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Human Cytomegalovirus Upregulates NF-κB Activity by Transactivating the NF-κB p105/p50 and p65 Promoters

ANDREW D. YUROCHKO,[1]* TIMOTHY F. KOWALIK,[2] SHU-MEI HUONG,[1] AND ENG-SHANG HUANG[1,3,4]

Lineberger Comprehensive Cancer Center,[1] Department of Immunology and Microbiology,[4] and Department of Medicine,[3] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295, and Department of Genetics, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710[2]

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During human cytomegalovirus (HCMV) infection, a series of regulated events take place following virus binding and entry into the cell, including the upregulation of cellular transcription factors, such as NF-κB, which play an essential role in the viral life cycle. We show here that NF-κB message is induced during HCMV infection and that the induction is biphasic, suggesting an initial induction at immediate-early (IE) times and a second round of induction at early times. This hypothesis is supported by experiments using cyclohexamide, which showed that the first tier of induction was drug insensitive, while the second tier was drug sensitive. We then show that virus binding alone is sufficient to stimulate NF-κB DNA binding activity, supporting its role in the initial induction of NF-κB. To begin to elucidate the mechanism(s) for the second tier of NF-κB regulation, we examined promoter constructs from the NF-κB subunits (p105/p50 and p65) for responsiveness following HCMV infection. HCMV infection transactivated the p105/p50 and p65 promoters. The viral IE proteins (IE1-72, IE2-55, and IE2-86) are expressed during the time we see NF-κB induction, so we examined their role in NF-κB induction. The IE1-72, IE2-55, and IE2-86 proteins transactivated the p65 promoter, while only the IE2-55 protein transactivated the p105/p50 promoter. The p105/p50 promoter has NF-κB sites; therefore, upregulation could also be caused by an autoregulatory mechanism. The p65 promoter, however, has been demonstrated to contain only SP1 sites. To investigate the potential role of SP1, we examined nuclear extracts from HCMV-infected cells. Here, we show that there is a biphasic increase in SP1 activity during viral infection and that there is apparently an absolute requirement for SP1 in the transactivation of the p65 promoter. In conclusion, we suggest a model in which the initial induction of NF-κB occurs through viral modulation of cellular factors and the sustained levels of NF-κB induction are regulated by a combination of cellular and viral factors.

Human cytomegalovirus (HCMV) is a double-stranded DNA virus in the herpesvirus family with a genome of over 240 kb (13). It is a ubiquitous virus that infects >60% of the general population and as much as 100% within some populations and/or geographic areas. HCMV is rarely associated with severe clinical symptoms in immunocompetent individuals. However, in immunocompromised individuals and transplant patients and during pregnancy, HCMV infection can manifest itself in severe and often fatal conditions (for a review, see reference 44). In addition, HCMV has been implicated as a co-etiological agent in cervical cancer (78) and has been found associated with a wide range of other tumors (44). One of the common events that takes place during viral infection, and which probably leads to many of the clinical symptoms seen following infection, is the interaction of the virus with the host, i.e., the interplay between cellular and viral factors. This interplay is critical for the regulation of viral gene expression, replication, and maturation and virion release.

During HCMV infection, a coordinated cascade of events takes place. Included in the cascade is the orderly induction of viral genes: immediate-early (IE), early, and then late (63). The IE genes are the first viral genes expressed, and their expression is critical for the regulation of early and late genes. Furthermore, the expression of this first set of genes is heavily dependent on host cell transcription factors. There are now several reported regions in the HCMV genome encoding IE factors (reviewed in reference 81): the major IE (MIE) factor (81), US3 (81), UL36-38 (81), and the TRS1/US22 family (80). The most extensively studied region is the MIE region, which is regulated by the MIE promoter (MIEP) (reviewed in references 29 and 44). Within a few hours postinfection (hpi), a series of overlapping spliced mRNAs are transcribed from the MIEP. The predominant species are a 1.95-kb RNA from the IE1 region (UL123) encoding a protein of 72 kDa (IE1-72, or IE72) and 1.70- and 2.25-kb RNAs from the IE2 region (UL122) encoding proteins of 55 (IE2-55, or IE55) and 86 (IE2-86, or IE86) kDa, respectively (81). IE1-72, IE2-55, and IE2-86 are important transcriptional regulators with multiple functions essential for subsequent viral and cellular gene expression and viral replication. IE1-72 stimulates transcription from HCMV promoters as well as heterologous viral and cellular TATA and TATA-less promoters (6, 9, 16, 25, 30, 36, 53, 82, 86, 87). The mechanism of IE1-72 transactivation may involve the induction of NF-κB (16, 75) or other, unknown processes. IE2-86, on the other hand, is a DNA-binding protein with at least five described DNA binding sites (2, 18, 48, 56, 60, 76). IE2-86 is a promiscuous transactivator of both viral and cellular promoters (9, 23, 30, 35, 36, 41, 53, 59, 64, 69, 76, 84, 87) and may transactivate many of these promoters by associating with the TATA-binding protein within the basal

* Corresponding author. Mailing address: Rm. 117, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, CB# 7295, Chapel Hill, NC 27599-7295. Phone: (919) 966-4323. Fax: (919) 966-4303. Electronic mail address: YUROCHKO @MED.UNC.EDU.

transcription component, TFIID (11, 27, 35, 48, 49, 59, 79). In addition, IE2-86 synergizes with IE1-72 in transactivating a variety of promoters (24, 30, 36, 41, 53, 59, 64, 82, 84, 87) and autorepresses its own promoter (15, 40, 58, 60, 67–69, 82). IE2-55 has been reported to act as a negative regulator of IE1-72 and IE2-86 function by competing with the other IE proteins for host cell factors or interaction with the promoter complex (59) or as a positive regulator of promoter activity (6, 30). Concomitant with this cascade of viral gene expression is an upregulation of cellular genes including genes encoding transcription factors, enzymes needed for viral replication, and factors needed for cell cycle regulation (reviewed in references 1 and 29). An induction of cellular transcription factors is one of the hallmarks of HCMV infection. Of paramount importance to the initial induction and continued expression of the IE genes and also probably early and late genes is the regulation of cellular transcription factors, such as NF-κB (16, 29, 55, 75). NF-κB induction during viral infection is not limited to HCMV infection, as the induction of NF-κB activity occurs in other herpesvirus and nonherpesvirus infections (17, 37, 72).

