JOURNAL OF VIROLOGY, June 1997, p. 4638–4648 Vol. 71, No. 6
0022-538X/97/$04.00+0

Induction of the Transcription Factor Sp1 during Human Cytomegalovirus Infection Mediates Upregulation of the p65 and p105/p50 NF-κB Promoters

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Received 22 January 1997/Accepted 10 March 1997

During human cytomegalovirus (HCMV) infection, the promoters for the classical NF-κB subunits (p65 and p105/p50) are transactivated. Previously, we demonstrated that the viral immediate-early (IE) proteins (IE1-72, IE2-55, and IE2-86) were involved in this upregulation. These viral factors alone, however, could not account for the entirety of the increased levels of transcription. Because one of the hallmarks of HCMV infection is the induction of cellular transcription factors, we hypothesized that one or more of these induced factors was also critical to the regulation of NF-κB during infection. Sp1 was one such factor that might be involved because p65 promoter activity was upregulated by Sp1 and both of the NF-κB subunit promoters are GC rich and contain Sp1 binding sites. Therefore, to detail the role that Sp1 plays in the regulation of NF-κB during infection, we initially examined Sp1 levels for changes during infection. HCMV infection resulted in increased Sp1 mRNA expression, protein levels, and DNA binding activity. Because both promoters were transactivated by Sp1, we reasoned that the upregulation of Sp1 played a role in p65 and p105/p50 promoter activity during infection. To address the specific role of Sp1 in p65 and p105/p50 promoter transactivation by HCMV, we mutated both promoters. These results demonstrated that the Sp1-specific DNA binding sites were involved in the virus-mediated transactivation. Last, to further dissect the role of HCMV in the Sp1-mediated induction of NF-κB, we examined the role that the viral IE genes played in Sp1 regulation. The IE gene products (IE1-72, IE2-55, and IE2-86) cooperated with Sp1 to increase promoter transactivation and physically interacted with Sp1. In addition, the IE2-86 product increased Sp1 DNA binding by possibly freeing up inactive Sp1. These data supported our hypothesis that Sp1 was involved in the upregulation of NF-κB during HCMV infection through the Sp1 binding sites in the p65 and p105/p50 promoters and additionally demonstrated a potential viral mechanism that might be responsible for the upregulation of Sp1 activity.

Human cytomegalovirus (HCMV) is rarely associated with clinical symptoms in immunocompetent individuals. However, in immunocompromised individuals, in transplant patients, during pregnancy, and in certain other instances, HCMV infection can manifest itself in severe and often fatal conditions (for a review, see reference 27). During HCMV infection, an organized cascade of events must take place in order to generate a productive infection. This cascade involves an ordered regulation of viral genes starting with the immediate-early (IE) genes, followed by the early genes and finally the late family of genes (44). The viral IE gene products are the earliest family of regulators expressed, and these gene products are essential for the induction of the early and late gene families. The expression of these vital IE genes is predominantly, if not completely, dependent on host cell transcription factors. This requirement for host cell factors is hypothesized to be the reason for the massive upregulation of cellular transcription factors following HCMV infection.

One of these essential transcription factors induced during HCMV infection is NF-κB. Previously, it was demonstrated that infection results in a rapid induction of nuclear NF-κB DNA binding activity by a receptor/ligand-mediated process and an increase in the steady-state message levels for the two classical NF-κB subunits, p50 and p65 (6, 38, 51, 64, 65). Classical NF-κB, a member of the *rel* family of transcription factors, is a heterodimeric complex made up of two subunits, a 50-kDa (p50, NF-κB1) protein and a 65-kDa (p65, RelA) protein (for reviews, see references 18, 52, and 57). Under nonstimulatory conditions, NF-κB is found in the cytosol in an inactive complex with an inhibitor termed IκB (1, 2, 18, 25, 52, 57). IκB functions by blocking the nuclear localization signal of NF-κB, thereby preventing its mobilization to the nucleus (5, 20, 66). Stimulation results in the release of NF-κB from IκB, mobilization to the nucleus, and subsequent activation of NF-κB-inducible genes (18, 52, 57).

The importance of NF-κB for the virus stems from its critical role in the regulation of the major IE promoter (MIEP) (9, 51) and consequently the regulation of the essential HCMV IE genes (for a review of the IE genes, see reference 55). The MIEP and many other viral promoters have consensus NF-κB binding sites which have been demonstrated to be critical to their regulation (9, 21, 51). NF-κB is also utilized by other herpesviruses and nonherpesviruses (10, 24, 49). The essential role for NF-κB during HCMV infection is further underscored by the fact that to date, no other stimulus has been documented to upregulate p65 message expression (38, 65). During HCMV infection, NF-κB is modulated in two tiers. Initially, there is a release of preformed stores within minutes of a virus-cell interaction in a protein synthesis-independent manner (6, 63–65), followed by the de novo synthesis of new prod-

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uct via p65 and p105/p50 promoter transactivation (65). The second tier of regulation, the de novo synthesis of new NF-κB, appears to occur by at least two important and distinct mechanisms and is dependent on protein synthesis (65). The first mechanism involves the HCMV IE genes (IE1-72 or IE72, IE2-55 or IE55, and IE2-86 or IE86 [for a review, see reference 55]) which are expressed during the time frame in which we see NF-κB upregulation and can transactivate the p65 and p105/p50 promoters (65). Second and equally important is the role of cellular transcription factors in the regulation of these promoters (65).

