Modulation of Human Cytomegalovirus Immediate-Early Gene Enhancer by Mitogen-Activated Protein Kinase Kinase Kinase-1

Bin Sun, Greg Harrowe, Christoph Reinhard, Corinne Yoshihara, Keting Chu, and Shaoqiu Zhuo*

Chiron Corporation, 4560 Horton Street, California 94608-2916

Abstract   The immediate-early (IE) promoter of human cytomegalovirus (HCMV) constitutes a primary genetic switch, which determines the progression of viral infection. Earlier reports by others have shown mitogen-activated protein kinase kinase kinase-1 (MEKK1) to be able to up-regulate HCMV-IE promoter through downstream mitogen-activated protein kinase (MAPK) pathways. However, we noticed that the activation of the HCMV-IE promoter by constitutively active MEKK1 (MEKK1-TRU) might not be through the MAPK pathways. Using a HCMV-IE enhancer/promoter (-522 to $+72$) driving a luciferase reporter, we demonstrated that the downstream MAPK activation actually repressed the up-regulation of the promoter by MEKK1 in CHO-K1 and human 293 cells. We further found that the up-regulation of HCMV-IE promoter by MEKK1 could be in great extent suppressed by over-expression of IκBα. Deletion of the NFκB/rel sites in the HCMV-IE enhancer region by mutagenesis proportionally reduced the transcriptional activation by MEKK1-TRU, whereas deletion of the ATF/CREB binding sites or cyclic AMP response elements (CRE) had no effects. Furthermore, the NFκB/rel deletion mutant also showed repression on the basic transcription activity of the HCMV-IE promoter. Our results indicate that the NFκB/rel sites are not only responsible for the modulation of HCMV-IE enhancer activity by MEKK1 but also control the basic transcription activity of the HCMV-IE promoter. On the other hand, the four consensus CRE sites were found to have no function in the activation of the promoter by MEKK1. J. Cell. Biochem. 83: 563–573, 2001.   © 2001 Wiley-Liss, Inc.

Key words: transcription regulation; PKA; MAP kinases; NF-κB; CREB

The mitogen-activated protein (MAP) kinase signaling cascades are evolutionarily conserved cellular signal transduction pathways. These signaling pathways respond to extracellular stimuli such as growth factors, cytokines, serum, and stresses, and regulate physiological processes including cell growth, differentiation, oncogenic transformation, immune responses, and apoptosis [reviewed by Ip and Davis, 1998; Schaeffer and Weber, 1999]. The MAP kinase cascades are organized as a core signaling module. There are typically three protein kinases, MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK), and MAP kinase, in the module although it may be extended upstream [Schaeffer and Weber, 1999]. The upstream kinases sequentially phosphorylate and thus activate the downstream components to transmit the signals. The substrates of activated MAP kinases include other protein kinases and transcription factors that further transduce the signals to modulate gene expression and cellular function. In the mammalian system, six different MAPK signaling modules have been described to date [Schaeffer and Weber, 1999]. An individual signaling module receives different stimuli and activates different substrates. Among them, Erk, JNK, and p38 MAPK are the best-characterized modules.

The human cytomegalovirus immediate early (HCMV-IE) gene encodes two transcription factors, IE1 p72 and IE2 p86, both composed of sequence-specific DNA-binding and activator domains. The expression of IE products is required for HCMV replication, and the activation of HCMV-IE is modulated by an upstream enhancer region (base position -55 to -550) [reviewed by Fortunato and Spector, 1999]. The HCMV-IE enhancer is one of the strongest and most complex regulatory regions for RNA pol

II-directed transcription [Foecking and Hofstetter, 1986] and contains arrays of five 19-, four 18-, and three 21-bp repeats that are consensus binding sites for ATF/CREB, NF-κB/rel, and YY1, respectively [Thomsen et al., 1984; Boshart et al., 1985]. In addition, the HCMV-IE enhancer region has a number of consensus and non-consensus Sp1 sites and one binding site each for serum response element (SRE), CCAAT/enhancer binding protein (C/EBP), ETS factor, AP1, and the retinoic acid receptor RAR-RXR family members [Meier and Stinski, 1996].

The immediate early events of HCMV infection are mediated by virus-host interactions [Fortunato and Spector, 1999]. Many of the host transcription factors involved in controlling HCMV gene expression are regulated by phosphorylation. Erk MAPK activity is sustained for at least 8 h post CMV infection [Rodems and Spector, 1998]. It was also found that HCMV infection of human fibroblasts resulted in a dramatic increase in p38 MAPK phosphorylation [Johnson et al., 1999]. Furthermore, an up-regulation of the HCMV-IE enhancer/promoter activity by stress-activated MAPKs was recently demonstrated [Bruening et al., 1998]. In contrast, we showed that the activation of the HCMV-IE enhancer/promoter by constitutively active MEKK1 (MEKK1-TRU) is not through the MAPK pathways in our routine protein expression experiments. By over-expression of kinase members of the MAPK pathway in CHO-K1 and human 293 cells, we found that Erk2, p38, and JNK1 all repressed the MEKK1-TRU dependent activation of HCMV-IE enhancer/promoter. Therefore, we sought to investigate the signaling pathway and target regulatory sites for MEKK1 mediated up-regulation of the HCMV-IE enhancer/promoter.

METHODS

Cell Lines and Reagents

Most of the experiments were carried out in the CHO-K1 cell line. The starting passage of the cell line is at number 26 and the cells used were not over eight additional passages. Some of the critical experiments were repeated in human 293 cells. The starting passage of 293 cell-line is unknown and no more than 10 additional passages of the cells were used. The CHO-K1 cell line was maintained in HAM's F-12 medium with 10% fetal bovine serum and 35 µg/ml L-proline and the 293 cell line in DMEM with 10% fetal bovine serum and 2 mM L-glutamine. Lipofectamine and Plus reagent were from Gibco-BRL. Dual-Luciferase® Reporter Assay System is the product of Promega. Antibodies against MEKK1, JNK, and IKKβ were purchased from Pharmingen. Antibodies against MEK1, p38, and Erk were from Transduction Laboratories. Antibodies against MKK7 and FLAG-tag were from Santa Cruz Biotechnology and Sigma, respectively.