NF-κB, originally identified by Sen and Baltimore (77), is a member of the *rel* family of transcription factors (for a review, see reference 4). It is a heterodimeric complex made up of two subunits (3): a 50-kDa protein (p50) (32, 51) and a 65-kDa protein (p65) (62, 66, 73). Under nonstimulatory conditions, NF-κB is stored in the cytosol in an inactive form by one of two potential mechanisms: (i) complexed with an inhibitor, IκB (IκBα or MAD3/IκB [33, 38]), which blocks the nuclear localization signal of NF-κB (8, 28, 90), thereby preventing its mobilization to the nucleus, or (ii) in an unprocessed form in which the COOH-terminal end of the p50 precursor, p105, acts like IκB by masking the nuclear localization signal (57, 61, 65, 71). To free active NF-κB from this inactive cytosolic store, the heterodimeric NF-κB has to be released from the IκB. This release has been reported to occur in at least two different ways. In the first reported case, NF-κB complexed with IκBα is freed by phosphorylation of IκBα by an unidentified kinase followed by proteolysis of IκBα and release of NF-κB (31, 39, 50). The process by which phosphorylation leads to degradation is unclear at the present time, although it occurs very rapidly after stimulation of cells with either mitogens or cytokines (7, 20). The second reported mechanism occurs when NF-κB is held via the COOH-terminal portion of p105. In this scenario, an unidentified protease cleaves the COOH-terminal portion of the p105 protein, releasing the active NF-κB complex (61, 71). In either case, when NF-κB is freed of its inhibitor and its nuclear localization signal is unmasked (8, 28, 65, 90), it can translocate to the nucleus and transactivate NF-κB-responsive promoters.

Recent studies have suggested that the initial activation of the MIEP in a productive infection is dependent on NF-κB (16, 75). This conclusion is based on the observation that HCMV maximally induces nuclear NF-κB DNA binding activity at very early times after infection (10, 55, 75). Furthermore, the NF-κB sequences contained in the MIEP are transactivated upon infection, the NF-κB binding to these sequences apparently occurs before binding to other transcription factors, and this NF-κB activity is not sensitive to cyclohexamide (CHX) (75). Previously, it was demonstrated that HCMV infection results in a rapid induction of nuclear NF-κB DNA binding activity (55). This initial and very rapid increase in nuclear NF-κB activity appears to result from the release of preformed cytosolic NF-κB heterodimers (55). The exact mechanism for the HCMV-induced release of cytosolic NF-κB is not known but could involve either the phosphorylation and degradation of IκB or the cleavage of the IκB-like COOH-terminal portion of p105. It appears that there is not an increase in the processing of the p50 precursor, p105 (54), suggesting instead that the initial increase in NF-κB occurs through the disruption of the NF-κB–IκB complex. We know that the IκBα protein is dysregulated during HCMV infection (55) and that there is an HCMV virion-associated kinase that can phosphorylate IκBα in vitro (88). These data suggest a pathway by which NF-κB is rapidly translocated to the nucleus and consequently utilized for viral and cellular IE gene induction. In addition to the increase in NF-κB binding activity, an increase in the steady-state message levels for the NF-κB subunits, p105/p50 and p65, in human foreskin fibroblasts during HCMV infection has also been demonstrated (55). The increase in mRNA levels correlated with the sustained increase in NF-κB activity that was seen during the course of infection. We hypothesize that there are two tiers of NF-κB regulation: (i) an initial release of preformed stores and (ii) de novo synthesis of new NF-κB molecules. To date, there has been no reported induction of p65 message (only constitutive expression) except during HCMV infection. Our data therefore suggest that HCMV uses a novel mechanism for the induction of p65, underscoring its vital role in the viral life cycle. To further elucidate the mechanism(s) of this transcriptional regulation of NF-κB and to determine whether this increase in activity is due to an upregulation of the promoters for the NF-κB subunits, p50 and p65, we investigated the regulation of the p105/p50 and p65 promoters during HCMV infection. We report here that HCMV infection results in the upregulation and transactivation of the p105/p50 and p65 promoters. In addition, we show that the IE genes of HCMV are capable of transactivating the p105/p50 and p65 promoters and that cellular factors such as SP1 and, as reported before, NF-κB (19, 83), which is induced upon HCMV infection, are also responsible for the observed transactivation.

MATERIALS AND METHODS

Virus and cells. HCMV Towne strain was passaged in HEL fibroblasts as described previously (42). For all experiments involving infected cells, cells were infected at a multiplicity of infection of 2 and absorbed for 90 min at 37°C. Free virus was washed off, and the cells were cultured for the desired length of time in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) at 37°C in a 5% CO_2 incubator. For the experiments in which *Drosophila* SL2 cells were used, cells were cultured in Schneider's *Drosophila* medium supplemented with 12% fetal bovine serum (HyClone) at room temperature with continuous stirring. In experiments in which CHX was used, 50 μg of CHX per ml was added to the cells, which were then incubated for 1 h prior to the addition of virus. CHX was also added during the viral absorption stage and for the length of incubation. In some experiments, gradient-purified HCMV was used to infect cells as previously described (43).

RNA isolation and Northern blot analysis. Total cellular RNA from the time course of infected HEL fibroblasts was collected in 4 M guanidinium isothiocyanate solution and isolated by cesium chloride equilibrium centrifugation. Total cellular RNAs from equal numbers of cells (from mock infection through 24 hpi) were electrophoresed on a 1% denaturing formaldehyde agarose gel and transferred overnight to nitrocellulose (Immobilon-NC; Millipore, Bedford, Mass.). The Northern (RNA) blotting data presented are from representative experiments, with the data shown from multiple probes of the same blot. The nitrocellulose blots were probed with ^{32}P-labeled cDNA sequences specific for the p105/p50 (51) and p65 (73) subunits of NF-κB. The probes were labeled by nick translation, Sephadex column purified, and then hybridized to the blots overnight at 42°C. The blots were then washed to a stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 56°C and developed with intensifier screens at −70°C.