This previous study (65), although suggesting that cellular factors were involved, did not examine in detail the nature of their specific involvement. Thus, we next undertook a study to examine the role that cellular factors played in the regulation of the two primary rel family members induced during infection, p65 and p105/p50. The role that NF-κB plays in this promoter regulation might be a logical starting point for investigation because it has been shown to autoregulate the p105/p50 promoter (12, 56); however, because it does not transactivate the p65 promoter (60), other cellular transcription factors must play a critical role in the regulation of the NF-κB subunit promoters, particularly the p65 promoter. Our previous work implied that Sp1 might be an important player in the regulation of the p65 promoter (65). In addition, the p65 and p105/p50 promoter regions are GC rich and contain putative Sp1 binding sites (12, 56, 60). In fact, the p65 promoter may contain only Sp1 binding sites (60). Therefore, we chose to focus the present study on determining whether the transcriptional upregulation of p65 and p105/p50 following HCMV infection (38, 65) might be mediated by Sp1. Sp1 is a transcription factor that binds to specific GC-rich elements known as GC boxes (15, 16, 22), is derived from a single gene product (33, 50), and is heavily posttranslationally modified (7, 28, 29). The mechanisms involved in the control of Sp1 activity remain largely uncharacterized, although there have been several recent reports describing potential regulatory products (8, 35, 41, 42, 58, 59). Like NF-κB, Sp1 is an important player in the regulation of cellular transcription and is utilized in the regulation of both herpesvirus and nonherpesvirus gene products (17, 28, 31, 32, 50, 67). As presented in this work, an upregulation of Sp1 mRNA, protein, and DNA binding occurred during HCMV infection, the induction of both NF-κB promoters (the p65 and p105/p50 promoters) was significantly upregulated by Sp1, and the Sp1 sites in these promoters were shown to be necessary for promoter transactivation during infection. We also showed a link between the HCMV IE gene products and Sp1 regulation, suggesting a model whereby the viral induction of Sp1 plays a regulatory role in the second tier of NF-κB regulation and thus helps maintain the cellular environment necessary for the continuation of the viral gene cascade.

MATERIALS AND METHODS

HCMV and cells. Human embryonic lung (HEL) fibroblasts were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, Md.) at 37°C in a 5% CO_2 incubator prior to use. HCMV Towne strain was passaged as previously described (26). For all experiments in which HEL fibroblasts were infected, a multiplicity of infection (MOI) of 2 to 3 was used. Cells and virus were incubated for 90 min at 37°C in a 5% CO_2 incubator, and the free virus was then washed off (we have termed this time zero [t_0]), and the cells were cultured for the desired length of time in Eagle's minimal essential medium supplemented with heat-inactivated 4% fetal bovine serum (GIBCO BRL) at 37°C in a 5% CO_2 incubator. The cells were harvested at the times stated in Results. For experiments involving the Sp1-negative *Drosophila* Schneider line 2 (SL2) cells, cells were cultured in Schneider's *Drosophila* medium (GIBCO BRL) supplemented with 12% fetal bovine serum (GIBCO BRL) at room temperature with continuous stirring as described previously until needed (65). Cells were then plated at 5×10^6, incubated overnight, and used for transfections as stated below.

GST Fusion protein purification. The glutathione *S*-transferase (GST) fusion proteins were collected and harvested as stated previously (19). Briefly, GST fusion proteins were purified from bacterial lysates and bound to glutathione-Sepharose 4B beads (Pharmacia Biotech, Piscataway, N.J.). For the GST-IE fusion proteins, the beads were washed five times with an ice-cold 1% Triton X-100 (Sigma, St. Louis, Mo.)–phosphate-buffered saline solution. The GST fusion proteins were then eluted with a 20 mM reduced glutathione (Boehringer Mannheim Corporation, Indianapolis, Ind.)–100 mM Tris-HCl (pH 8.0) solution, dialyzed, and stored at −70°C until needed. The construction and characterization of the GST–IE1-72, GST–IE2–55, and GST–IE2-86 products, as well as the GST alone were described previously (19). The GST-Sp1 used in these experiments was attached to the glutathione-Sepharose 4B beads (Pharmacia) and then used immediately for investigation of protein-protein interactions. The GST-Sp1 fusion protein was a generous gift from T. D. Gilmore (54).

Nuclear extract isolation. Briefly (14, 38, 65), an infection time course of HEL fibroblasts was collected and 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 [NP-40], 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma], 2 mM phenanthroline [Sigma], 250 μM dichloroisocoumarin [Sigma], 100 μM E-64 [Sigma], 10 μM pepstatin A [Sigma]). The cytoplasmic samples were collected by centrifugation and removal of the cytoplasmic supernatant. The nuclear extract was then isolated by washing the remaining pellet with the above-mentioned cytoplasmic buffer (without NP-40) and then incubating the sample for 10 min on ice with the nuclear isolation buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol, 2 mM phenanthroline, 250 μM dichloroisocoumarin, 100 μM E-64, 10 μM pepstatin A). These extracts were spun, and the supernatant was collected and then stored at −70°C.

RNA isolation and Northern blot analysis. Total cellular RNA was harvested from infected HEL fibroblasts at various times, mock through 96 h postinfection (hpi), in a 4 M guanidine isothiocyanate solution and isolated by cesium chloride equilibrium centrifugation. Equal cell number of total cellular RNA was electrophoresed on a 1% denaturing formaldehyde agarose gel and transferred to a nitrocellulose membrane (Immobilon-NC; Millipore, Bedford, Mass.). The Northern blot analysis was repeated three times, and the data presented are from a representative experiment. The blots were incubated overnight at 42°C with a nick-translated (Boehringer Mannheim), Sephadex column (Boehringer Mannheim)-purified, [α-^{32}P]dATP (ICN, Irvine, Calif.)-labeled 2.1-kb cDNA sequence specific for Sp1 (an *Xho*I fragment digested from the pPacSp1 expression plasmid [13]). The blots were washed to a stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 56°C, developed, and examined by autoradiography.

Western blot analysis. Lysate for Western blot analysis was collected and harvested in two different ways: (i) whole-cell lysate from a time course of infected HEL fibroblasts was harvested in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, scraped, boiled, and then stored at −20°C; or (ii) cytoplasmic and nuclear extracts as described above were mixed 1:2 in an SDS-PAGE buffer, boiled, and then stored at −20°C. Samples were electrophoresed on an SDS–6% polyacrylamide gel, and then the proteins were transferred overnight to nitrocellulose (Immobilon-P; Millipore). Protein concentrations were determined, and equal protein amounts were added to each lane. The blots were blocked with a 5% skim milk–0.1% Tween 20 solution for 1 h and incubated with the primary antibody (1:2,000 anti-human Sp1; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) for 1 h. Next, the blots were washed three times, incubated with the secondary antibody for 1 h (Sigma), washed three times, incubated with the developing agent, and developed according to the enhanced chemiluminescence protocol (Amersham Life Sciences, Arlington Heights, Ill.).

