5 volumes of ice-cold Nonidet P-40 lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl$_2$, 0.5% Nonidet P-40, 0.15 mM spermine, and 0.5 mM spermidine) for 10 min. Nuclei were pelleted for 5 min at 3000 rpm and washed twice with washing buffer (100 mM NaCl, 50 mM Tris (pH 8.0), 3 mM MgCl$_2$, 0.15 mM spermine, and 0.5 mM spermidine) and then nuclei were collected at 3000 rpm for 5 min. Nuclease Digestion, Restriction Enzyme Digestion, and Southern Blot Analysis—For DNase I digestion, nuclei were resuspended in DNase I digestion buffer (100 mM NaCl, 50 mM Tris (pH 8.0), 3 mM MgCl$_2$, 0.15 mM spermine, 0.5 mM spermidine, 1 mM CaCl$_2$) at 2 × 10$^8$ nuclei/ml. 100-µl aliquots of nuclei were digested with increasing concentrations of DNase I, typically 0, 0.04, 0.06, and 0.08 µg at 37 °C for 10 min. The reactions were stopped by addition of 10 µM EDTA, 100 µl of DNase I digestion buffer, 100 µg of proteinase K, and 0.2% SDS and incubated at 37 °C overnight. For DNA purification, the samples were extracted once with phenol/chloroform and once with chloroform. The aqueous phase was digested with 20 µg of RNase A for 2 h at 37 °C, extracted with phenol/chloroform and chloroform, and then precipitated with ethanol, and DNA was redissolved in water. The 25-µg aliquots of genomic DNA from DNase I-treated nuclei were digested overnight with 50 units of EcoRI and SacI. Digested DNA was loaded onto 8% agarose gel. Gel was then soaked in denaturing buffer (0.6 M NaCl, 0.4 M NaOH) for 45 min and in neutralizing buffer (1.5 mM NaCl, 0.1 M Tris base, 0.04 mM Tris-HCl) for another 45 min and transferred to a nylon membrane. As the probe for DNase I hypersensitivity, PCR product was labeled with$32$P by random priming. The membrane was prehybridized and hybridized with the$32$P-labeled probe.

RESULTS

Isolation and Characterization of mkp-M Gene—In order to analyze the mouse mkp-M gene, a mouse genomic library was screened with MKP-M cDNA as the probe. We isolated 14 positive clones and characterized them by Southern blot analysis, restriction enzyme mapping, and nucleotide sequencing. The structure of the mkp-M gene including the intron/exon junctions is schematically shown in Fig. 1A. The mkp-M gene contained 7 exons; the consensus ATG that corresponded to the translational start codon was located at the distal part of exon 2 (Fig. 1A).

Primer extension analysis was applied to map the transcriptional start site of the mkp-M gene. A synthesis oligonucleotide complementary to 5′ end of the previously reported MKP-M cDNA was hybridized to poly(A)+ RNA from LPS stimulated RAW264.7 cells and extended by reverse transcription. Two transcriptional start sites were identified by comparing the transcribed products with the sequence ladder from the same primer (Fig. 1B). The first proximal transcriptional start site was located 229 bp upstream of the 5′ end of the cDNA described previously (5). The second transcriptional start site was 115 bp upstream to the first site. To confirm these sites, several other oligonucleotides designed from different parts of MKP-M cDNA and mkp-M genomic DNA were also used for primer extension producing compatible results (data not shown). The nucleotide of the proximal transcriptional start site was designed as +1 throughout this paper (Fig. 1C).

The Structural and Functional Analysis of the 5′-Upstream Region of the Mouse mkp-M Gene—The nucleotide sequence upstream of the transcriptional start sites was searched for the potential binding sites of transcriptional factors using Genome Exploring and Modeling software (7). As shown in Fig. 1C, neither TATA nor CAAT boxes were identified in the 5′-upstream region for each transcriptional initiation site. However, the region between the two initiation sites is very rich in GCs (GC content, 78%).