Transfection and CAT assays. DNA (10 μg of each plasmid) was transfected into HEL fibroblasts by the calcium phosphate procedure. Cells were then incubated overnight, washed twice in phosphate-buffered saline, and then either infected with HCMV at a multiplicity of infection of 2 as stated above or mock infected (left uninfected). The cells were then incubated for an additional 48 h prior to being harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (34). CAT activity was normalized by using equal protein amounts per sample. In addition, cells were cotransfected with 5 μg of a β-galactosidase expression plasmid and the harvested lysate was

assayed for β-galactosidase activity as a means to equalize for transfection efficiency. Levels of activity were quantified by measuring the [^{14}C]chloramphenicol in acetylated forms compared with unacetylated forms by liquid scintillation. The CAT constructs used in the investigation of p105/p50 promoter activity (pHSCAT [the p105/p50 promoter] and pSSRCAT [the p105/p50 promoter in reverse orientation]) were gifts from A. S. Baldwin, Jr. (19). The backbone vector alone (pUCCAT) was used as a negative control (19). The p65 promoter-CAT construct (pKBCAT) was a gift of K. Ueberla and W. A. Haseltine (85). As a negative control, the backbone vector alone pCAT Basic (Promega, Madison, Wis.) was used. Additionally, in some studies the positive control pIECAT/pHD101CAT3 (22) was used. For cotransfection experiments, 10 μg each of the p105/p50 or p65 promoter-CAT construct was cotransfected with 10 μg each of the pcDNA3-IE1-72, pcDNA3-IE2-55, or pcDNA3-IE2-86 expression plasmids or control plasmid. The pcDNA3 expression plasmids were constructed by digesting our Rous sarcoma virus (RSV)-driven expression plasmids (pRC/RSVIE1-72, pRC/RSVIE2-55, and pRC/RSVIE2-86) with *Eco*RI-*Xba*I (Boehringer Mannheim, Indianapolis, Ind.) for pRC/RSVIE1-72 and *Hin*dIII-*Xba*I (Boehringer Mannheim) for pRC/RSVIE2-55 and pRC/RSVIE2-84 to isolate the various HCMV IE genes. These products were then ligated to an *Eco*RI- or *Hin*dIII-*Xba*I-digested pcDNA3 vector (Invitrogen, San Diego, Calif.) containing a mutant MIEP lacking the negative-regulatory *cis*-regulatory sequence. The original RSV-driven expression plasmids were also used, and similar results were seen (data not shown). Two additional constructs were used in the transfection of the insect cells: the SP1 expression plasmid ppacSP1 (21) and the SP1 frameshift mutant pFXSP1 (91). In addition to the controls stated above, Western blot (immunoblot) analyses were performed with harvested lysates from the cotransfected cells to confirm expression of the various IE products and SP1. Antibodies used include anti-IE1 antibody and anti-IE2 antibody (specific for both isoforms) (27) and anti-SP1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). All CAT assays were repeated at least three times.

Nuclear extract isolation. Nuclear extracts were made as previously described (26, 55, 86). Briefly, mock-infected and infected HEL fibroblasts were collected by first scraping the cells with a rubber policeman and centrifuging the cells. The collected cell pellets were then incubated for 4 min on ice with a cytoplasmic isolation buffer (10 mM HEPES [pH 7.6], 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [Sigma, St. Louis, Mo.], 2 mM phenanthroline [Sigma], 250 μM dichloroisocoumarin [Sigma], 100 μM E-64 [Sigma], and 10 μM pepstatin A [Sigma]). The samples were centrifuged, and the nuclear pellet was isolated by removing the supernatant containing the cytoplasmic extract. The cytoplasmic extracts were not used in this study. The nuclear pellet was washed in the cytoplasmic buffer without Nonidet P-40, spun, and incubated for 10 min on ice with nuclear isolation buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, 2 mM phenanthroline, 250 μM dichloroisocoumarin, 100 μM E-64, and 10 μM pepstatin A). These extracts were then spun, and the supernatant was collected and stored at −70°C.

Electrophoretic mobility shift assays (EMSAs) and gel shift assays. Briefly (for details, see reference 5), collected nuclear extracts were incubated for 15 min in binding buffer (10 mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) containing 7.5 mM $MgCl_2$, 0.1 μg of poly(dI-dC), and a ^{32}P-labeled wild-type GC box (CCTTTTTAAGGGGCGGGGCTT) or mutant GC box (CCTTTTTAAGGTTCGGGGTT) double-stranded oligonucleotide probe for the experiments examining SP1 activity and a wild-type major histocompatibility complex κB binding site (CCTTTTTTTTTAGGGGACTT TCCGA) double-stranded oligonucleotide probe for experiments examining NF-κB activity. The annealed double-stranded oligonucleotide probes with T overhangs and C ends were labeled by filling in the recessed 3′ ends of the oligonucleotides with [α-^{32}P]dATP (ICN, Irvine, Calif.) by using Klenow fragment (Boehringer Mannheim), subjected to a chase with cold dATP and dGTP, and then finally G-25 Sephadex (Boehringer Mannheim) column purified. The samples were then electrophoresed on a 5% polyacrylamide gel, dried, and developed with intensifier screens at −70°C. Antibodies and/or peptides (antibody and control peptides; Santa Cruz Biotechnology, Inc.) were used to supershift the specific complexes of interest (data not shown) by pretreating the extracts for 1 h at 4°C with antibody or antibody plus peptide (preincubated overnight at 4°C according to company protocol).

RESULTS

HCMV infection results in the upregulation of p105/p50 and p65 messages. To more exactly determine the pattern of mRNA expression of the NF-κB subunits, p105/p50 and p65, particularly at IE times of infection, HEL fibroblasts were harvested at various times after HCMV infection and then analyzed for steady-state levels of mRNA by Northern blot analysis. The general pattern of expression of these genes was similar to the previously reported increase in p105/p50 and p65 expression following infection of primary foreskin fibroblasts (55). This time course, however, was more extensive, especially

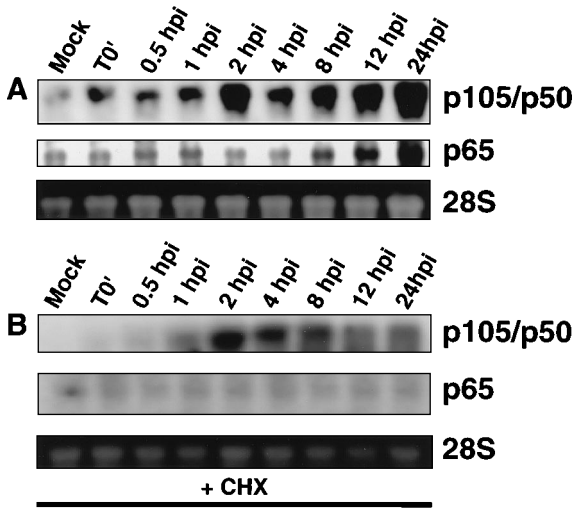

FIG. 1. HCMV infection results in the upregulation of the message for the NF-κB subunits, p105/p50 and p65. (A) Northern blot analysis of an HCMV infection time course of HEL fibroblasts. Lane Mock, uninfected cells; lane T0′, cells harvested immediately following a 90-min viral absorption stage. The blots were hybridized with nick-translated ^{32}P-labeled cDNA probes specific for p105/p50 and p65. Also shown as a control is the 28S ribosome for each lane. (B) Northern blot analysis of a time course of HCMV-infected HEL fibroblasts treated with CHX. The Northern blot analysis was repeated, and the data shown are from a representative experiment. Lanes are as described for panel A.