Plasmids

CMV firefly luciferase reporter vector was constructed by replacement of the SV40 promoter region in pGL3 Control Luciferase Reporter Vector (Promega) with the HCMV-IE enhancer/promoter (-521 to $+72$). The promoter contains all factor binding sites except the SRE site since the SRE sites do not play pivotal roles in the regulation of HCMV-IE transcription [Fortunato and Spector, 1999]. For the control vector, only the minimal promoter (-33 to $+3$) was inserted into the region. NFκB/rel binding site deletion mutants were constructed by changing the essential GGG to TCG. CRE sites were destroyed by changing TGAC to ACCG for the ATF/CREB mutant constructs. Different NFκB/rel binding sites are defined as the followings: NFκB/rel binding site at -422 to -413 (NFκB-a), NFκB/rel site at -271 to -262 (NFκB-b), NFκB/rel site at -166 to -157 (NFκB-c), and NFκB/rel site at -103 to -94 (NFκB-d). Similarly, different CRE sites are defined as the followings: CRE site at -463 to -456 (CREB-a), CRE site at -410 to -403 (CREB-b), CRE site at -327 to -319 (CREB-c), and CRE site at -141 to -134 (CREB-d). For each type of binding site, four mutants were made. These mutants have different number of deletion sites (1 to 4 sites). NFκB-d, NFκB-bc, NFκB-bcd, and NFκB-abcd represent that 1, 2, 3, and 4 NFκB sites are deleted by mutagenesis and the lower case letters indicate the position of deletion. Similarly, CREB-b, CREB-bc, CREB-bcd, and CREB-abcd indicate the number and position of the deletion for CRE sites. Renilla (sea pansy) luciferases vector, pRL-TKluc(R) (Promega), was used as the internal control. Constitutively active rat MEKK1 (rMEKK1-TRU) expression vector, pFc-MEKK1-TRU, and the catalytic subunit of protein kinase A (PKA) expression vector, pFcPKA, were originally purchased from

Stratagene. Expression vectors for nuclear factor CREB and IκBα, pcDNA3.1/GS/CREB, and pcDNA3.1/GS/MAD3, were obtained from Invitrogen. Other expression vectors were constructed as following. Human MEK1, MKK6, MKK7, IKKβ, JNK1, p38, and rat Erk1 were cloned into the pCGN vector [Tanaka and Herr, 1990] equipped with a HA-tag in frame at the N-terminus. Human MKK3, MKK4, p38, p38β2, p38δ and p38γ were cloned into pcDNA3.1 expression vector (Invitrogen) with Flag-tag. All constitutively active or dominant negative mutants were generated from the original clones by using the Quik Change™ Site-directed Mutagenesis Kit (Stratagene). All constructs were confirmed by DNA sequencing. All mutants are named as follows: MEKK1-TRUKR (K432R), MEK1EE (S218E, S222E), MKK3EE (S189E, T193E), MKK4ED (S257E, T261D), MKK7EE (S271E, T275E), JNK1AF (T183A, Y185F), Erk2AF (T183A, Y185F), p38AF (T180A, Y182F), p38β2AF (T180A, Y182F), p38δKM (K54M), p38γAF (T182A, Y184F), IKKβAA (S177A, S181A), IKKβEE (S177E, S181E). All clones are human except for MEKK1 and Erk2 which are both from rat.

Transient DNA Transfection and Luciferase Assays

CHO-K1 or 293 cells in exponential phase were seeded in 12-well plates at 2×10^5 cells/well and incubated at 37°C for 24 h. Before transfection, the cells were washed with 1 ml of Opti-MEM I culture medium (Gibco-BRL) and incubated in 0.4 ml of the same medium for at least 1 h. Cells were then transfected with Lipofectamine Plus (Gibco-BRL) according to the manufacturer's instructions. Transfected cells were incubated at 37°C for 40 ± 3 h without changing the medium. Cells were washed with PBS once before harvesting. Luciferase assays were carried out with the Dual-Luciferase® Reporter Assay System (Promega) following manufacturer's protocols. Experiments were carried out in triplicate samples and average values were presented. Luciferase activities were expressed in the ratio of Firefly/Renilla to normalize the transfection efficiency. In some cases, the values were normalized into the percentage of controls. Each experiment was repeated at least twice.

SDS–PAGE and Western Blot

After luciferase activity assays, the triplicate cell extracts were pooled and centrifuged. Supernatants were concentrated with Centricon 10 (Amicon) and recovered with SDS loading buffer. Samples were run on 4–20% gradient SDS Tris-glycine gels (Novex) and electro-transferred onto nitrocellular membranes. The membranes were then blotted in 5% non-fat milk and probed with primary antibodies at 0.5–2.0 μg/ml in PBS plus 2% milk overnight. After rinsed with PBS for 3–4 times (about 5–10 min each time), the membranes were further incubated with second antibodies coupled to horseradish peroxidase for 45 min and extensively washed with PBS plus 0.025% Tween 20 for three times (5–10 min each time). Specific protein bands were detected with ECL (Amersham Life Science) as suggested by manufacturer.

RESULTS

Activation of HCMV-IE Enhancer/Promoter by MEKK1-TRU

The HCMV-IE enhancer region contains AP1 and ATF/CREB consensus binding sites (CRE sites) [Sambucetti et al., 1989]. The CRE sites have been shown to play an important role in maintaining the basal activity of the IE promoter [Hunninghake et al., 1989; Chang et al., 1990; Niller and Hennighausen, 1990]. The ATF family is targeted by p38 and, in some cases, by JNK for phosphorylation and regulation [Schaeffer and Weber, 1999]. Recently, it has been suggested that MEKK1 might activate HCMV-IE enhancer through the stress-activated MAPKs, p38, and JNK [Bruening et al., 1998]. In addition, it was observed that HCMV infection could induce phosphorylation of p38 but not JNK [Johnson et al., 1999]. Also, it was found that there is sustained Erk activity early during HCMV infection [Rodems and Spector, 1998]. Since MEKK1-TRU can activate more than one pathway [Schlesinger et al., 1998], we expressed different constitutively active MAPK kinases to further characterize different MAPK pathways in the regulation of the HCMV-IE enhancer/promoter. In CHO-K1 cells, only MEKK1-TRU and the constitutively active MEK1 (MEK1EE) showed 9- and 6-fold activation of Firefly Luciferase, respectively, compared with the control (Fig. 1A). The constitutively active MKK3, MKK4, and MKK7 (MKK3EE, MKK4EE, and MKK7EE) constructs demonstrated either no significant activation or a slight repression of the enhancer/

promoter activity, despite the comparable expression of the proteins indicated by Western blotting analysis (Fig. 1A). To ensure that the activation signal by MEKK1-TRU is through the HCMV-IE enhancer/promoter, control reactions were carried out under the same conditions with SV40 pGL3control or a HCMV-IE minimal promoter (-33 to $+3$) reporter vectors. As shown in Figure 1B, there was no activation for the control vectors (data for HCMV-IE minimal promoter reporter vector not shown) following the over-expression of MEKK1-TRU and MEK1EE. Furthermore, the kinase activity-reduced mutant (MEKK1-TRUKR) had a reduced effect under the same conditions. The same experiments were performed in 293 cells and similar results were obtained except that the activation by MEKK1-TRU was less (about 4−8 fold) and there was no significant activation by MEK1EE (data not shown).