Because applying conventional PCR methods to this region was difficult most likely because of the high GC content, the Advantage®-GC 2 PCR kit (Clontech) was used to generate a series of 5′-deletion constructs to identify the region required for mkp-M gene activation. These 5′-deletion constructs were cloned into a promoter-less luciferase reporter vector. The activities of the mkp-M promoter constructs were first determined by transient transfection into RAW264.7 cells. Each of the reporter plasmids showed the proper basal promoter function, but none showed the inducible activity by LPS (data not shown). These plasmid constructs were then stably integrated into RAW264.7 cells by co-transfecting a G418-resistant plasmid for drug selection. Several independent stable clones for each construct were isolated. In stably integrated RAW264.7 cells, some constructs showed LPS-driven induction of luciferase activities (Fig. 2A). The highest inducible promoter activity was obtained from the longest pGL3-1958 construct. Subsequent deletion of pGL3-1958 resulted in moderately reduced promoter activity, but no significant differences were detected among those four deletion constructs (pGL3-1958, −1488, −875, and −252). In contrast, the responsiveness to LPS treatment was completely abrogated when the construct was deleted to −135 (pGL3-135).

Roles of E Box and CRE Consensus Elements in LPS-induced MKP-M Transcription—As shown in Fig. 2A, the region between −252 and −135 seemed critical for the activation of mkp-M promoter by LPS. The sequence analysis within this region demonstrated the presence of consensus elements for E box, CRE, and AP-2.

In order to examine the involvement of these elements in LPS responsiveness, each site was mutated by site-directed mutagenesis on the pGL3-252 wild type mkp-M promoter construct, and the mutated constructs were stably integrated into RAW264.7 cells. In stably integrated RAW264.7 cells, mutation of either E box or CRE consensus element significantly reduced fold induction by LPS. The responsiveness to LPS was completely abrogated by E box/CRE double mutation (Fig. 2B).

We next examined if DNA-protein binding within this region is involved in the activation of mkp-M promoter by EMSA. Nuclear extracts prepared from untreated and LPS-treated RAW264.7 cells were incubated with a$32$P-labeled probe spanning the mkp-M promoter region between −252 and −135. EMSAs revealed the presence of two protein complexes binding to the probe in an LPS-dependent manner, and the formation of the protein-DNA complexes was practically absent in unstimulated cells (Fig. 3A). To determine the DNA sequences responsible for these complexes, cold-target competition was carried out with 50-fold excesses of E box, CRE, or AP-2 consensus nucleotide sequences. As shown in Fig. 3A, both complexes were abrogated by the addition of 50-molar excesses of either unlabeled CRE or E box-specific oligonucleotides, whereas the other cold target consensus element tested, AP-2, was incapable of inhibiting either complex.

We next addressed whether an antibody against CREB could modify the protein-DNA complexes. As shown in Fig. 3B, the inducible protein complexes were efficiently shifted with the anti-CREB antibody, indicating that both DNA-protein complexes contain CREB as a component. Furthermore, synthetic oligonucleotides specific to CRE and E box also formed protein-DNA complexes when they were incubated with nuclear extracts from LPS-treated RAW264.7 (Fig. 3C). In contrast their mutated versions did not. These results strongly indicated that both CRE and E box consensus sequences are functional as binding sites for transcriptional factors.

DNase I Hypersensitivity Sites in the 5′-Upstream Region of the Mouse mkp-M Gene—Transcriptional activation of a gene is often accompanied by chromatin remodeling leading to the appearance of DNase I hypersensitivity (HS) sites (10–12). Thus we searched for the HS site in the MKP-M 5′-regulatory
Fig. 1. Characterization of mkp-M gene. A, the mkp-M gene was characterized by restriction enzyme mapping and Southern blot analyses. Solid boxes represent exons, and the horizontal lines indicate introns. The position of the first methionine is also shown. B, primer extension analysis for mapping of the transcriptional start sites of the mkp-M gene. The transcribed product (P) was run along with sequencing ladder from the same primer on a 6% polyacrylamide/urea gel. The asterisks indicate the two transcribed products. C, the nucleotide sequence of the 5′-flanking region of the mouse mkp-M gene, two transcriptional start sites (TSS), are shown and the proximal transcriptional start site was assigned as +1. Potential binding sites for transcription factors are indicated by boldface capital letters. Arrows identify the 5′ end of deletion promoter constructs.
region before and after LPS stimulation. The increasing amounts of DNase I was used to cleave the nuclei from RAW264.7 cells, and then the genomic DNA was completely digested with EcoRI and SalI and subjected to Southern blot analysis. The probe used for the assay was derived from the 3'-H11032 end of the 5-kb genomic EcoRI/SalI fragment containing the mkp-M promoter region. As shown in Fig. 4, two smaller DNA fragments were observed by Southern probe only in the LPS-treated cells. By determining the size of these DNA fragments, the regions of HS were around 1500 and 1522 from the proximal transcriptional start site. Although the contribution of the 1500 HS in the MKP-M transcription is not clear, the site around 1522 coincides with the locations of E box and CREB-binding sites.