with regard to IE times, and demonstrates that the increase in p105/p50 expression was biphasic and that the increase in p65 expression was slightly delayed in HEL fibroblasts compared with that in foreskin fibroblasts. From Fig. 1A, it can be seen that the expression of the p105/p50 and p65 messages increased following infection. The initial increase in p105/p50 expression could be detected as early as 2 hpi, followed by a decrease at 4 hpi and a subsequent increase again by 12 hpi. p105/p50 expression reached a maximum by 24 hpi and remained elevated past 72 hpi (data not shown). Furthermore, there is a noticeable increase between mock-infected cells and infected cells harvested at T0′ (after 90 min of incubation with virus), suggesting that virus binding and internalization (described below) are sufficient to induce the first tier of NF-κB induction. p65 is usually constitutively expressed; however, upon HCMV infection, we saw a noticeable increase at 8 to 12 hpi, with a maximum reached by 24 hpi. As with p105/p50 expression, after 24 hpi, p65 levels remained elevated for the duration of the time course (data not shown).

To determine if the initial or sustained increase in p105/p50 and p65 messages required de novo protein synthesis, we infected HEL fibroblasts in the presence of CHX (Fig. 1B). As can be seen in Fig. 1B, the initial peak of induction of p105/p50 mRNA by 2 hpi still occurred and the original autorad showed a slight increase at T0′ to 0.5 hpi, indicating that virus binding or absorption in the absence of protein synthesis is sufficient for the initial increase of this transcript. This contrasted with the second tier of p105/p50 induction, which was blocked by CHX treatment. There was no additional increase in p105/p50 message seen. However, a slight decrease in this transcript by 12 and 24 hpi can be seen, and this correlated with the beginning of a decrease in cell viability. There was no induction of p65 expression following CHX treatment, only a very low level of constitutive expression. These data suggest that infection results in the early release of free NF-κB, which in turn upregulates NF-κB-responsive genes, such as the p105/p50 gene,

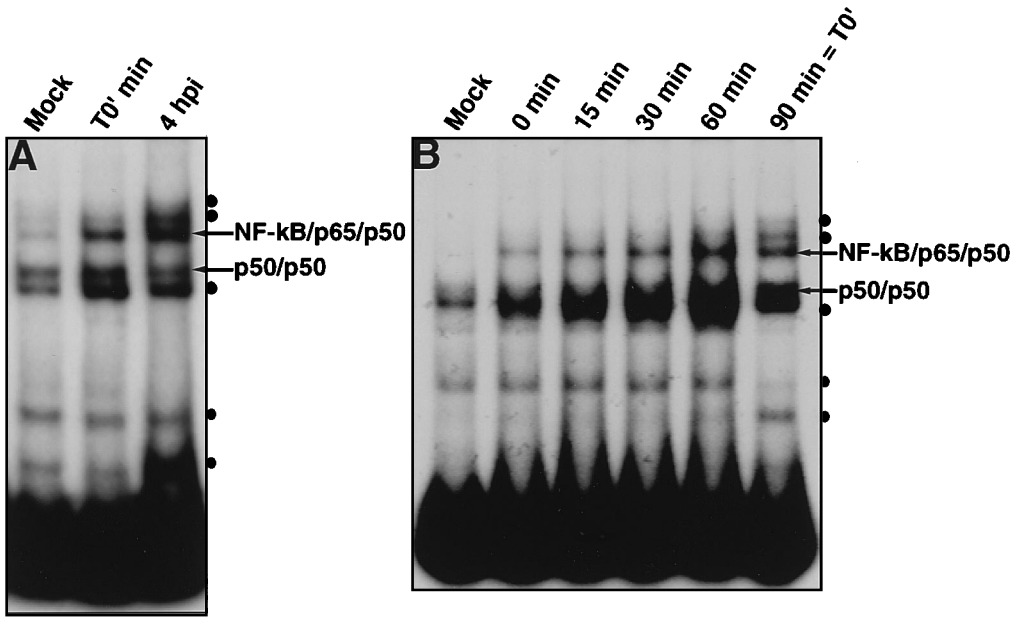

FIG. 2. HCMV binding results in NF-κB activation. (A) EMSA of mock-infected-cell nuclear extracts and extracts created at T0′ or 4 hpi. The T0′ time point represents an extract made immediately after the 90-min virus absorption stage. The mock sample was handled in the same way as the T0′ sample except that virus was not included. (B) EMSA of nuclear extracts processed at the indicated times after virus addition. The 0- to 60-min time points indicate harvesting during the viral absorption period while the 90-min sample was equivalent to T0′ in panel A and other figures. Shifted complexes representing NF-κB p65-p50 and p50-p50 are indicated. The other bands represent nonspecific DNA binding complexes (filled circles) (54, 55). This experiment was repeated, and representative results are shown.

and then the newly synthesized NF-κB further upregulates additional message expression. Viral replication is not required for gene induction, as ganciclovir had no effect on the kinetics of message expression (89).

Rapid induction of NF-κB DNA binding activity upon HCMV infection. Given that the Northern analyses suggested that transcripts for at least p105/p50 increased during the 90-min virus absorption stage and had done so in a manner independent of protein synthesis, we wished to determine if NF-κB DNA binding activity increased at very early times after virus addition. As has been previously shown (16, 55, 75), protein-DNA bound complexes specific for NF-κB were increased by 4 hpi (Fig. 2A). The NF-κB-specific complexes were also induced in the T0′ sample, which represents the 90-min viral absorption-binding period. There were low levels of p50-p50 and p65-p50 complexes visible in the mock-infected sample.

To determine the earliest point in which NF-κB p65-p50 binding activity is induced by HCMV, we harvested a series of nuclear extracts from HEL fibroblasts which were incubated with HCMV from a "true" 0 min (representing the time required to coat a roller bottle with the cells with virus and then harvest them) to 90 min (our T0′ time point). As shown in Fig. 2B, increased p65-p50 activity was apparent at 0 min, and this increase continued through the 90-min time course. Little or no p65-p50 activity was detected in the mock-infected sample. To ensure that viral binding and not a possible contaminating cytokine in the viral supernatant was inducing the mobilization of NF-κB to the nucleus, we used gradient-purified virus for all the experiments (43). This very rapid activation of NF-κB activity would be prior to viral IE protein synthesis and suggests that virus binding or absorption was sufficient for NF-κB activation.