Repression of the MEKK1-TRU Dependent HCMV-IE Enhancer Activity by JNK, p38α, and Erk MAPKs

To further characterize the activation of HCMV-IE enhancer/promoter by MEKK1-TRU, we co-expressed human JNK1, p38α, and rat Erk2, and their dominant negative mutants with MEKK1-TRU. Failure to activate the HCMV-IE enhancer/promoter by MKK4 and MKK7 indicates that MEKK1-TRU may not function through the JNK pathway. Indeed, when expressed alone, none of the MAP kinases had any effects on the transcriptional activity of the HCMV-IE promoter (data not shown). When co-expressed with MEKK1-TRU, unexpectedly, JNK1 almost completely suppressed the activation of the enhancer by MEKK1-TRU whereas the dominant negative mutant greatly enhanced the activation (Fig. 2A). Similar results were observed with p38α and Erk2 although the repression was somewhat less profound. Their dominant negative mutants also potentiated the activation by MEKK1 (Fig. 2A). Since MEK1-EE was shown to activate the transcription of HCMV-IE promoter as well (Fig. 1A), to further clarify the role of Erk MAPK in the activation of HCMV-IE promoter, we co-expressed Erk2 with MEK1EE in CHO-K1 cells. Similarly, the co-expression of Erk2 resulted in reduction of the activation of HCMV-IE enhancer/promoter by MEK1EE (Fig. 2B). Its dominant negative mutant had slightly positive effects. The results suggest
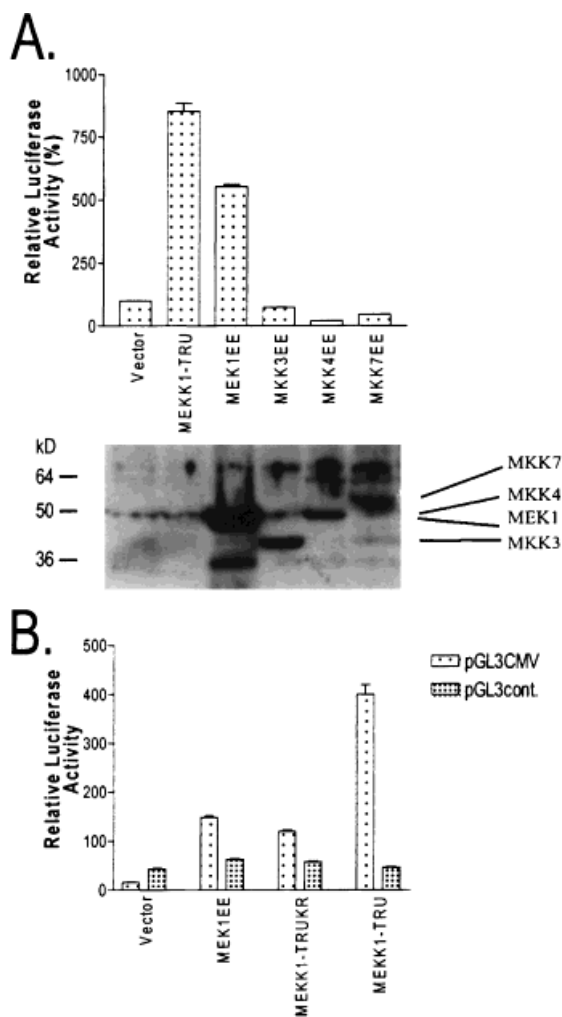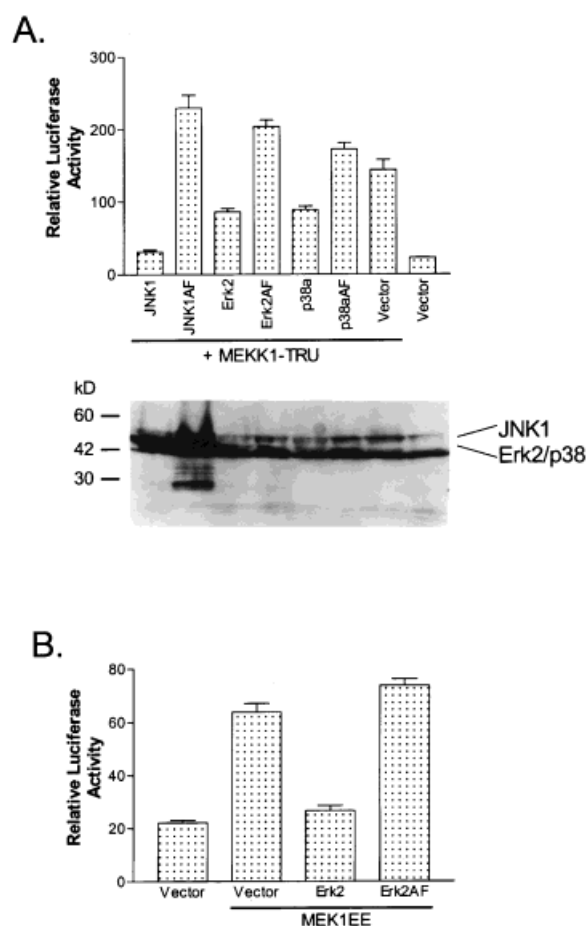

Fig. 1. Activation of HCMV-IE enhancer by MEKK1 and MKK. **A**: CHO-K1 cells in 12-well plates were transfected with 0.02 µg of pGL3CMVlucF, 0.008 µg of pRL-TKlucR, and 1.4 µg of vector or kinase DNA per well. Since two different cloning vectors (pCGN and pcDNA3.1) were used here, each kinase was further normalized to the percentage of the control vectors (vector = 100%). Each point of the luciferase activity is the average of a triplicate assay. After the assay, the three extracts were pooled. Western blot was run as described in the method section. Protein expression was confirmed with a mixture of primary antibodies including rabbit anti-human MEKK1 (Phar-Mingen), mouse anti-human MEK1 (Transduction Laboratories), rabbit anti-Flag Tag (Sigma), and goat anti-mouse MKK7 (Santa Cruz Biotechnology). MKK3 and MKK4 were tagged with Flag-peptide at N-terminals and recognized by anti-Flag antibody. The affinity of anti-MEKK1 is relatively low and can only be detected when other bands are over exposed. **B**: CHO-K1 cells in 12-well plates were transfected with 0.02 µg of pGL3CMVlucF (lightly dotted bars) or pGL3-Control (densely dotted bars), 0.01 µg of pRL-TKlucR, and 1.4 µg of vector or kinase DNA per well. Cells were harvested 40 h after transfection and luciferase signals were detected by using Dual-Luciferase® Reporter Assay System from Promega as described in the experimental section. Luciferase activities were expressed in the ratio of Firefly/Renilla to normalize the transfection efficiency.