EMSAs. Nuclear extracts were incubated for 15 min in a binding buffer (10 mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) plus 7.5 mM $MgCl_2$, 0.1 μg of poly(dI-dC) (Pharmacia Biotech), and a ^{32}P-labeled wild-type GC box (5′-CCTTTTTAAGGGGCGGGGCTT-3′) or a mutant GC box (5′-CCTTTTTAAGGTTCGGGGCTT-3′) double-stranded oligonucleotide probe for the experiments examining Sp1 DNA binding (3, 65). The samples were electrophoresed on a 5% polyacrylamide gel, dried, and developed with intensifier screens at −70°C. For the electrophoretic mobility shift assays (EMSAs) in which the GST fusion proteins were added, equal protein amounts of the different fusion proteins and GST alone were incubated with the above-mentioned products for the same length of time. Titrations of the fusion products and different incubation conditions were done to determine the optimal conditions for these experiments (data not shown). The oligonucleotide probes containing T overhangs and C ends were labeled by filling in the recessed 3′ ends with [α-^{32}P]dATP (ICN) by using Klenow enzyme (Boehringer Mannheim), chased with unlabeled dATP and dGTP, and then finally Sephadex (Boehringer Mannheim) column purified. An anti-Sp1 antibody (Santa Cruz Biotechnology) with or without the appropriate blocking peptide (Santa Cruz Biotechnology) was used to supershift the specific complexes of interest by pretreating the extracts for 15 min at 4°C with antibody or antibody plus peptide (preincubated overnight at 4°C as specified by the supplier).

Transfection and CAT assays. For the transfection-infection experiments, 10 μg of plasmid DNA (full-length and mutant promoter constructs) was transfected into HEL fibroblasts by the calcium phosphate procedure and incubated overnight (65). The cells were then washed twice in phosphate-buffered saline and either (i) infected at an MOI of 2 to 3 and incubated for an additional 48 h or (ii) incubated for an additional 48 h without virus and treated as mock-infected cells. For cotransfection experiments, 10 μg of promoter chloramphenicol acetyltransferase (CAT) construct (full length or mutant) was cotransfected along with 10 μg of either the Sp1 expression plasmid, pPacSp1 (13), or the negative control Sp1 frameshift mutant, pFXSp1 (67), and then incubated for 48 h. In addition, in some experiments 10 μg of DNA from each of the HCMV IE constructs (pcDNA3-IE1-72, -IE2-55, and -IE2-86 [65]) was cotransfected along with the pPacSp1 or pFXSp1 construct. Titrations of the various expression plasmids were done to determine a dose-response curve (data not shown). In all transfection experiments, equal amounts of DNA were always added to all plates of cells. After incubation, cells were harvested and assayed for CAT activity. CAT activity was normalized by using equal protein amounts per sample, as well as assaying for β-galactosidase activity as a means to equalize for transfection efficiency. Levels of activity were analyzed by counting the [^{14}C]chloramphenicol (Dupont NEN, Boston, Mass.) in acetylated forms and comparing these results to those for the unacetylated forms. The full-length CAT constructs used in this investigation, pKBCAT (the p65 promoter [60, 65] and pHSCAT (the p105/p50 promoter [12, 65]), were described before. The backbone vectors alone (pCAT Basic [Promega, Madison, Wis.] and pUCCAT [12]) were used as a negative control. Additionally, 5′-3′ deletion constructs and site-directed mutants were made. For construction of the p65 promoter deletion constructs (p65F1CAT, p65F3CAT, p65F5CAT, and p65F6CAT [this construct also contains a site-directed mutation in the last GC box]), PCR primer pairs were determined from the p65 promoter sequence (60) and then used to make the different mutants. For the construction of the p65-site-directed mutant (p65F1ΔΔCAT), mutated nested primer pairs were constructed (mutations were made in the specific GC-box sequences) and used to make this construct. These constructs were then purified and ligated by using a T4 DNA ligase (GIBCO BRL) to a linearized pCAT Basic (Promega). See Fig. 4E for schematic diagrams of the constructs. The p105/p50 promoter deletion constructs were made by enzymatic digestion of the full-length promoter (pSpSCAT, *Sph*I; pHiSCAT, *Hin*fI; pASCAT, *Apo*I) followed by purification and ligation to linearized pUCCAT (12). The p105/p50-site-directed mutant (pHSLUCΔSp1) was made and luciferase activity was determined as previously described (11). See Fig. 5E for diagrams of the constructs. In addition to the controls stated above, Western blot analyses were performed on harvested lysates from the cotransfected cells to confirm equal expression of Sp1 or for expression of the IE gene products.

In vitro interactions. Transcription-translation of the HCMV IE proteins (IE1-72, IE2-55, and IE2-86) was done by using the Promega TnT coupled reticulocyte lysate system for [^{35}S]methionine labeling (Promega) as specified by the manufacturer. The purity of the products was examined prior to use on an SDS–10% polyacrylamide gel and subsequently visualized by autoradiography. For examination of the in vitro interactions, Sepharose 4B beads (Pharmacia) complexed with GST-Sp1 were washed three times with ELB$^+$ buffer (0.25 M NaCl, 0.1% NP-40, 0.05 M HEPES [pH 7.0], 0.001 M PMSF, 0.005 M EDTA, 0.5 mM DTT) and then incubated with the in vitro-translated IE products for 1 h at 4°C. The complexes were washed five times with ELB$^+$ buffer and boiled in Laemmli sample buffer. Proteins were then resolved by electrophoresis through a SDS–10% polyacrylamide gel and visualized by autoradiography.