Trichostatin A, a Histone Deacetylase Inhibitor, Induced mkp-M Gene Activation—Restructuring of the chromatin is often associated with histone acetylation. In order to determine a possible role of histone modification in the regulation of mkp-M gene activation, we treated RAW264.7 cells with a histone deacetylase inhibitor, trichostatin A (TSA), and we investigated its effects on mkp-M gene expression. In the Northern blot analysis, MKP-M mRNA level was potently increased by TSA treatment (Fig. 5A). This result prompted us to investigate the effect of TSA on mkp-M promoter activity by treating RAW264.7 stable clones with TSA. Consistent with Northern blot analysis, TSA treatment of RAW264.7 cells with MKP-M luciferase reporters (pGL3-1958, -1488, -875, and -252) stably integrated resulted in the induction of luciferase activity 5–6-fold over untreated cells. The induction was completely lost for the shortest promoter construct, pGL3-135 (Fig. 5B). As in LPS stimulation, the responsiveness to TSA treatment was also significantly reduced by the mutation of either E box or CRE-binding element (Fig. 5B). Moreover, combined treatment of these cells with TSA and LPS increased the fold induction of the luciferase activity in an additional manner (Fig. 5B and C). Taken together, these results strongly implied that histone acetylation may be involved in the regulation of mkp-M gene activation.

LPS Treatment Caused Acetylation of Histone H3 and H4 at the mkp-M Promoter—As mentioned above, both LPS and TSA
could induce the mkp-M promoter-mediated luciferase activation when reporter plasmids were stably integrated but not when they were transiently transfected in RAW264.7 cells. It may be because transiently transfected necked DNA was not efficiently packed into chromatin, as it is well established that modifying chromatin structure through acetylation of histone needs proper chromatin structure. In order to determine whether histone acetylation actually occurs at the endogenous mkp-M gene, we utilized ChIP assay using antibodies specific to acetylhistone H3 or acetylhistone H4. The presence of the mkp-M gene in the immunoprecipitated chromatin was analyzed by PCR using a pair of radiolabeled primers specific to the CREB/E box-binding region (Fig. 6A). The ChIP assay results showed that LPS stimulation potently induced the acetylation of both histone H3 and H4 at the mkp-M promoter, whereas neither acetylated histone H3 nor H4 was detected in non-stimulated cells (Fig. 6B). These results suggest that the regulation of the endogenous mkp-M gene activation is associated with the acetylation of histone H3 and H4 at the mkp-M promoter.

To confirm the specificity of our ChIP assay results, we determined the acetylation level of histone H3 and H4 on the mkp-M gene more 5'-upstream of the E box/CREB-binding region (Fig. 6A). A pair of primers specific to the −1970 to −1755 of the mkp-M gene were used to analyze the acetylation status of histone H3 and H4 using the same DNA templates. Although the basal acetylation of histone H3 and H4 was detected, LPS stimulation did not increase their acetylation at this region (Fig. 6B). These results, along with the HS data (Fig. 4), indicate that acetylation of histones and chromatin remodeling occurs at a short stretch of DNA around the E box/CRE site.