that MEK1 may contribute to the activation of HCMV-IE in CHO-K1 cells through some mechanism other than the Erk pathway. Furthermore, MEK1EE does not increase the transcriptional signals of luciferase in 293 cells significantly (data not shown).
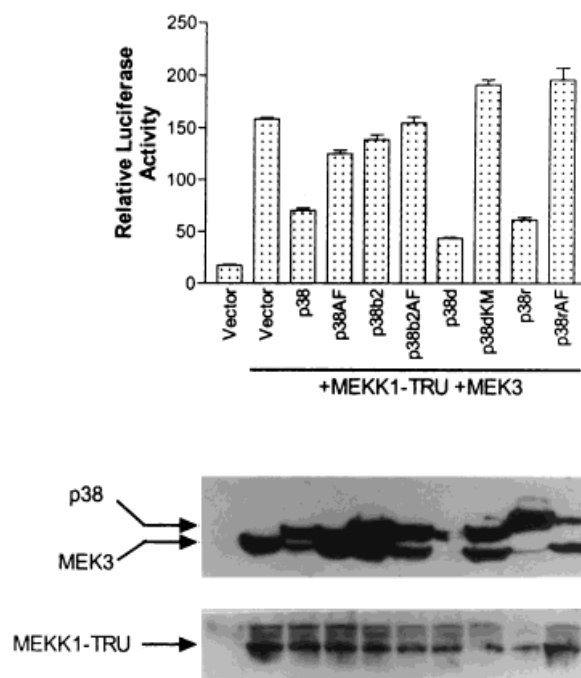
Effects of Expression of p38 MAPK Isoforms on the Up-Regulation of HCMV-IE Enhancer by MEKK1-TRU

Stress activated-MAPKs, p38 and JNK, were showed to up-regulate the activity of HCMV-IE promoter [Bruening et al., 1998]. It was also observed that HCMV infection could induce phosphorylation of p38 and that the p38 inhibitor, SB202190 (FHPI), could retard HCMV permissive infection of HEL cells [Johnson et al., 1999]. However, we found that expressing MKK3EE had no effect on the transcription activity of HCMV-IE promoter (Fig. 1A). To further examine the effects of p38 kinases on the up-regulation of HCMV-IE enhancer by MEKK1-TRU, we co-expressed four p38 kinase isoforms and their dominant negative mutants with both MKK3 and MEKK1-TRU. Co-expression of p38, p38β2, p38δ, or p38γ or their dominant negative mutants in CHO-K1 with MEKK1-TRU and MKK3 resulted in suppression of the HCMV-IE enhancer activity by the wild-type enzymes except for p38β2 (Fig. 3). The Western blot analysis showed similar expression levels for

Fig. 2. Repression of MEKK1 mediated activation of HCMV-IE enhancer by MAPK. CHO-K1 cells in 12-well plates were transfected with 0.02 µg of pGL3CMVlucF, 0.008 µg of pRL-TKlucR, and 1.4 µg of vector or kinase DNA per well. Of the 1.4 µg kinase DNA are 0.7 µg MEKK1-TRU plus 0.7 µg of MAPKs or their dominant negative mutants. Transfection and luciferase activity determination are described as in Methods. Luciferase activities were expressed in the ratio of Firefly/Renilla to normalize the transfection efficiency. Extracts were pooled and subjected to Western analysis as described in Methods. The blot was analyzed with a primary antibody mixture of mouse anti-human JNK1/JNK2 (PharMingen), mouse anti-rat Erk2, and mouse anti-human p38 (Transduction Laboratories).


Fig. 3. Effects of expressing p38 MAPK isoforms on the up-regulation of HCMV-IE enhancer by MEKK1-TRU. CHO-K1 cells in 12-well plates were transfected with 0.02 µg of pGL3CMVlucF, 0.008 µg of pRL-TKlucR, and 1.4 µg of vector or kinase DNA per well. The 1.4 µg of kinase DNA was composed of 0.4 µg of MEKK1-TRU, 0.2 µg of MKK3EE, and 0.8 µg of p38 isoform. For controls, empty cloning vectors were used to replace the kinases. Cells were cultured, harvested, and lysed and luciferase activities were determined as described in Methods. The values were normalized with Renilla luciferase activities. Extracts were pooled and subjected to Western analysis as described in Methods. The blot was first analyzed with rabbit anti-human MEKK1 (PharMingen) and then with anti-Flag antibody. All p38 isoform and MEK3 constructs were tagged with Flag peptide at N-terminals.

most proteins (Fig. 3). All dominant negative mutants had no significant effects. Both kinase active and inactive forms of p38β2 were also found to have no significant effects on the HCMV-IE enhancer activity in CHO-K1 cells. This is consistent with the reports that p38β family is only activated by MKK6, and not by MKK3 [Enslen et al., 1998; Keesler et al., 1998]. Replacement of MKK3 with MKK6 in a similar experiment resulted in 64% inhibition of the MEKK1-TRU mediated luciferase signal by p38β2 (data not shown). This confirms that repression of the MEKK1-TRU mediated activation of HCMV-IE promoter requires activated p38.

Effects of IκB Kinase β (IKKβ) and IκBα on the Activation of HCMV-IE Promoter by MEKK1-TRU

Human CMV-IE enhancer/promoter contains NF-κB/rel consensus binding sites and responds to inflammatory factors. MEKK1 has been shown to phosphorylate and activate both IKKα and IKKβ [Lee et al., 1997]. It is possible that MEKK1-TRU may activate the HCMV-IE enhancer through the activation of IκB kinases. To investigate this possibility, we over expressed human IKKβ, IKKβAL (truncation of the ATP binding domain and thus a kinase inactive form), and IKKβEE (mutation of Ser177 and Ser181 to glutamic acid and thus a constitutively active form) in CHO-K1 cells. In the absence of MEKK1-TRU, no significant activation of HCMV-IE enhancer by IKKβ and IKKβAL can be detected (Fig. 4A). The consti-