RESULTS

Sp1 message and protein levels were increased in HEL fibroblasts following HCMV infection. Our previous data suggested that in addition to the role of the viral IE products, virally induced host cell factors, such as Sp1, also provided an important contribution to the activation of the NF-κB promoters (65). Therefore, to further our study on the regulation of the NF-κB promoters, we first addressed whether Sp1 levels were altered during infection and thus could be a cellular candidate for the induction of the p65 and p105/p50 promoters during HCMV infection. A time course of whole-cell RNA from mock through 96 hpi was examined by Northern blot analysis. From Fig. 1A, it can be seen that there was an increase in the 8.2-kb Sp1 message (33, 50) by as early as what we have previously termed t_0 (90 min after virus addition, the viral absorption stage), and this increase continued until late times of infection. The two- to threefold increase that was seen in Sp1 message levels at t_0 parallels the early increase seen in p105/p50 message levels (65). Maximum steady-state levels of Sp1 mRNA were detected at 24 to 72 hpi, with a 10-fold increase in message levels by 48 hpi. Next, changes in Sp1 protein levels after HCMV infection were analyzed. Whole-cell and nuclear lysates were isolated and then examined by Western blot analysis with an anti-Sp1 monoclonal antibody (Fig. 1B). These results demonstrated that Sp1 protein levels increased following infection in a biphasic pattern, with an early peak at t_0 to 4 hpi and then a second peak beginning at 24 hpi. The increase in Sp1 protein levels reached a maximum of 10- to 20-fold at 48 hpi. The changes in Sp1 protein levels in the whole-cell lysates paralleled the changes seen in the nuclear lysates, the exception being at 96 hpi, when a significant decrease in Sp1 nuclear levels was detected. This apparent discrepancy can be explained by the results of Western blot analysis of cytosolic extracts collected from cells at these late times of infection. Significant amounts of Sp1 was found in the cytosolic extracts at late times after infection (48 to 96 hpi [data not shown]). In both whole-cell and nuclear lysates, beginning at 24 hpi (and beyond) and to a lesser extent at the t_0 to 4-hpi peak, equal amounts of the slower (phosphorylated)- and faster (unphosphorylated)-migrating species of Sp1 were seen compared to the other time points.

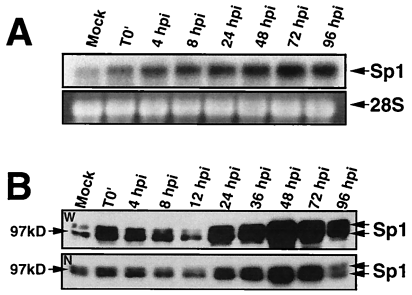

FIG. 1. HCMV infection results in the upregulation of the message and protein for the transcription factor Sp1. A time course of HCMV infection (MOI of 2 to 3) of HEL fibroblasts was performed. (A) A Northern blot probed with a nick-translated ^{32}P-labeled cDNA probe specific for Sp1. As a control, the 28S ribosome for each lane is shown. (B) Western blot analysis of whole-cell (W) and nuclear (N) lysates. An anti-Sp1 antibody (Santa Cruz Biotechnology) was used to probe the blots. The two species of Sp1 are marked on the right, and the appropriate molecular weight marker is indicated on the left. Lanes: Mock, uninfected cells; T0′, cells harvested immediately following a 90-min viral absorption stage. The other lanes represent the time the cells were harvested after t_0. The assays were repeated, and results of a representative experiment is shown.

HCMV infection also upregulates Sp1 DNA binding activity. To investigate whether HCMV-mediated upregulation of Sp1 resulted in elevated Sp1 DNA binding activity, EMSAs were performed. The results of the Sp1 DNA binding activity presented in this report (Fig. 2) are from an expanded time course using the same conditions as were used for Fig. 1. A biphasic increase in Sp1 DNA binding following infection was seen, with one peak at 4 hpi (lane 3; a ≥5-fold increase in activity) and a second peak beginning at 24 hpi (lanes 6 to 10; a 5- to 20-fold increase, depending on the time point) that remained elevated throughout the course of infection. There was a slight decrease in DNA binding detected at late times of infection (lane 10; 96 hpi) supporting the results of the Western blot analysis (Fig. 1B). This correlation is not surprising, as all of the lysates prepared were investigated both for protein levels by Western blot analysis and for DNA binding by EMSA. The extracts were also tested for the ability to bind to a mutant probe (lane 11) and to be supershifted by anti-Sp1 antibodies (lanes 12 and 13) to confirm the specificity of the bound products. As shown in Fig. 2, lane 11, there was no detectable binding of the 48 hpi extract to a mutant GC-box probe. The results were similar for all time points tested (data not shown). Last, as seen in Fig. 2, lane 12, the majority of the shifted

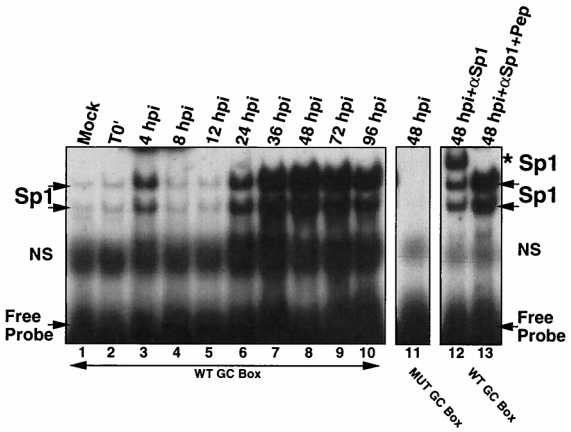

FIG. 2. HCMV infection results in the upregulation of Sp1 DNA binding activity in a biphasic pattern. Shown is EMSA of a time course of Sp1 DNA binding activity during HCMV infection of HEL fibroblasts. Lanes: Mock, uninfected cells; T0′, the 90-min viral absorption step; 3 to 10, the times the cells were harvested after t_0; 11, binding to a mutant probe; 12 and 13, the supershift and the appropriate blocking peptide (Pep) control lanes. Binding of the HEL nuclear extracts to a wild-type (WT) consensus GC box (5′-CCTTTTTAAGGG GCGGGGCTT-3′) (lanes 1 to 10, 12, and 13) or a mutant (MUT) GC box (5′-CCTTTTTAAGGTTCGGGGCTT-3′) (lane 11) is shown. The specific Sp1, supershifted Sp1 (*Sp1), and nonspecific (NS) binding activities are marked. Competition experiments also confirmed that the activity was bona fide GC box binding (data not shown). The data shown are from a representative experiment.

complex is bona fide Sp1, as shown by the supershifted complex in the presence of the anti-Sp1 antibodies. The appropriate blocking peptide confirmed the specificity of the anti-Sp1 antibody used (compare lanes 12 and 13). Again, the results of the supershift experiments were similar for all time points tested (data not shown). The identity of the minor GC-box binding protein(s) (also induced during infection) is unknown and could represent other Sp family members (23, 36).