p38 MAPK Is Involved in the Transcriptional Induction of MKP-M—We reported previously that p38 MAPK is necessary for LPS-mediated mkp-M gene expression in RAW264.7 cells using a specific inhibitor of p38 MAPK, SB208580 (5). To determine whether the inhibition of p38 MAPK pathway had any effect on LPS-induced mkp-M promoter, we pretreated RAW264.7 stable clones of reporter construct with SB208580. As expected, the inhibition of p38 MAPK pathway by pretreatment of the stable clones with SB208580 completely abrogated mkp-M promoter-driven luciferase activity by LPS (Fig. 7A). Thus the activation of p38 pathway is essential for mkp-M promoter activation by LPS.
DISCUSSION

In our previous report (5) we demonstrated that gene expression of a dual specificity phosphatase, MKP-M, is rapidly induced by LPS or synthetic lipid A stimulation in mouse macrophage cell lines. MKP-M has strong substrate specificity toward JNK, and the increased level of MKP-M is at least partially responsible for the down-regulation of LPS-mediated JNK activation and TNF-α secretion. Thus MKP-M seems important in regulating the excessive inflammatory response in Gram-negative bacterial infection. Interestingly, unlike other MAP kinase phosphatases, MKP-M mRNA in macrophages is not significantly induced by treatment with pro-inflammatory cytokines such as TNF-α, interferon-γ, IL-2, or IL-15, suggesting a unique regulatory mechanism of the mkp-M gene expression (5).

In this report, we describe the isolation of the mkp-M genomic clone and characterized its transcriptional initiation sites and the 5′-regulatory region. The mkp-M gene covers ~55 kb and consists of 7 exons (Fig. 1A). It utilizes two transcriptional start sites (Fig. 1, B and C). The 5′-flanking region of the mkp-M promoter lacks either TATA or CAAT box but is rich in GC content. The GC-rich/TATA less promoters have been found among the inducible and tissue-specific genes, such as the urokinase-type plasminogen activator receptor (13), CD7 (14), and pac-1 (17), and some of these genes contain multiple start sites like mkp-M. Interestingly, both of the two transcriptional start sites are indispensable for mkp-M gene transcription, as the deletion of either site caused the loss of the basal promoter activity (data not shown).

Our promoter functional analyses showed that the upstream region reaching up to −252 from the proximal start site is sufficient for the fully inducible expression of the mkp-M gene by LPS (Fig. 2A). Further deletion to −135 abrogated the induction, indicating the region between −252 and −135 confers LPS responsiveness to the mkp-M promoter. Nucleotide sequencing analysis of this region identified consensus binding sequences for three transcription factors that may be involved in the induction of mkp-M gene expression: E box, CRE, and AP-2 (Fig. 1C). Mutation of the E box and/or CRE consensus elements is capable of forming protein-DNA complexes in the LPS-treated macrophage cell line. Addition of either E box or CRE consensus oligonucleotide as a non-radioactive competitor effectively inhibited the formation of the two complexes, whereas the AP-2 consensus oligonucleotide showed no inhibitory effect (Fig. 3A). Furthermore, an oligonucleotide specific to either E box or CRE consensus element was capable of forming protein-DNA complexes (Fig. 3C). These lines of evidence strongly indicate that both E box and CRE contribute to mkp-M gene activation by LPS.

Our ChIP assay of DNA products from immunoprecipitation reactions was carried out as described under “Experimental Procedures.”

In this report, we describe the isolation of the mkp-M genomic clone and characterized its transcriptional initiation sites and the 5′-regulatory region. The mkp-M gene covers ~55 kb and consists of 7 exons (Fig. 1A). It utilizes two transcriptional start sites (Fig. 1, B and C). The 5′-flanking region of the mkp-M promoter lacks either TATA or CAAT box but is rich in GC content. The GC-rich/TATA less promoters have been found among the inducible and tissue-specific genes, such as the urokinase-type plasminogen activator receptor (13), CD7 (14), and pac-1 (17), and some of these genes contain multiple start sites like mkp-M. Interestingly, both of the two transcriptional start sites are indispensable for mkp-M gene transcription, as the deletion of either site caused the loss of the basal promoter activity (data not shown).