tutively active form, IKKβEE, stimulated the basal activity about 0.5–2 folds depending on the individual experiment and cell type used. Under similar conditions, MEKK1-TRU usually stimulated the activity by about 9–12-fold. However, when co-expressed with MEKK1-TRU, IKKβEE additionally potentiated the enhancer activation by MEKK1-TRU (Fig. 4B).
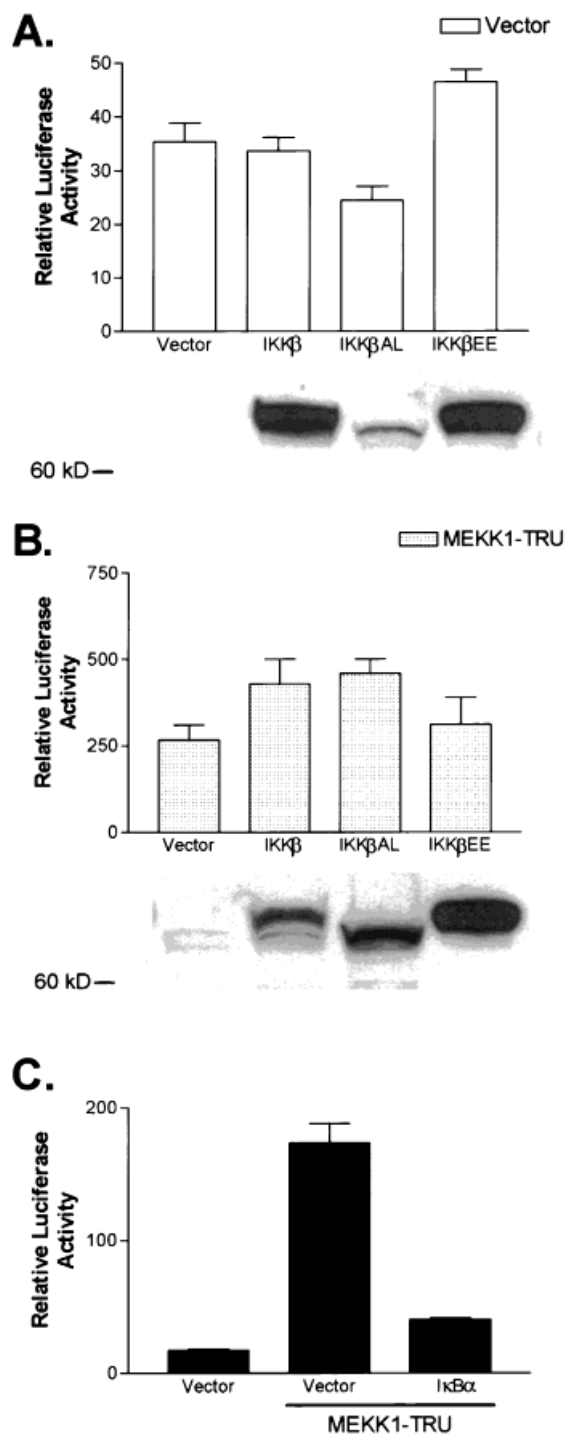
Fig. 4. Effects of IKKβ expression on the transcriptional activity of HCMV-IE enhancer. CHO-K1 cells in 12-well plates were transfected with 0.02 μg of pGL3CMVlucF, 0.008 μg of pRL-TKlucR, and 0.4 μg of pCMV vector or MEKK1-TRU DNA per well. Also included in individual transfection is 1.0 μg of IKKβ, IKKβAL, IKKβEE or pCGN vector. Transfection conditions and luciferase activity determination are the same as in Figure 1A. Cells were cultured, harvested, and lysed, and luciferase activities were determined as described in Figure 1A. The values were normalized with Renilla luciferase activities. The extracts were subjected to Western analysis using rabbit anti-human IKKβ (PharMingen). **A:** Co-expressed with pCMV vector. **B:** Co-expressed with MEKK1-TRU. **C:** CHO-K1 cells in 12-well plates were transfected as following. Transfection medium for each well contained 0.02 μg of pGL3CMVlucF, 0.008 μg of pRL-TKlucR, and 1.4 μg of DNA, which consisted of 1.4 μg vector, 1.0 μg vector plus 0.4 μg MEKK1-TRU or 0.2 μg vector plus 0.4 MEKK1-TRU and 0.8 μg IκBα. Cells were cultured, harvested, and lysed, and luciferase activities were determined as described in Methods. The values were normalized with Renilla luciferase activities.

An unusual observation is that both of the inactive forms of IKKβ, the ATP-domain truncation and the Ser177Ala/Ser181Ala double mutation (data not shown), did not inhibit the activation of HCMV-IE enhancer by MEKK1-TRU and, instead, activated the enhancer activity similar to the wild-type enzyme and the constitutively active mutant. This indicates that the additional activation of the HCMV-IE enhancer by IKKβ in the presence of MEKK1-TRU might not involve its kinase activity.

Since the above results indicate that the activation of all three MAPK pathways may actually have inhibitory effects, it seems unlikely that MEKK1-TRU activates the HCMV-IE enhancer through the CRE or AP-1 sites. Although the dominant negative mutants of IKKβ could not inhibit the MEKK1-TRU mediated activation of the HCMV-IE enhancer, it is still possible that the enzyme may function through the NF-κB/rel sites by other mechanisms. To determine whether the NF-κB/rel sites are essential for the MEKK1-TRU mediated activation of HCMV-IE enhancer, we tried to block the activity by over expression of IκBα. In cells, IκBα functions to retain NF-κB in the cytoplasm and thus inhibits its DNA binding activity [Whiteside and Israël, 1997]. Despite the dominant negative mutants of IKKβ having the opposite effects on the MEKK1-TRU mediated activation of HCMV-IE enhancer, over expression of IκBα blocked the activation by about 65–75% (Fig. 4C). These results suggest that the up-regulation of HCMV-IE enhancer activity by MEKK1-TRU may be associated with the NF-κB/rel sites.

Deletion of NFκB/rel and ATF/CREB Sites on the HCMV-IE Enhancer and its Effects on the Transcriptional Activation by MEKK1-TRU

To further clarify the roles of NFκB/rel and ATF/CREB binding sites (CRE sites) on the MEKK1-TRU mediated HCMV-IE promoter activation, we generated a series of mutants that have some or all of NFκB/rel or CRE sites deleted. HCMV-IE enhancer contains four NFκB/rel sites and five CRE sites [Fortunato and Spector, 1999]. Of five CRE sites, one of them (-67 to -60) is only 85% conserved and not removed in our studies. Figure 5A shows that decrease of NFκB/rel sites on HCMV-IE enhancer region reduces the potential of the construct to be activated by MEKK1-TRU. For some unknown reason, the HCMV-IE enhancer

activity was completely abolished when three NFκB/rel sites were deleted. Further deletion of the last NFκB site on the construct at -422 to -413 recovered some activity instead. On the other hand, deletion of up to four CRE sites had no significant effects on the MEKK1-TRU mediated HCMV-IE promoter activation (Fig. 5B). The results are consistent with that of the above experiments. MEKK1-TRU requires the NFκB/rel and not the CRE sites to activate the HCMV-IE promoter.