Sp1 strongly transactivates the p65 and p105/p50 promoters. The work described above demonstrated that HCMV infection significantly increased Sp1 levels. It did not, however, demonstrate that the increase in Sp1 was directly related to the increase in p65 or p105/p50 promoter activity that we have seen (65), even though the GC-rich nature of both promoters (12, 56, 60), the lack of other identifiable transcription factor binding sites besides Sp1 in the p65 promoter (60), and the lack of p65 promoter responsiveness to NF-κB (60), suggesting the involvement of other factors such as Sp1, might indicate a link between the two. Therefore, to directly demonstrate that a link exists between Sp1 and p65 and p105/p50 promoter activity, the full-length p65 and p105/p50 promoter-CAT constructs (pKBCAT and pHSCAT, respectively) were cotransfected into *Drosophila* SL2 cells (which lack endogenous Sp1) along with either an Sp1 expression plasmid, pPacSp1 (13), or an Sp1 frameshift mutant, pFXSp1 (67). These experiments demonstrated that Sp1 was capable of strongly transactivating both promoters (Fig. 3A). There was a 40-fold induction of the p65 promoter (pKBCAT) and over a 30-fold induction of the p105/p50 promoter (pHSCAT) when Sp1 was present (pPacSp1); however, little or no CAT activity was observed in the presence of the Sp1 frameshift control (pFXSp1). A titration of the Sp1 expression plasmid along with the frameshift mutant was also performed and showed that as the levels of Sp1 increased, the activities of the p65 and p105/p50 promoters (Fig. 3B and C, respectively) also increased. Western blot analysis of the harvested lysate confirmed the presence of the Sp1 protein in the extracts. These results thus demonstrated that these two NF-κB promoters were highly responsive to Sp1 in a dose-dependent manner.

Sp1 binding sites influence the regulation of the NF-κB promoters. To demonstrate whether Sp1 plays a direct role in the p65 and p105/p50 promoter regulation during infection, 5′-3′ deletion constructs and site-directed mutants were made for both promoters as stated in Materials and Methods. These promoter mutants were investigated for activity in both cotransfection experiments (with or without Sp1) using *Drosophila* SL2 cells and transfection-infection experiments using HEL fibroblasts.

(i) The p65 promoter. Because of the sole reported presence of putative Sp1 sites in the p65 promoter (60), Sp1 responsiveness of the p65 promoter was investigated first via cotransfection of SL2 cells (Fig. 4A) and then via transfection-infection of HEL fibroblasts (Fig. 4B) with the full-length p65 promoter and various promoter deletion constructs. These results demonstrated a clear role for Sp1 in the regulation of this promoter. As shown in Fig. 4A, the ability of cotransfected Sp1 to positively transactivate the p65 promoter mapped to the GC-rich regions which contained the Sp1 DNA binding sites (Fig. 4E). Although other potential Sp1 binding motifs reside within the p65 promoter region, the centrally located site (−232 bp) conferred the majority of the Sp1 responsiveness, as there was an 86% reduction in the ability of Sp1 to positively modulate the p65 promoter when this site was deleted (Figure 4A; compare p65F3CAT and p65F5CAT). In contrast to the cotransfection experiments, most of the virally mediated activity was located between −575 and −178, suggesting that both the furthest 5′ Sp1 site and the centrally located site were involved in the induction of the reporter construct during infection. In both the cotransfection experiments and the transfection-infection experiments, little activity resided in the third Sp1 site (proximal to the start site), as a construct in which a site-directed mutation was generated in this third GC box (p65F6CAT) had no significant difference in activity compared to the unmutated construct (p65F5CAT). In addition, a site-directed mutant construct in which both the −377 bp and the −232 bp Sp1 sites were mutated (p65F1ΔΔCAT) was made to specifically examine the role of the important Sp1 sites that were documented to be responsible for activity in Fig. 4A and B. As shown in Fig. 4C and D, there was a significant decrease in promoter activity when the first two documented GC boxes were mutated (p65F1ΔΔCAT) compared to the full-length promoter (pKBCAT) or the construct in which the GC boxes were unaltered (p65F1CAT). There was over a 60% decrease in activity when the point mutant construct was examined in cotransfection experiments in SL2 cells following the addition of pPacSp1 (Fig. 4C) and nearly a 90% decrease in activity during HCMV infection (Fig. 4D). There was a detected low level of activity in the p65F5CAT and p65F6CAT constructs following viral infection. These regions, however, failed to respond to Sp1-mediated transactivation, suggesting that the low level of promoter activity observed during the transfection-infection studies was not a result of virally induced Sp1 but rather was a result of another factor(s) interacting through a region near the start of transcription or the result of nonspecific viral transactivation which is commonly observed in HCMV-infected cells.

(ii) The p105/p50 promoter. In classic NF- ± KB, one of the predominant *rel* family complexes induced during HCMV infection (65), p65 never functions without its cognate counterpart p50; thus, it is equally important to examine the role of Sp1 in the regulation of the p105/p50 promoter. Therefore, the potential role of Sp1 in the p105/p50 promoter was also investigated by promoter mutation analysis in cotransfection (Fig.