Our promoter functional analyses showed that the upstream region reaching up to −252 from the proximal start site is sufficient for the fully inducible expression of the mkp-M gene by LPS (Fig. 2A). Further deletion to −135 abrogated the induction, indicating the region between −252 and −135 confers LPS responsiveness to the mkp-M promoter. Nucleotide sequencing analysis of this region identified consensus binding sequences for three transcription factors that may be involved in the induction of mkp-M gene expression: E box, CRE, and AP-2 (Fig. 1C). Mutation of the E box and/or CRE consensus elements is capable of forming protein-DNA complexes in the LPS-treated macrophage cell line. Addition of either E box or CRE consensus oligonucleotide as a non-radioactive competitor effectively inhibited the formation of the two complexes, whereas the AP-2 consensus oligonucleotide showed no inhibitory effect (Fig. 3A). Furthermore, an oligonucleotide specific to either E box or CRE consensus element was capable of forming protein-DNA complexes (Fig. 3C). These lines of evidence strongly indicate that both E box and CRE contribute to mkp-M gene activation by LPS.

Our ChIP assay of DNA products from immunoprecipitation reactions was carried out as described under “Experimental Procedures.”

In this report, we describe the isolation of the mkp-M genomic clone and characterized its transcriptional initiation sites and the 5′-regulatory region. The mkp-M gene covers ~55 kb and consists of 7 exons (Fig. 1A). It utilizes two transcriptional start sites (Fig. 1, B and C). The 5′-flanking region of the mkp-M promoter lacks either TATA or CAAT box but is rich in GC content. The GC-rich/TATA less promoters have been found among the inducible and tissue-specific genes, such as the urokinase-type plasminogen activator receptor (13), CD7 (14), and pac-1 (17), and some of these genes contain multiple start sites like mkp-M. Interestingly, both of the two transcriptional start sites are indispensable for mkp-M gene transcription, as the deletion of either site caused the loss of the basal promoter activity (data not shown).

Our promoter functional analyses showed that the upstream region reaching up to −252 from the proximal start site is sufficient for the fully inducible expression of the mkp-M gene by LPS (Fig. 2A). Further deletion to −135 abrogated the induction, indicating the region between −252 and −135 confers LPS responsiveness to the mkp-M promoter. Nucleotide sequencing analysis of this region identified consensus binding sequences for three transcription factors that may be involved in the induction of mkp-M gene expression: E box, CRE, and AP-2 (Fig. 1C). Mutation of the E box and/or CRE consensus elements is capable of forming protein-DNA complexes in the LPS-treated macrophage cell line. Addition of either E box or CRE consensus oligonucleotide as a non-radioactive competitor effectively inhibited the formation of the two complexes, whereas the AP-2 consensus oligonucleotide showed no inhibitory effect (Fig. 3A). Furthermore, an oligonucleotide specific to either E box or CRE consensus element was capable of forming protein-DNA complexes (Fig. 3C). These lines of evidence strongly indicate that both E box and CRE contribute to mkp-M gene activation by LPS.

Our ChIP assay of DNA products from immunoprecipitation reactions was carried out as described under “Experimental Procedures.”

In this report, we describe the isolation of the mkp-M genomic clone and characterized its transcriptional initiation sites and the 5′-regulatory region. The mkp-M gene covers ~55 kb and consists of 7 exons (Fig. 1A). It utilizes two transcriptional start sites (Fig. 1, B and C). The 5′-flanking region of the mkp-M promoter lacks either TATA or CAAT box but is rich in GC content. The GC-rich/TATA less promoters have been found among the inducible and tissue-specific genes, such as the urokinase-type plasminogen activator receptor (13), CD7 (14), and pac-1 (17), and some of these genes contain multiple start sites like mkp-M. Interestingly, both of the two transcriptional start sites are indispensable for mkp-M gene transcription, as the deletion of either site caused the loss of the basal promoter activity (data not shown).