Characterization of NFκB/rel and ATF/CREB Binding Site Deletion Mutants

It has been documented that the CRE sites play an important role in maintaining the basal

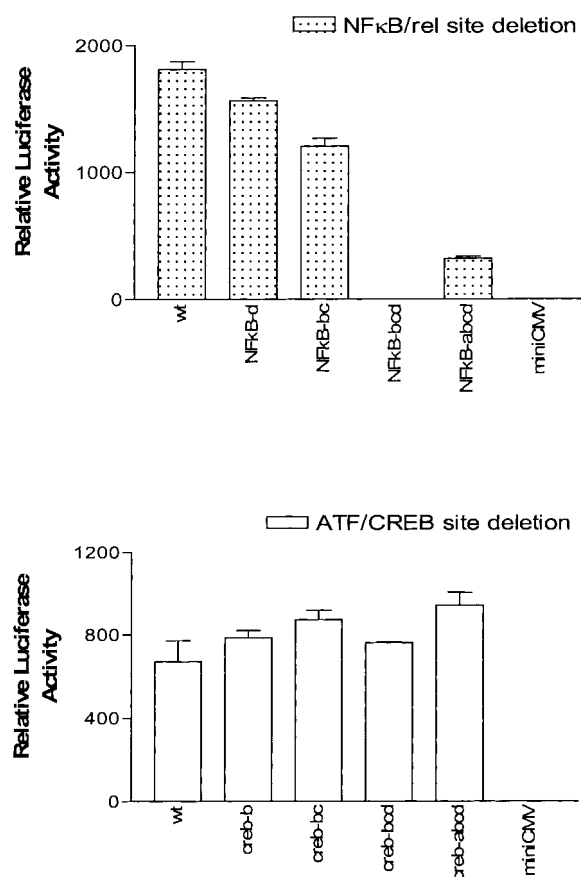**Fig. 5.** MEKK1-TRU-mediated activation of HCMV-IE promoter with different NFκB/rel or ATF/CREB binding site deletion. CHO-K1 cells in 12-well plates were transfected with 0.13 μg of different reporting vectors and 0.008 μg of pRL-TKlucR plus 1.4 μg of vector or MEKK1-TRU DNA per well. Cells were harvested for 40 h after transfection and luciferase signals were detected by using Dual-Luciferase® Reporter Assay System from Promega as described in the experimental section. Luciferase activities were expressed in the ratio of Firefly/Renilla to normalize the transfection efficiency. **A:** Deletion mutants for NFκB/rel site. **B:** Deletion mutants for ATF/CREB site.

activity of the HCMV-IE promoter [Hunninghake et al., 1989; Chang et al., 1990; Niller and Hennighausen, 1990] and that the NF-κB/rel sites respond to factors induced by HCMV virions, cytokines, and the IE1 protein itself [Cherrington and Mocarski, 1989; Sambucetti et al., 1989; Boldogh et al., 1990]. It is interesting to examine the effects of deletion of these transcription factor binding sites on the non-induced (basal) transcription activity of HCMV-IE promoter. To our surprise, only the NFκB/rel deletion mutant (NFκB-abcd) reduced its transcription activity and no effects were observed for the CRE deletion mutant (CREB-abcd) (Fig. 6A). However, it should be born in mind that there is still a less conserved (~85%) ATF/CREB binding site not mutated in the CREB-abcd construct. Since no phenotype was observed for the CRE mutations, the CREB-abcd mutant was further characterized for its response to the activation mediated by PKA. PKA phosphorylates CREB on Ser-133 and activates its DNA binding [reviewed by Shaywitz and Greenberg, 1999]. Because NFκB/rel deletion affects the basal transcriptional activity, the induced activation of HCMV-IE promoter needs to be normalized with the respective basal activity. Figure 6B,C show that MEKK1-TRU-induced promoter activation was reduced by deletion of the NFκB/rel sites and PKA-induced promoter activation was reduced by deletion of the CRE sites. No effects were observed vice versa. This suggests that the CRE sites are only involved in the activation by PKA and not involved in the activation by MEKK1-TRU.

Since all three MAPKs were shown to repress the MEKK1-TRU induced activation of HCMV-IE promoter (Figs. 2 and 3), the four-CRE-site deletion mutant (CREB-abcd) was used to characterize these inhibitory effects. No differences between wt and CRE deletion mutant were observed (data not shown) when the experiments in Figure 2A were repeated using this construct. This suggests that the repression of MEKK1 mediated HCMV-IE promoter activation by MAPKs may not be through the ATF/CREB transcription factor binding sites.

Comparison of the HCMV-IE Promoter Activation Mediated by MEKK1-TRU and IKKβEE

Both MEKK1-TRU and IKKβEE were found to up-regulate HCMV-IE promoter. MEKK1 was reported to activate IKKβ [Lee et al., 1997].
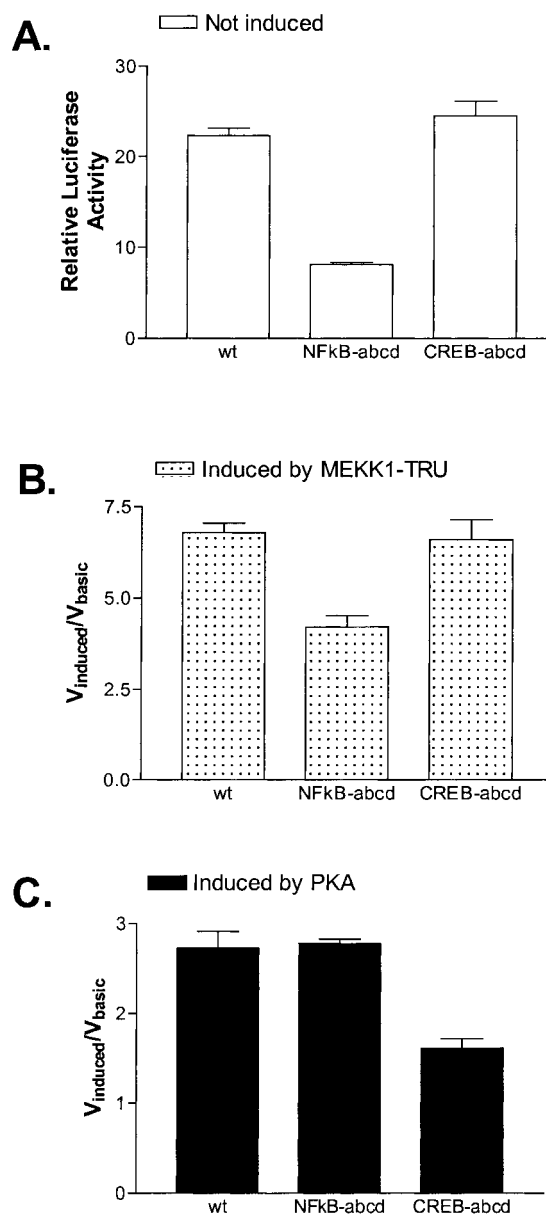
Fig. 6. Effects of deleting NFκB/rel and ATF/CREB binding sites on pGL3CMVlucF. CHO-K1 cells in 12-well plates were transfected with 0.02 μg of different reporting vectors and 0.008 μg of pRL-TKlucR plus 0.5 μg of vector or MEKK1-TRU/PKA DNA per well. Final total DNA is about 1.0 μg/well for all transfection reactions. Vector DNA was used as the balancing DNA. **A:** Non-induced activities. **B:** Induced with MEKK1-TRU and normalized with the respective non-induced activity. **C:** Induced with PKA and normalized with the respective non-induced activity. Experiment conditions are described in Methods.