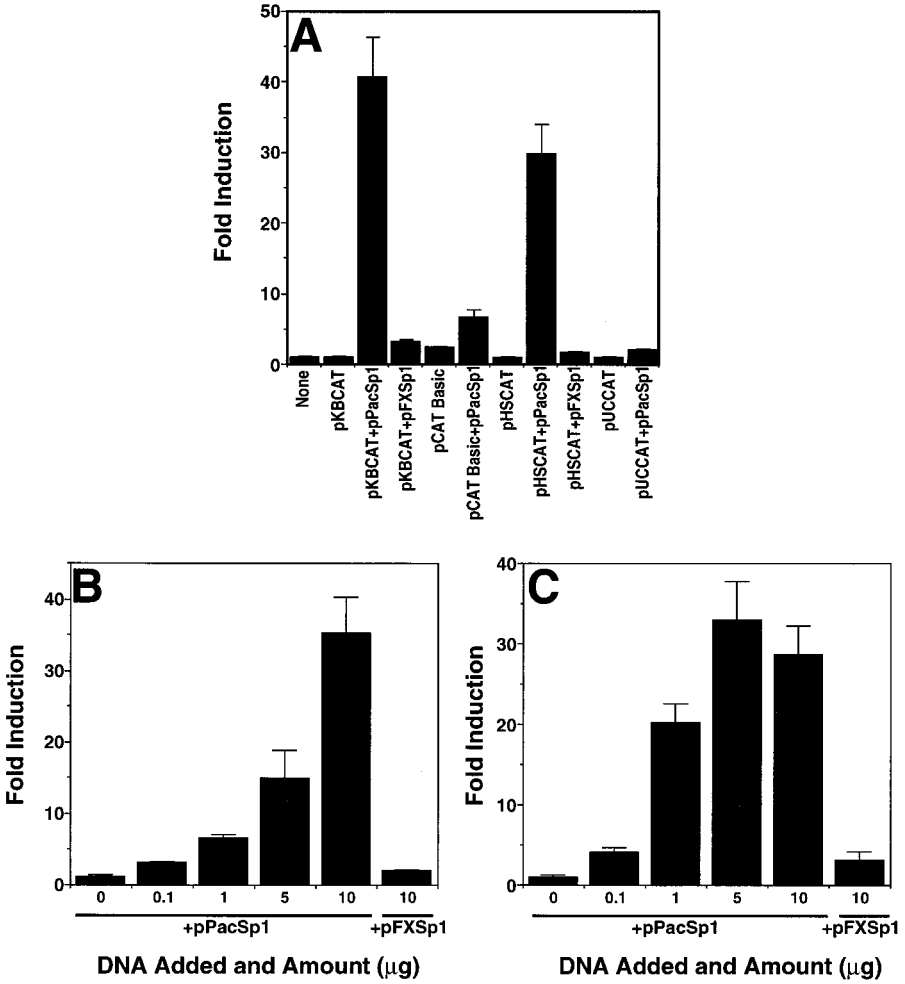

FIG. 3. The p65 and p105/p50 promoters are upregulated by Sp1. CAT assays were performed on harvested *Drosophila* SL2 cells that were transfected with the various constructs or combination of constructs listed. Equal amounts of total plasmid DNA were used for all samples. (A) Comparison of the effects of Sp1 on the p65 and p105/p50 promoters. (B) Effect of the titration of the Sp1 expression construct along with the control mutant construct on the p65 promoter. (C) Effects of the titration of the Sp1 expression construct and the control mutant construct on the p105/p50 promoter. The constructs used are defined as follows: None, no DNA; pKBCAT, the p65 promoter; pCAT Basic, the vector-alone control for the p65-promoter; pHSCAT, the p105/p50 promoter; pUCCAT, the vector-alone control for the p105/p50 promoter; pPacSp1, an Sp1 expression plasmid; and pFXSp1, a frameshift Sp1 mutant. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector-alone controls. The data shown are from a minimum of three replicate experiments.

5A and C) and transfection-infection (Fig. 5B and D) experiments. As shown in Fig. 5A, p105/p50 promoter activity correlated with the loss of the Sp1-responsive elements. There was nearly a 10-fold decrease in pASCAT activity compared to that of the full-length pHSCAT reporter. When the pHiSCAT construct (deletion of the NF-κB, PEA2, and AP1 sites) was used in cotransfection experiments, there was a 58% decrease in Sp1-mediated p105/p50 transactivation compared to the activity of the pSpSCAT construct. Because there are no Sp1 sites in this region, the decrease was most likely not due to a loss of direct Sp1-DNA interaction but probably due to a loss of an interaction between Sp1 and NF-κB that has previously been shown to promote transactivation (41, 46, 47, 53, 54). There was also over a 50% decrease in activity when the Sp1 site in the pHiSCAT construct was deleted (Fig. 5A; compare pHiSCAT and pASCAT). Like the cotransfection experiments, the transfection-infection studies displayed a decrease in p105/p50 promoter activity when pASCAT activity was compared to the activity of full-length pHSCAT construct. A decrease in activity was also seen when the NF-κB sites were deleted (pSpSCAT compared with pHiSCAT activity). The presence of activity in the minimal pASCAT construct following infection was similar to the results obtained for the minimal p65 construct, p65F6CAT, further confirming our hypothesis that viral infection heightened overall transcription. There was not a significant difference between the activities in the pASCAT and pHiSCAT constructs during infection; however, when a construct in which a documented Sp1 site in this region was mutated by site-directed mutagenesis (pHSLUCΔSp1 [11]) was used, there was detected a significant decrease in activity in both the cotransfection (66% decrease) and transfection-infection (51% decrease) experiments compared to the full-length promoter (pHSLUC). Together, these studies demonstrate that the cellular transcription factor Sp1 plays an important role in the transactivation of both the p65 and p105/p50 promoters and that one of the physiological results of the elevated Sp1 levels during HCMV infection is the transcriptional upregulation of the NF-κB-encoding genes.

The IE gene products (IE1-72, IE2-55, and IE2-86) cooperated with Sp1 to upregulate promoter activity. Previously, it