Infection upregulates the p105/p50 promoter. To examine if the increase in message levels of p105/p50 correlated with the increase in the activity of the promoter (diagrammed in Fig. 3A), we transfected the p105/p50 promoter-CAT constructs into HEL fibroblasts and then infected the cells with HCMV. The various p105/p50 promoter-CAT constructs were previously described by Cogswell et al. (19). The data presented in Fig. 3B showed that following HCMV infection there was a greater than fivefold increase in p105/p50 promoter (pHSCAT) activity in comparison with the negative controls, including the vector alone (pUCCAT) and the p105/p50 promoter in reverse orientation (pSSRCAT). There was no CAT activity detected in any of the mock-infected cells. As a positive control, pIECAT/pHD101CAT3 (22) showed nearly a 35-fold induction compared with the vector alone (data not shown). The induction of the p105/p50 promoter following HCMV infection correlated with the increase in p105/p50 message expression that we saw.

HCMV infection also upregulates the p65 promoter. Next, we examined whether the increase in p65 message was due to the upregulation of its promoter (85). Using the p65 promoter-CAT construct (Fig. 4A) (85), we performed transfection-infection experiments. From the data presented in Fig. 4B, it can be seen that following infection there was an increase in p65 promoter CAT activity (pKBCAT) of over 25-fold compared with that in cells with the vector, pCAT Basic, or no DNA or with the activity seen in mock-infected cells. A similar induction was seen with the positive control (pIECAT/pHD101 CAT3; data not shown). The low activity in the p65 promoter in mock-infected cells probably represents the basal constitutive activity that normally occurs with the p65 promoter.

HCMV IE genes play a role in the regulation of the p105/p50 and p65 promoters. To examine the potential role of the MIE gene products in the regulation of the p105/p50 and p65 promoters, we cotransfected the expression plasmid pcDNA3-IE1-72, pcDNA3-IE2-55, or pcDNA3-IE2-86 with the p105/p50 or p65 promoter-CAT construct or the corresponding control construct. The data presented in Fig. 5 demonstrate that all three IE gene products transactivated the p65 pro-

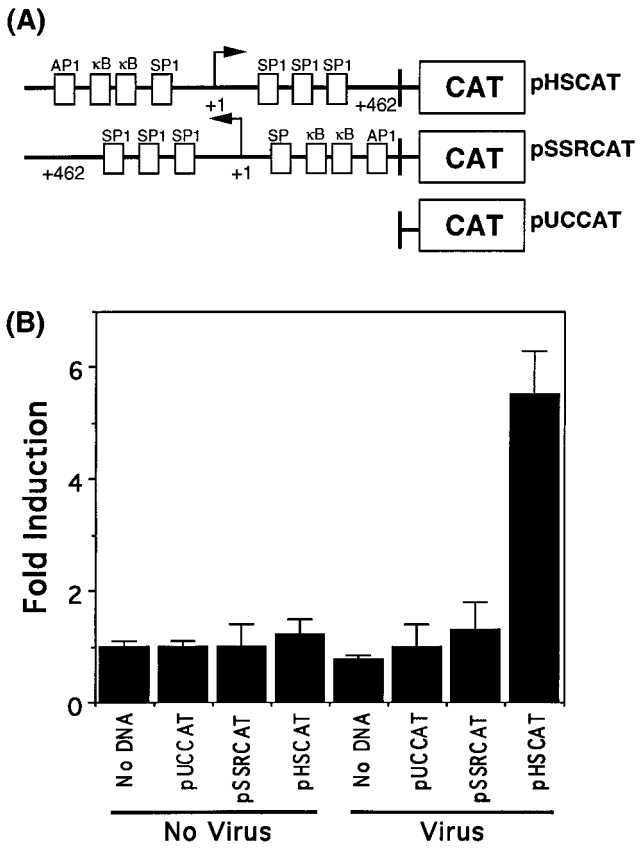

FIG. 3. Infection transactivates the NF-κB p105/p50 promoter. (A) Diagram of the p105/p50 promoter-CAT constructs used and some of the potential transcription factor binding sites (19, 83). (B) CAT assay of HEL fibroblasts transfected with the various p105/p50 promoter constructs with and without HCMV infection (Virus and No Virus, respectively). The constructs used in these experiments are defined as follows: pUCCAT, vector alone; pSSRCAT, p105/p50 promoter in reverse orientation; pHSCAT, p105/p50 promoter; and No DNA, cells alone. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector-alone controls. All CAT assays were repeated.

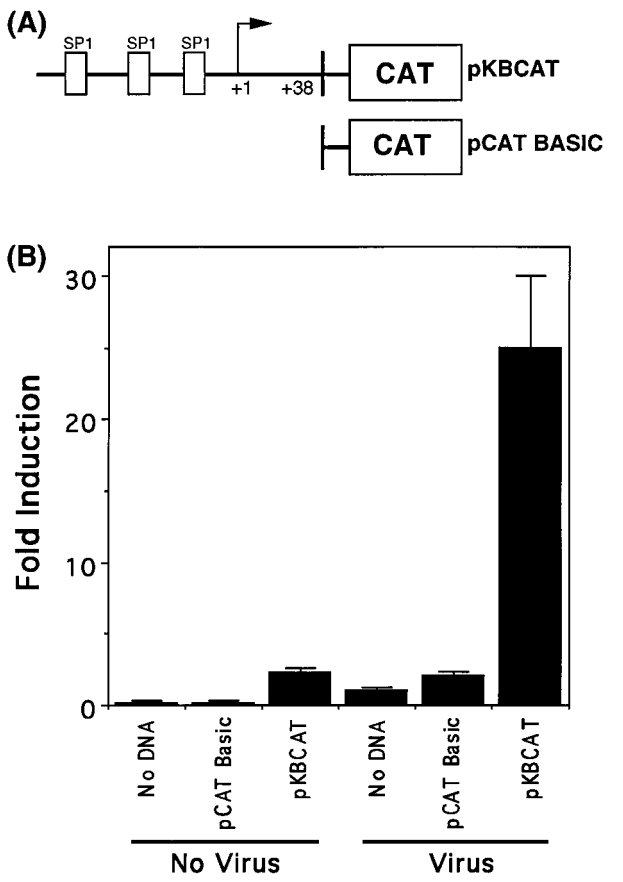

FIG. 4. Infection transactivates the NF-κB p65 promoter. (A) The p65 promoter-CAT constructs used, with the potential transcription factor binding sites marked (85). (B) CAT assay of HEL fibroblasts transfected with the various p65 promoter constructs with and without HCMV infection (Virus and No Virus, respectively). The constructs used are defined as follows: pCAT Basic, vector alone; pKBCAT, p65 promoter; and No DNA, cells alone. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector-alone controls. All CAT assays were repeated.

moter, while only the IE2-55 product transactivated the p105/p50 promoter. Specifically, IE1-72 transactivated the p65 promoter (pKBCAT) but not the p105/p50 promoter (pHSCAT). There was a greater than fivefold activation of the p65 promoter in comparison with the negative controls. The spliced IE2-55 gene product transactivated both the p105/p50 and the p65 promoters. There was a nearly 5-fold activation of the p105/p50 promoter and a >30-fold induction of the p65 promoter. The low-level transactivation of the p105/p50 promoter by IE2-55 was consistently seen. In contrast, IE2-86 significantly transactivated only the p65 promoter (nearly 15-fold). There may be a slight transactivation of the p105/p50 promoter by IE2-86. Western blot analysis of harvested lysate confirmed the presence of the IE gene products (data not shown). Furthermore, titration of the various expression plasmid constructs demonstrated a dose response for each (data not shown). The differential effects of the two IE2 isoforms suggest that they indeed have unique functions during infection, and, furthermore, the actions of IE2-55 are consistent with its function as a bona fide transcription factor, as others have recently shown (6, 30).