However, the activation pattern of HCMV-IE promoter by MEKK1-TRU and IKKβEE is very different. Much higher activation was observed for MEKK1-TRU than for IKKβEE. Figure 7 shows the normalized activation for

MEKK1-TRU and IKKβEE with both the wild type and NFκB/rel deletion promoters under the same experimental conditions. With the wild type HCMV-IE promoter, the luciferase activity was elevated about 17-folds over the basal level in MEKK1-TRU transfected CHO-K1 cells while only 2-folds of the elevation was observed for IKKβEE. When all of the NFκB/rel sites were deleted, no luciferase activity increase for IKKβEE transfected cells was detected. However, there were still about 5.7-folds of enhancement in luciferase activity for MEKK1-TRU transfected cells. The results suggest that about 1/3 of the luciferase transcription under the HCMV-IE promoter stimulated by MEKK1-TRU come from some pathway other than NFκB/rel.

DISCUSSION

The MEKK family members respond to a diverse array of extracellular stimuli ranging from growth factors to DNA damaging sensors and, thus, are important for cells to sense exposure to various environmental signals. Another important aspect of this family is that they can transmit the extracellular signals to more than one downstream pathway. For example, MEKK1, the best characterized MEKK member, has been shown to activate not only the JNK, Erk, and p38 MAPK pathways but also both IKKα and IKKβ [Schlesinger
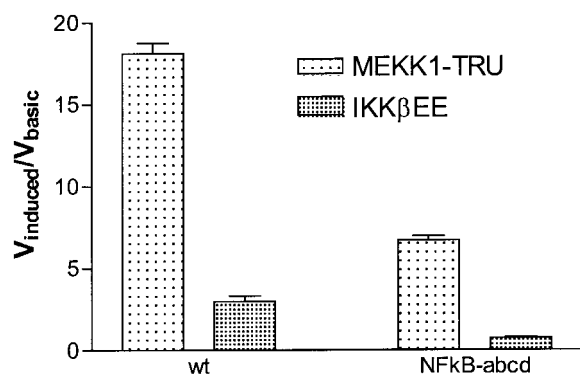
Fig. 7. Comparison of activation of HCMV-IE promoter induced by MEKK1-TRU and IKKβEE. CHO-K1 cells in 12-well plates were transfected with 0.02 μg of different reporting vectors and 0.008 μg of pRL-TKlucR plus 1.0 μg of vector, MEKK1-TRU or IKKβEE DNA per well. Final total DNA is about 1.0 μg/well for all transfection reactions. Cells were transfected as described in experimental section. Firefly luciferase activity was first normalized with Renilla luciferase activity for transfection efficiency and then normalized again with the respective non-induced activity for the folds of activation. Values of about 1.0 represent no activation.

et al., 1998]. On the other hand, the human CMV-IE enhancer/promoter region contains both CRE and NF-κB/rel sites and either of these pathways will respond to MEKK1-TRU activation. Indeed, we as well as others [Bruening et al., 1998] have shown that over-expression of constitutively active MEKK1 results in significant elevation of HCMV-IE enhancer activity (Fig. 1). However, further exploration of downstream signal transduction shows that the enhancer activation by MEKK1-TRU may not be through the MAPK pathways. First, the downstream constitutively active kinases except MEK1 are not able to maintain the activation of the enhancer (Fig. 1A). Second, co-expression of JNK1, Erk2 or p38 with MEKK1-TRU leads to either inhibition by the MAP kinases or further activation by their dominant negative mutants or both (Figs. 2 and 3). Third, deletion of CRE sites on the HCMV-IE enhancer region does not affect the transcriptional enhancement by MEKK1-TRU (Fig. 6B). The other possibility is that MEKK1-TRU activate HCMV-IE enhancer through the NF-κB pathway. MEKK1 has been shown to activate both IKKα and IKKβ, and therefore NF-κB [Schlesinger et al., 1998]. Although the experiments of expressing IKKβ and its dominant negative mutant are not conclusive, over-expression of IκBα did block 65–75% of the HCMV-IE enhancer activity stimulated by over-expression of MEKK1-TRU (Fig. 5C). Furthermore, deletion of NFκB/rel sites on the HCMV-IE enhancer region proportionally reduces the transcriptional activation by MEKK1-TRU (Fig. 6A). However, several lines of evidence suggest that MEKK1-TRU may not necessarily activate the enhancer through the activation of IκB kinases. IKK forms heterodimers in cells [Rothwarf and Karin, 1999]. Activation or inhibition of one component of the kinases could activate or inhibit the whole complex. In the absence of MEKK1-TRU, expression of constitutively activated IKKβ (IKKβEE) does activate HCMV-IE enhancer transcription (Fig. 5A). However, the activation is only about 1–2 fold (2-fold maximum observed). Under similar conditions, MEKK1-TRU stimulates about 9–25 fold higher activity above the uninduced level (depending on the individual experiment). The same results were obtained in 293 cells (data not shown). Most importantly, the dominant negative IKKβ (IKKβAL and IKKβAA) does not block the

stimulation of HCMV-IE enhancer activity by MEKK1-TRU. In contrast, all forms of IKKβ potentiate the enhancer activation by MEKK1-TRU about 1–2 fold (Fig. 5B). The folds of the increased activation are the same as that by IKKβEE in the absence of MEKK1-TRU. Although the mechanism is unclear, the data suggest that the activation of the HCMV-IE enhancer by MEKK1 and IKKβ is complementary. Possibly, IKK pathway only controls the basic activity of the HCMV-IE promoter and MEKK1 has more roles. Our results only show that the NFκB/rel sites are important for MEKK1 in up-regulation of HCMV-IE transcription. However, it may not be the only pathway for MEKK1 in regulating the HCMV-IE promoter. This can be seen in the experiment results of NFκB/rel site deletion mutant. In the absence of NFκB/rel sites, the HCMV-IE promoter can no longer be activated by expression of IKKβEE whereas partial activation can still be observed for expression of MEKK1-TRU (Fig. 7). Consistently, expression of mutated IκBα without the phosphorylation sites did not improve any inhibition results (data not shown). Complete suppression of the HCMV-IE enhancer activation by MEKK1-TRU is never observed for IκBα and the maximal inhibition is about 75%.