Our promoter functional analyses showed that the upstream region reaching up to −252 from the proximal start site is sufficient for the fully inducible expression of the mkp-M gene by LPS (Fig. 2A). Further deletion to −135 abrogated the induction, indicating the region between −252 and −135 confers LPS responsiveness to the mkp-M promoter. Nucleotide sequencing analysis of this region identified consensus binding sequences for three transcription factors that may be involved in the induction of mkp-M gene expression: E box, CRE, and AP-2 (Fig. 1C). Mutation of the E box and/or CRE consensus elements is capable of forming protein-DNA complexes in the LPS-treated macrophage cell line. Addition of either E box or CRE consensus oligonucleotide as a non-radioactive competitor effectively inhibited the formation of the two complexes, whereas the AP-2 consensus oligonucleotide showed no inhibitory effect (Fig. 3A). Furthermore, an oligonucleotide specific to either E box or CRE consensus element was capable of forming protein-DNA complexes (Fig. 3C). These lines of evidence strongly indicate that both E box and CRE contribute to mkp-M gene activation by LPS.
The E box element has the consensus sequence that is recognized by the members of the bHLH-ZIP transcription factor that include Myc (18), Max (19), upstream stimulatory factors 1 and 2 (20, 21), as well as ZEB (22). The protein-protein interaction within the bHLH-ZIP results in the formation of a dimer at the E box that leads to either the activation or repression of gene transcription (18).

The CRE consensus element is recognized by various transcription factors including CREB (23), ATF (24), c-Jun (25), and c-Fos (26). Notably, the combination of E box and CRE sites is not unique to the mkp-M gene. As a matter of fact, many mammalian genes contain both of these sequences in their proximal promoters, and the synergy between these two elements has been reported in transcriptional activation of several genes such as rat c′fos (27), murine and human transferrin (28), and transforming growth factor-β2 (29).

The competition assays of EMSA showed that the oligonucleotide corresponding to either E box or CRE consensus element abrogated the formation of the two protein-binding complexes, whereas the mutated versions of those lost the inhibitory effect (Fig. 3, A and C). Although we have not identified the protein binding to E box motif, the supershift assay using a specific antibody showed that CREB is in both of the DNA-protein complexes (Fig. 3B). Thus, it is reasonable to assume that CREB forms a complex with another regulatory protein binding to E box motif, and these two proteins synergistically activate transcription of mkp-M gene. In contrast, AP-2 does not seem to share any regulatory function. This is rather different from another DSP, PAC-1 (30). Regulation of PAC-1 expression in T cells in response to v-raf- and v-ras-induced signals is mediated by AP-2 and E box. Deletion of AP-2-binding site dramatically decreased pac-1 gene transcription, whereas mutation of E box had no effect in some cell lines.

Notably, in transient transfection assays, DNA fragments containing the 5′-upstream region of mkp-M gene showed proper basal promoter function but did not respond to LPS stimulation in RAW264.7 cells. The same constructs revealed significant inducibility by LPS when they were stably integrated in the genome, implying that LPS responsiveness of mkp-M promoter requires the proper chromatin structure. When DNA is packaged into nucleosome, it often fails to provide a recognition code for the transcription factors (31–34), and many transcription factors recognize chromatin templates with lower affinity or do not bind them at all (35). Also nucleosomal biochemistry can influence transcription factor accessibility to DNA (36). The structural changes in chromatin may accompany the acetylation of histone H3 and H4 by histone acetyltransferases and is associated with transcriptional activation, whereas deacetylation by histone deacetylases is associated with gene repression. Inducing the global hyperacetylation of cellular histones with TSA, a specific inhibitor for histone deacetylases, resulted in the up-regulation of endogenous MKP-M and mkp-M promoter reporter gene in RAW264.7 cell line (Fig. 5A). ChIP assays showed that LPS potentially induced acetylation of both histone H3 and H4 at the mkp-M promoter (Fig. 6B). These observations further support the crucial roles of chromatin modifications played by histone acetylation for mkp-M gene activation. Interestingly, the promoter activation of MKP-1, another DSP, has been shown to associate with histone H3 acetylation after arsenite treatment, and its gene expression is also induced by TSA (37). Thus MKP-M is a second DSP member whose gene expression has been shown to be positively regulated by histone acetylation. It is of note that the acetylation of histone and the inhibition of histone deacetylases by TSA are also associated with gene repression for some genes such as ETS transcription factor PU.1 (38).