SP1 activity increases during infection. Interestingly, the p65 promoter does not contain any NF-κB sites, only three potential SP1 binding sites (GGCGGG) (Fig. 4A) (85), suggesting that an SP1-related mechanism may occur during HCMV infection to upregulate p65 expression. The p105/p50 promoter also has several potential SP1 sites. To examine if SP1 could play a role in the upregulation of NF-κB, EMSAs were performed with nuclear extracts harvested from HEL fibroblasts at various times postinfection. The extracts were probed with either a wild-type consensus SP1 or a mutant SP1 GC box (Fig. 6). The results show that there is biphasic induction of SP1 activity (Fig. 6A). There was a very rapid induction (within 4 hpi) in SP1 binding activity following infection. This increase in SP1 activity was followed by a decrease in binding activity by 8 hpi and then an increase through 24 hpi. Finally, by 48 hpi, SP1 binding activity reached maximum levels. On a longer exposure, one can see a low level of binding activity in the mock-infected, T0′, and 8-hpi lanes of approximately equivalent amounts (data not shown). The two detected bands have been seen previously by others and represent two different forms of SP1 (phosphorylated and unphosphorylated) (46). Competition experiments proved that the activity we detected was indeed bona fide GC box binding (data not shown). Additionally, supershifts were performed with anti-SP1 antibody to determine if the GC box binding was actually SP1 activity (data not shown). The results suggest that the two bands are

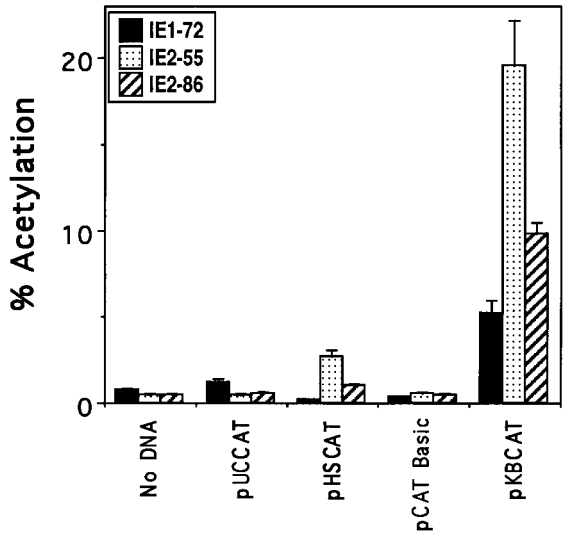

FIG. 5. Transactivation of the p105/p50 and p65 promoters by the HCMV MIE products. CAT assays were performed with HEL fibroblasts cotransfected with the various p105/p50 and p65 promoter-CAT constructs and the IE1-72, IE2-55, and IE2-86 expression plasmids (pcDNA3-IE1-72, pcDNA3-IE2-55, and pcDNA3-IE2-86, respectively) or the appropriate control constructs. The reporter constructs are as defined for Fig. 3 and 4. Percent acetylation was determined as described in Materials and Methods. All CAT assays were repeated.

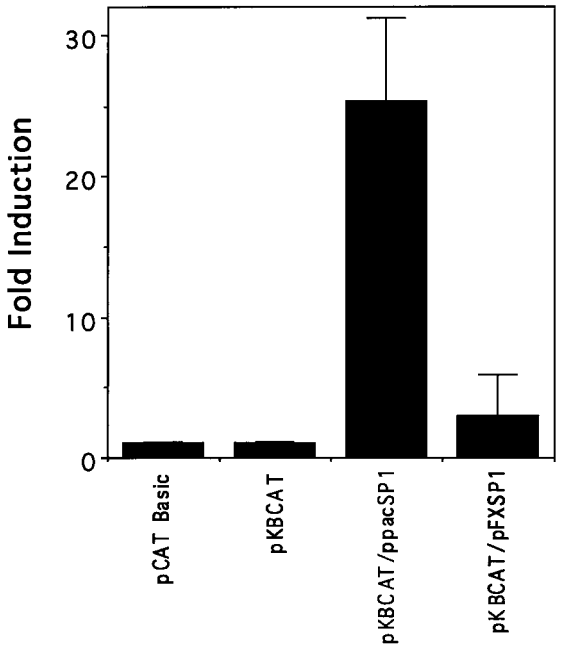

FIG. 7. The p65 promoter may require the SP1 transcription factor for transactivation. Results of CAT assays with *Drosophila* SL2 cells cotransfected with the p65 promoter and an expression plasmid containing either SP1 (ppacSP1) or a frameshift mutation (pFXSP1) are shown. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector-alone controls. CAT assays were repeated.

indeed SP1. Not all the binding activity was supershifted, suggesting that either the antibody is not the most effective supershifting antibody or other GC box binding proteins are also induced, such as SP2 or SP3 (52). No detectable SP1 binding activity was seen in Fig. 6B with the mutant GC box probe.

To examine if SP1 is important in the upregulation of the p65 promoter, we cotransfected *Drosophila* SL2 cells, which lack SP1, with the p65 promoter-CAT construct plus an SP1 expression construct, ppacSP1 (21), or a frameshift mutant, pFXSP1 (91). From the data (Fig. 7), it can be seen that there was no CAT activity detectable unless SP1 was present. When the ppacSP1 construct was cotransfected with the p65 promoter, there was a nearly 25-fold induction, and when the frameshift mutant, pFXSP1, was cotransfected or no additional construct was used, there was only a low level of activity. Western blotting analysis further confirmed the presence or absence of SP1 in the appropriate cellular lysates. These results strongly hint at a requirement for SP1 in the induction of the p65 promoter. To confirm these results, additional studies are under way to map the regions in the p65 promoter that are essential for regulation during HCMV infection.