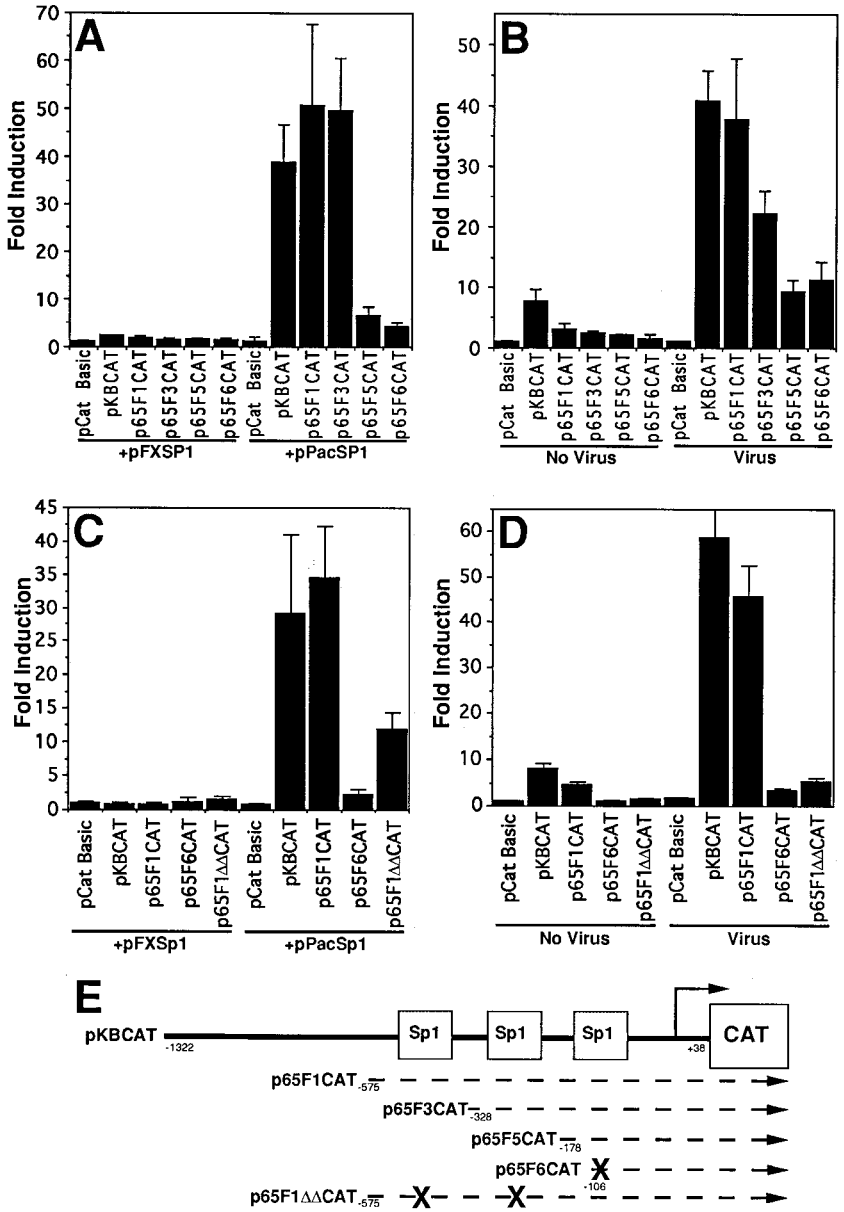

FIG. 4. Mutational analysis of the p65 promoter. Cotransfection and transfection-infection experiments using *Drosophila* SL2 cells and HEL fibroblasts were performed. (A) *Drosophila* SL2 cells were transfected with the various constructs listed plus or minus a functional Sp1 expression plasmid, and then CAT assays were performed. (B) CAT assays were performed on HEL fibroblasts transfected with the constructs listed and then either infected with HCMV (MOI of 2; Virus) or mock treated (No Virus). (C) *Drosophila* SL2 cells were transfected with the various constructs listed plus or minus a functional Sp1 expression plasmid. (D) HEL fibroblasts were transfected with the constructs listed and then either infected with HCMV or mock treated. (E) Diagrams of the p65 promoter and the various mutant constructs, with the known transcription factor binding sites indicated. The constructs used are defined as follows: pCAT Basic, vector alone; pKBCAT, the full-length p65-promoter; p65F1CAT, p65F3CAT, and p65F5CAT, 5′-3′ deletion constructs of the p65 promoter; p65F6CAT, a 5′-3′ deletion construct of the p65 promoter with the third Sp1 binding site mutated by site-directed mutagenesis; p65F1ΔΔCAT, a construct in which the first two Sp1 sites were mutated by site-directed mutagenesis; pPacSp1, an Sp1 expression plasmid; and pFXSp1, a frameshift Sp1 mutant. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector-alone controls. The data shown are from a minimum of three replicate experiments.

was shown that the HCMV IE gene products significantly transactivated the p65 promoter and to a lesser extent transactivated the p105/p50 promoter (64). Therefore, in an attempt to correlate our previously reported findings involving the regulation of the NF-κB subunit promoters by the IE gene products to the findings in the present work, on the role of Sp1 in the regulation of the NF-κB subunit promoters, we next examined whether the IE gene products could interact with Sp1 to promote increased levels of promoter transactivation. The data presented in Fig. 6A show that there was nearly a 40-fold increase p65 promoter activity (pKBCAT) following the addition of the Sp1 expression construct (pPacSp1) and that when the IE expression plasmids (pcDNA3-IE1-72, -IE2-55, and -IE2-86 [65]) were cotransfected along with Sp1, there was a further increase in activity (33, 200, and 100% increases following the addition of IE1-72, IE2-55, and IE2-86, respectively). There was little or no activity seen when the IE expression constructs were used in the absence of functional Sp1 (either