The acetylation of the histone tails disrupts and interferes with the higher order chromatin folding, promotes the solubility of chromatin at physiological ionic strength, and maintains the unfolded structure of the transcribed nucleosome allowing transcription factor binding (39, 40). The presence of HS sites commonly corresponds to regions in which the chromatin is open and accessible (51). Our HS mapping revealed at least two HS sites at the promoter region of mkp-M gene after LPS stimulation, one of which correspond to the location of the consensus sequences for E box/CRE.

Based on these data, we would like to propose a model in which the structural changes in chromatin allow CREB and other regulatory proteins to bind the mkp-M promoter (Fig. 7B). LPS-mediated gene remodeling has also been reported for several other genes. For example, LPS induction of IL-12 p40 gene expression has been reported to require remodeling of a promoter-encompassing nucleosome, and this process is dependent on Toll-like receptor 4 (41). The activation of c-Rel or other transcriptional factors is neither required nor sufficient for this remodeling, indicating there is a preceding mechanism for nucleosome remodeling. Importantly, a recent report (42) indicated that p38 MAPK, which is strongly activated by LPS, is responsible for the phosphoacetylation of histone H3 on promoters of a subset of stimulus-induced cytokine and chemokine genes. It is of note that MKP-M mRNA induction was effectively inhibited by a specific p38 kinase inhibitor in LPS-stimulated macrophages, whereas a specific inhibitor of MEK had no inhibitory effect, indicating that p38 kinase but not extracellular signal-regulated kinase is required for mkp-M gene activation by LPS (5). We showed consistently in this report that pretreatment of RAW264.7 stable clones with SB208530 abrogated mkp-M promoter-driven luciferase activity by LPS (Fig. 7A). Thus it seems reasonable to presume that p38 kinase activated by LPS induces phosphorylation of histones leading to their subsequent acetylation and nucleosome remodeling at the mkp-M promoter.

In conclusion, acetylation of histone H3 and H4 is induced at the mkp-M promoter in LPS-stimulated macrophages. This leads to nucleosome remodeling and subsequent recruitment of transcriptional factors including CREB to the E box/CRE elements of mkp-M promoter. Induced MKP-M plays an important role in down-regulating JNK activity and cytokine secretion (5). Thus LPS utilizes nucleosome remodeling as a transcriptional activation mechanism not only for cytokine and chemokine genes such as IL-12 p40 but also for genes of signal regulators such as mkp-M.

Acknowledgments—We thank Keiko Itano and Ayumi Nishikawa for technical assistance.