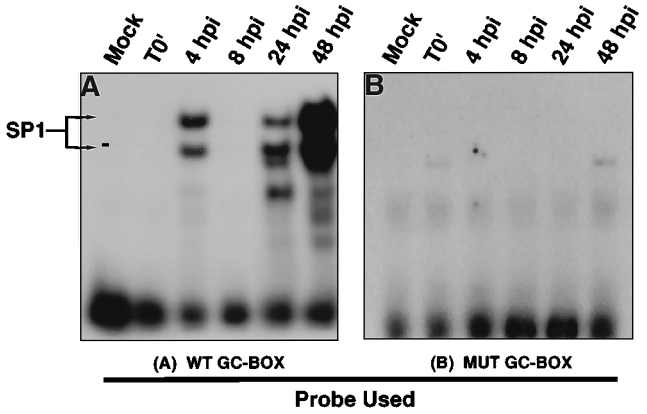

FIG. 6. HCMV infection upregulates SP1 DNA binding activity. (A) EMSA of nuclear extracts from a time course during HCMV infection of HEL fibroblasts. The EMSA probe used was a wild-type (WT) consensus GC box (CCTTTTTAAGGGGCGGGGCTT). (B) EMSA of the same nuclear extract probed with a mutant (MUT) GC box (CCTTTTTAAGGTTCGGGGCTT). The data shown are from a representative experiment. Lanes Mock and T0′ are as defined for Fig. 1.

DISCUSSION

In this study, we have shown that the p105/p50 and p65 promoters of NF-κB are upregulated in HEL fibroblasts following HCMV infection. The activation of these promoters correlates with the increase in the steady-state message levels of p105/p50 and p65 seen after HCMV infection, suggesting that the increase seen in NF-κB levels in infected cells is the result of transcriptional upregulation of the p105/p50 and p65 genes, not just the release of preformed cytosolic stores of NF-κB. Thus, our current data along with previously reported work begin to unravel the multiple pathways utilized by HCMV to regulate NF-κB activity. We have shown that there are two tiers to the regulation of NF-κB: (i) an initial release of existing cytosolic stores, probably via the dysregulation of IκBα, which is CHX insensitive, and (ii) an increase in p50 and p65 molecules through the de novo synthesis of new message and protein, which is CHX sensitive. The increase in new NF-κB molecules itself probably occurs by several mechanisms: autoregulation by NF-κB, an increase in SP1 DNA binding activity, and transactivation by the HCMV IE genes, IE1-72, IE2-86, and IE2-55. The multiple steps of NF-κB regulation that the data suggest are outlined in a model presented in Fig. 8.

The more specific question, then, is how does HCMV infec-

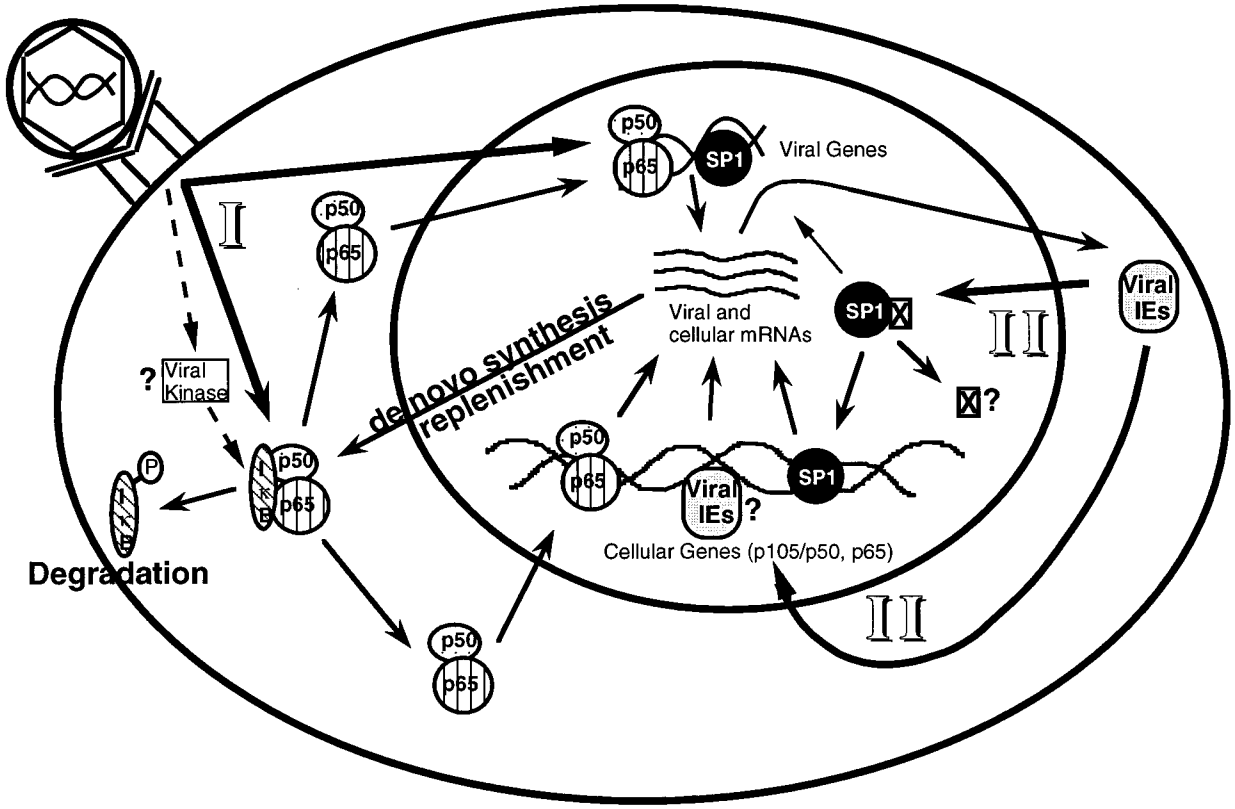

FIG. 8. Potential pathways for induction of p50 and p65 during HCMV infection. (I) The initial binding of HCMV to the cell results in a very rapid induction of free nuclear NF-κB by the phosphorylation of and degradation of IκBα via an unknown cellular pathway. Another possible player in the phosphorylation and subsequent degradation of IκBα is a virion-associated kinase that has been previously shown to phosphorylate IκBα in vitro (88). The translocation of NF-κB to the nucleus results in the upregulation of numerous cellular genes, including the p50 subunit of NF-κB and its inhibitor IκBα, and viral gene products in a process independent of protein synthesis. This increase represents the first tier of NF-κB regulation. (II) Viral gene products could by some unknown mechanism free up other nuclear factors, allowing the induction of cellular genes (such as p65 and SP1), or by acting as transcriptional activators themselves could activate cellular and/or various viral gene products. The action of viral gene products and the de novo synthesis of new NF-κB molecules represent the second tier of NF-κB regulation.