Our results clearly demonstrate that downstream activation of Jun, Erk or p38 pathway actually represses the enhancement effects of MEKK1 on HCMV-IE promoter. This is in conflict with the earlier observations [Bruening et al., 1998]. However, the conclusions of the above report are based on the effects of arsenite, which activates JNK and p38, and the p38 inhibitor SB203580. The authors never expressed any active MAPK enzymes in their studies and assumed that the MEKK1 mediated activation of HCMV-IE promoter is due to the activation of MAPKs. Although the authors did express dominant negative JNK in NIH 3T3 cells, the inhibition effects were not impressive. Activation of Erk and p38 MAPK during and post CMV infection [Rodems and Spector, 1998; Johnson et al., 1999] may actually be the responses of infected cells. This could be especially true for p38 family because it responds to many stress stimuli and CMV infection certainly causes stress. Many conclusions of p38 involvement are based on the results from chemical inhibitors. Due to the nature of chemical inhibitors (non-specific and

cross inhibition), we believe that the discrepancy between our results and others' are due to different approach. The repression mechanism by MAPK activation in the MEKK1 mediated enhancement of HCMV-IE promoter is not clear. C-Jun was reported as a negative regulator of basal and PKA-induced transcription from the ucp-1 promoter acting through this proximal CRE region [Yubero et al., 1998]. However, over-expression of CREB did not relieve the repression in the MEKK1 induced activation of HCMV-IE enhancer by JNK1 (data not shown). It seems unlikely that MAP kinases repress the induced promoter activity by activation of CREB. Inhibition of HCMV-IE promoter transcription activity by MAP kinases is only observed when the enhancer is activated by MEKK1-TRU. In the absence of MEKK1 activation, MAPKs have no effects on the basal (un-induced) transcription of the promoter (data not shown). Similar phenomenon was observed for glucocorticoid on the CRE in human glycoprotein hormone α-subunit promoter [Akerblom et al., 1988]. In transfection experiments the negative effect of glucocorticoid was only observed with cells in which the CRE were active. It was suggested that the repression was caused by overlapping target sites on the promoter. Although some of the CRE are closely adjacent to the NFκB/rel sites, this is excluded by the observation that the same repression was also observed in the CRE deletion mutant (CREB-abcd, data not shown). Down-regulation of NF-κB activity on Fas promoter by p38 was also observed by Ivanov and Ronai [2000] in human melanoma cells. Our observation suggests a possibility that the high MAPK activity in undifferentiated and transformed cells could be the cause for the latency observed in many CMV infections. However, the mechanism of the suppression by MAPK activation remains unclear. Further studies are being carried out to identify the downstream targets.

REFERENCES

Akerblom IE, Slater EP, Beato M, Baxter JD, Mellon PL. 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science 241:350–353.

Boldogh I, AbuBaker S, Albrecht T. 1990. Activation of proto-oncogenes : an immediate early event in human cytomegalovirus infection. Science 247:561–564.

Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41:521–530.

Bruening W, Giasson B, Mushynski W, Durham HD. 1998. Activation of stress-activated MAP protein kinases upregulates expression of transgenes driven by the cytomegalovirus immediate/early promoter. Nucleic Acids Research 26:486–489.

Chang YN, Crawford S, Stall J, Rawlins DR, Jeang KT, Hayward GS. 1990. The palindromic series I repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements. J Virol 64:264–277.

Cherrington JM, Mocarski ES. 1989. Human cytomegalovirus ie1 transactivates the alpha promoter-enhancer via an 18-base-pair repeat element. J Virol 63:1435–1440.

Enslen H, Raingeaud J, Davis RJ. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273:1741–1748.

Fortunato EA, Spector DH. 1999. Regulation of human cytomegalovirus gene expression. Adv Virus Res 54:61–128.

Foecking MK, Hofstetter H. 1986. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene 45:101–105.

Hunninghake GW, Monick MM, Liu B, Stinski MF. 1989. The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J Virol 63:3026–3033.

Ip YT, Davis RJ. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. Curr Opin Cell Biol 10:205–219.

Ivanov VN, Ronai Z. 2000. p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF-kappaB activity and fas expression. Oncogene 19:3003–3012.

Johnson RA, Huong SM, Huang ES. 1999. Inhibitory effect of 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole on HCMV DNA replication and permissive infection. Antiviral Research 41:101–111.

Keesler GA, Bray J, Hunt J, Johnson DA, Gleason T, Yao Z, Wang SW, Parker C, Yamane H, Cole C, Lichenstein HS. 1998. Purification and activation of recombinant p38

isoforms alpha, beta, gamma, and delta. Protein Expr Purif 14:221–228.

Lee FS, Hagler J, Chen ZJ, Maniatis T. 1997. Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. Cell 88:213–222.

Meier JL, Stinski MF. 1996. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology 39:331–342.

Niller HH, Hennighausen L. 1990. Phytohemagglutinin-induced activity of cyclic AMP (cAMP) response elements from cytomegalovirus is reduced by cyclosporine and synergistically enhanced by cAMP. J Virol 64:2388–2391.

Rodems SM, Spector DH. 1998. Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. J Virol 72:9173–9180.

Rothwarf DM , Karin M . 1999 . sigtrans;1999/5/re1. The NF-κB activation pathway: a paradigm in information transfer from membrane to nucleus. Science's STKE (26 Oct, 1999).

Sambucetti LC, Cherrington JM, Wilkinson GW, Mocarski ES. 1989. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J 8:4251–4258.

Schaeffer HJ, Weber MJ. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 19:2435–2444.

Schlesinger TK, Fanger GR, Yujiri T, Jonhson GL. 1998. The TAO of MEKK. Front Biosci 3:d1181–d1186.

Shaywitz AJ, Greenberg ME. 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861.

Tanaka M, Herr W. 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell 60:375–386.

Thomsen DR, Stenberg RM, Goins WF, Stinski MF. 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc Natl Acad Sci USA 81:659–663.

Whiteside ST, Israël A. 1997. I kappa B proteins: structure, function, and regulation. Semin Cancer Biol 8:75–82.

Yubero P, Barbera MJ, Alvarez R, Vinas O, Mampel T, Iglesias R, Villarroya F, Giralt M. 1998. Dominant negative regulation by c-Jun of transcription of the uncoupling protein-1 gene through a proximal cAMP-regulatory element: a mechanism for repressing basal and norepinephrine-induced expression of the gene before brown adipocyte differentiation. Mol Endocrinol 12:1023–1037.