REFERENCES
1. Camps, M., Nichols, A., and Arkinstall, S. (2000) FASEB J. 14, 6–16
2. Theodosiou, A., and Ashworth, A. (2002) Genome Biol. 3, 3009.1–3009.10
3. Masuda, K., Shima, H., Watanabe, M., and Kikuchi, K. (2001) J. Biol. Chem. 276, 39002–39011
4. Tanose, T., Yamamoto, T., Maeda, R., and Nishida, E. (2001) J. Biol. Chem. 276, 26629–26639
5. Matsuzaki, T., S. Yagihara, T., Johnson, T. R., Kraft, A. S., and Yoshikai, Y. (2001) Mol. Cell. Biol. 21, 6999–7009
6. Musikacharoen, T., Matsuzaki, T., Kikuchi, T., and Yoshikai, Y. (2001) J. Immunol. 166, 4516–4524
7. Quass, K., Frech, K., Kara, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
8. Schwenzer, R., Siemieniuk, K., Liptay, S., Schubert, G., Peters, N., Scheurich, P., and Wajant, H. (1999) J. Biol. Chem. 274, 19388–19374
9. Weinmann, A. S., Plevy, S. E., and Smale, S. T. (1999) Immunity 11, 665–675
10. Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1999) EMBO J. 18, 1409–1402
11. Hebbes, T. R., Clayton, A. L., Thorne, A. W., and Crane-Robinson, C. (1994) EMBO J. 13, 1823–1830
12. Urnov, F. D., and Wolfe, A. P. (2001) Mol. Endocrinol. 15, 1–16
13. Soravia, E., Grebe, A., De Luca, P., Helin, K., Suh, T. T., Degen, J. L., and Blasi, F. (1995) Blood 86, 624–635
14. Schanberg, L. E., Fleenor, D. E., Kurtzberg, J., Haynes, B. F., and Kaufman, R. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 603–607
15. Meeker, T. C., Loeb, J., Ayres, M., and Sellers, W. (1990) Mol. Cell. Biol. 10, 1680–1688
16. Tugores, A., Rubio, T., Rancano, C., and Alonso, M. A. (1997) DNA Cell Biol. 16, 245–255
17. Gerondakis, S., Economou, C., and Grumont, R. J. (1994) Genomics 24, 182–184
18. Ryan, K. M., and Birnie, G. D. (1996) Biochem. J. 314, 713–721
19. Blackwood, E. M., and Eisenman, R. N. (1991) Science 251, 1211–1217
20. Sawadogo, M., and Roeder, R. G. (1985) Cell 43, 165–175
21. Sirito, M., Lin, Q., Maity, T., and Sawadogo, M. (1994) Nucleic Acids Res. 22, 427–433
22. Postigo, A. A., Ward, E., Skeath, J. B., and Dean, D. C. (1999) Mol. Cell. Biol. 19, 7255–7263
23. Montminy, M. R., and Bilezikjian, L. M. (1987) Nature 328, 175–178
24. Lee, K. A., Hai, T. Y., SivaRaman, L., Thimmappaya, B., Hurst, H. C., Jones, N. C., and Green, M. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8355–8359
25. Bohmann, D., Roe, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987) Science 238, 1386–1392
26. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) Cell 54, 541–552
27. Di Rocco, G., Pennuto, M., Illi, B., Canu, N., Filocamo, G., Trani, E., Rinaldi, A. M., Possenti, R., Mandolesi, G., Sirinian, M. I., Jucker, R., Levi, A., and Nasi, S. (1997) Mol. Cell. Biol. 17, 1244–1253
28. Chaudhary, J., and Skinner, M. K. (1999) Endocrinology 140, 1262–1271
29. Scholz, B., Kingsley-Kallassen, M., and Rizzino, A. (1996) J. Biol. Chem. 271, 32373–32380
30. Grumont, R. J., Rasko, J. E., Strasser, A., and Gerondakis, S. (1996) Mol. Cell. Biol. 16, 2913–2921
31. Forsberg, E. C., and Bresnick, E. H. (2001) Bioessays 23, 820–830
32. Grunstein, M. (1997) Nature 389, 349–352
33. Strahl, B. D., and Allis, C. D. (2000) Nature 403, 41–45
34. Kadam, S., and Emerson, B. M. (2002) Curr. Opin. Cell Biol. 14, 262–268
35. Taylor, I. C., Workman, J. L., Schuetz, T. J., and Kingston, R. E. (1991) Genes Dev. 5, 1285–1298
36. Han, M., and Grunstein, M. (1988) Cell 55, 1137–1145
37. Li, J., Read, L. R., and Baker, M. D. (2001) Mol. Cell. Biol. 21, 501–510
38. Laribee, R. N., and Klemza, M. J. (2001) J. Immunol. 167, 5160–5166
39. Spencer, V. A., and Davie, J. R. (1999) Gene (Amst.) 240, 1–12
40. Tse, C., Sera, T., Wolfe, A. P., and Hansen, J. C. (1998) Mol. Cell. Biol. 18, 4629–4638
41. Weinmann, A. S., Mitchell, D. M., Sanjabi, S., Bradley, M. N., Hoffmann, A., Lou, H. C., and Smale, S. T. (2001) Nat. Immunol. 2, 51–57
42. Saccani, S., Pantano, S., and Natali, G. (2002) Nat. Immunol. 3, 69–75
Histone Acetylation and Activation of cAMP-response Element-binding Protein Regulate Transcriptional Activation of MKP-M in Lipopolysaccharide-stimulated Macrophages

Tipayaratn Musikacharoen, Yasunobu Yoshikai and Tetsuya Matsuguchi

J. Biol. Chem. 2003, 278:9167-9175.
doi: 10.1074/jbc.M211829200 originally published online January 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211829200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 20 of which can be accessed free at
http://www.jbc.org/content/278/11/9167.full.html#ref-list-1