tion result in the transactivation of these NF-κB subunits? As seen from the data presented in this paper, p105/p50 message levels are biphasic, with an early peak at 2 hpi and then a rise again after 12 hpi. This biphasic pattern of p105/p50 expression is similar to what is seen with the regulation of IκBα expression. Both IκBα (45) and p105/p50 (19, 83) have NF-κB sites in their promoters. NF-κB can and has been shown to act in an autoregulatory fashion to transactivate the p105/p50 promoter (19, 83). To account for the biphasic induction of the p105/p50 promoter, we hypothesize that the NF-κB-dependent transactivation initially occurs by the mobilization of preformed NF-κB to the nucleus by very early events, such as receptor-ligand interaction and subsequent signal transduction during viral absorption, and that the sustained levels of p105/p50 message are maintained by the continued synthesis of new NF-κB and degradation of IκBα. Our CHX studies support this hypothesis. In addition, it has been determined that an HCMV virion-associated kinase can phosphorylate IκBα in vitro (88), suggesting an additional virally regulated mechanism that may play a role in the aberrant regulation of IκBα that is seen during HCMV infection. This virion-induced regulation of IκBα would temporally occur after the initial virus- or ligand-induced signaling pathway. The very early induction of NF-κB during HCMV infection that we saw in fibroblasts is supported by the work of others with different cell types (10). Because there are SP1 sites in the p105/p50 promoter, the biphasic increase in SP1 that we see could also be responsible for part of the early and/or late induction of p105/p50. Other putative sites exist in the p105/p50 promoter, such as AP1, PEA 2, and PEA 3 (19); although their function in the regulation of the p105/p50 promoter has not been investigated, their role during viral infection cannot be ruled out. In addition, IE2-55 could play a role in the second tier of p105/p50 upregulation. The levels of p105/p50 promoter transactivation were not as large as those detected for the p65 promoter, suggesting that other mechanisms such as message stability may play a role in maintaining the high levels of p105/p50 transcript seen following infection. Future studies will be designed to address these issues.

p65 expression, which is usually constitutively expressed at low levels, increased following infection. Unlike the p105/p50 promoter, the p65 promoter has been reported to functionally contain only three SP1 sites upstream of the start site and no NF-κB sites (85). Because HCMV infection upregulated SP1 binding activity during the time frame in which we saw increases in p65 message, the mechanism for p65 upregulation by HCMV may occur at least partially through an SP1-mediated event. SP1 levels increase dramatically by 4 hpi, drop by 8 hpi, and then increase again to reach a maximum by 48 hpi. The role of SP1 in p65 induction was supported by transfection experiments with cells lacking SP1. In *Drosophila* SL2 cells, p65 induction was negative when experiments were performed without cotransfection of SP1. We are currently investigating the regions of the p65 promoter that are necessary during

HCMV infection. The IE genes also appear to play a role in this induction, although we cannot exclude the possibility of other mechanisms, such as message stability, contributing to the regulation of p65 during HCMV infection. It seems likely, however, that the ubiquitous nature of SP1 would allow other stimuli to activate SP1 and consequently activate p65, but this has not been shown to occur, with HCMV infection being the only reported stimulus to date in which p65 induction is detected. Furthermore, like p65, SP1 is generally considered to be constitutively present and to be refractory to activation. However, SP1 levels, both protein and mRNA, have been documented to increase during simian virus 40 (SV40) infection (46, 74), perhaps suggesting a mechanism utilized by certain viruses to upregulate the cellular genes necessary for survival. This mechanism may include the viral disruption of an inhibitory complex in which SP1 is stored (14) or the modification, glycosylation (47), or phosphorylation (46) of SP1. Interestingly, SV40 infection causes a preferential increase in the phosphorylated form of SP1 compared with the unphosphorylated form (46). The functional significance of the phosphorylated form of SP1 is not presently clear, although phosphorylation of SP1 occurs only after DNA binding by a DNA-dependent protein kinase (46). However, we do not see a preferential increase in the phosphorylated versus the unphosphorylated form of SP1, but rather we observe an increase in both species of SP1 (89). The reason for this difference is unknown but may result from the differential usage of SP1 in SV40- and HCMV-infected cells. Nevertheless, upregulation of SP1 is still apparently rare. The induction of both SP1 activity and p65 message supports our hypothesis that HCMV utilizes a novel mechanism for the regulation of p65.

The three MIE products, IE1-72, IE2-55, and IE2-86, were able to transactivate these cellular promoters in cotransfection experiments. IE1-72 and IE2-86 both transactivated the p65 promoter but not the p105/p50 promoter. IE1-72 and IE2-86 are known transactivators of many viral and cellular genes (6, 9, 12, 16, 23, 25, 30, 35, 36, 41, 53, 59, 64, 69, 76, 82, 84, 86, 87), so it was not surprising that they also transactivate this promoter. Their inability to upregulate the p105/p50 promoter was surprising, especially for IE2-86, which is known to transactivate most promoters tested, an exception being the MIEP, for which it acts as a repressor (15, 40, 58, 60, 67–69, 82). One additional role IE2-86 could play in the upregulation of the p65 promoter is through its direct interaction with SP1 (59). Perhaps an IE2-86–SP1 complex has a differential regulatory activity in promoters containing SP1-GC box sites. Furthermore, it is possible that virus-cell protein-protein interaction, such as IE2-86–SP1 interaction, is responsible for the unique HCMV induction of p65. IE2-55, on the other hand, transactivated both the p105/p50 and the p65 promoters, suggesting, as Baracchini et al. have reported (6), that IE2-55 is a positive regulator, at least in some cases, of promoter activity (6, 30). The regulation of the NF-κB promoters by IE2-55 could occur only at IE and early times because that was the time frame in which IE2-55 protein expression was seen (70). Lukac et al., however, have reported that IE2-55 acted as a negative regulator of promoter activity (59). The reasons for these differences are presently unclear, but the differences may be due to cell type or promoter specificity. The differential functions of the two IE2 isoforms provide additional evidence that they serve different functions during a permissive infection. In conclusion, the data support a hypothesis in which viral binding induces the first tier of NF-κB activity and this induced activity then upregulates cellular and viral IE gene expression. The newly synthesized viral IE products, in turn, act in a feedback mechanism to maximally activate the NF-κB subunits (the second tier of NF-κB regulation). Finally, it is this complex combination of cellular and viral products that ultimately is responsible for the induction of the cellular and viral genes necessary for viral replication and maturation.

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