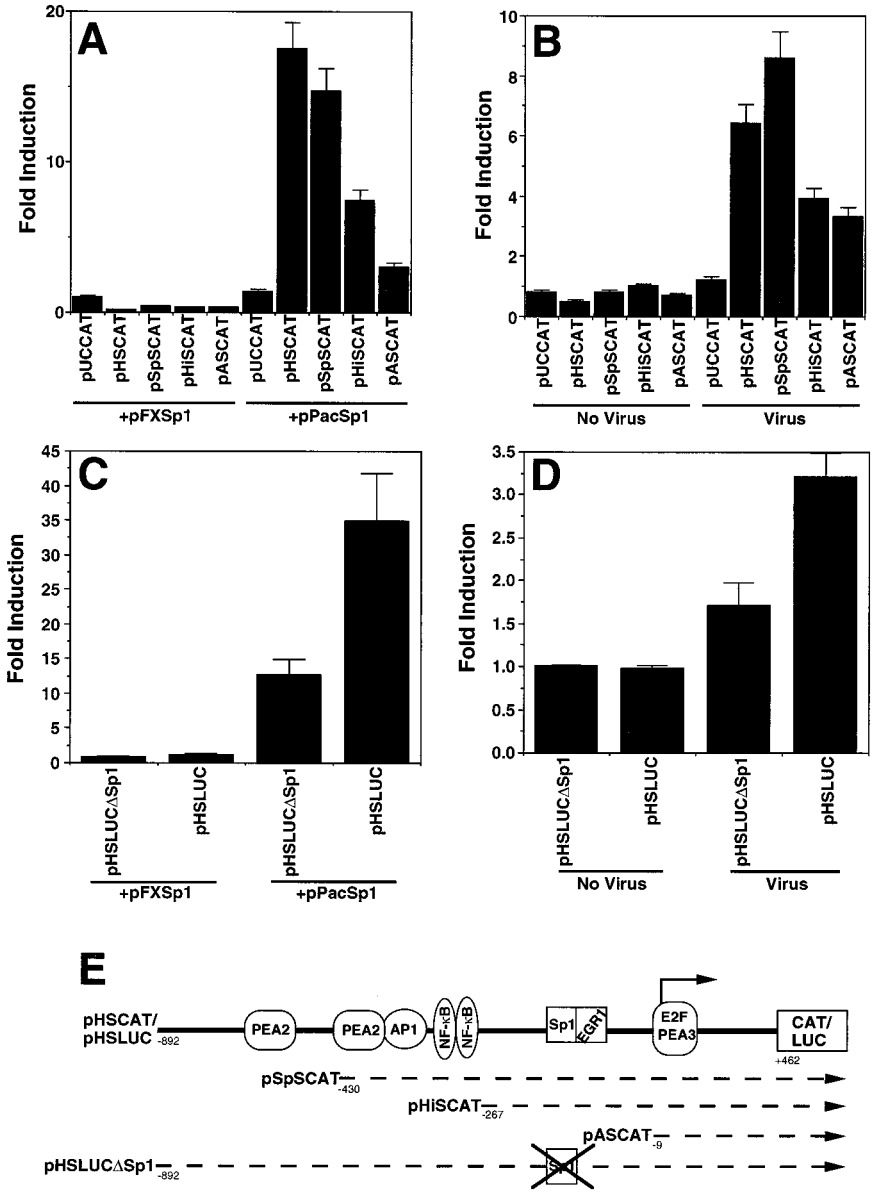

FIG. 5. Mutational analysis of the p105/p50 promoter. Cotransfections and transfection-infections of *Drosophila* SL2 cells and HEL fibroblasts were performed, and then the harvested lysates were examined for CAT activity. (A) *Drosophila* SL2 cells were cotransfected with the various constructs plus or minus a functional Sp1 expression plasmid (pPacSp1 or pFXSp1). (B) HEL fibroblasts were transfected with the various constructs and then either infected with HCMV (Virus) or mock treated (No Virus). (C) *Drosophila* SL2 cells were cotransfected with the various constructs plus or minus a functional Sp1 expression plasmid. (D) HEL fibroblasts were transfected with the various constructs and then either infected with HCMV or mock treated. (E) Diagram of the p105/p50 promoter, with the binding sites for various transcription factors indicated. The constructs used are defined as follows: pUCCAT, vector alone; pHSCAT, the full-length p105/p50 promoter; pSpSCAT, pHiSCAT, and pASCAT, 5′-3′ deletion constructs of the p105/p50 promoter; pHSLUCΔSp1, a full-length p105/p50 promoter in which a known Sp1 site was mutated by site-directed mutagenesis; pHSLUC, the full-length p105/p50 promoter; pPacSp1, a Sp1 expression plasmid; and pFXSp1, a frameshift Sp1 mutant. Fold induction represents the difference between the percent acetylation of the test samples and that of the vector alone controls. The data shown are from a minimum of three replicate experiments.

no Sp1 added or the addition of the mutant control expression construct, pFXSp1). In addition there was no activity when the control reporter construct (pCAT Basic) or the empty vector (pcDNA3) was used (data not shown). Similar results, although less striking, were seen when the p105/p50 promoter construct (pHSCAT) was used (data not shown), supporting our previous findings that the IE constructs appear to play a larger role in the upregulation of the p65 promoter than the p105/p50 promoter and that the IE2-55 gene product is a bona fide transactivator of this promoter (65).

IE86 increases Sp1 DNA binding activity. Because the documented increase in Sp1 DNA binding activity during infection may not be solely the result of increased levels of Sp1 but also the result of the release of Sp1 bound to an inhibitor as was suggested by Chen et al. (8), we wanted to examine if an interaction between the various IE proteins and Sp1 occurs and to determine if the IE products could function similarly to pRB by freeing up inactive Sp1 and thus promoting increased Sp1 DNA binding. To perform these experiments, GST-IE fusion proteins were added to the EMSA reaction buffer along

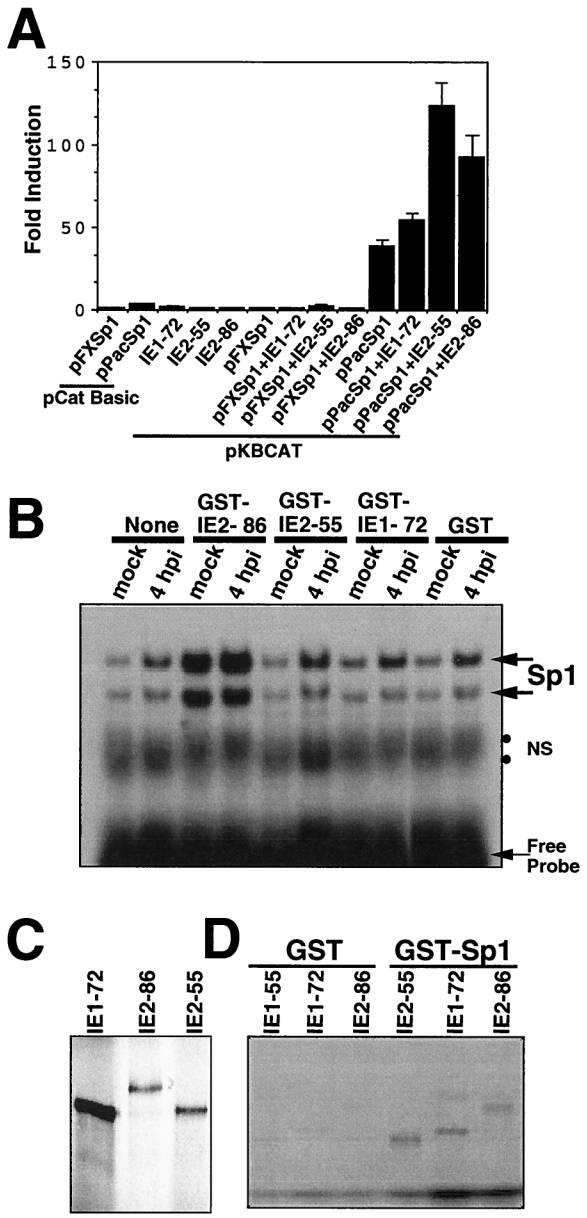

FIG. 6. Role of the HCMV IE gene products in the regulation of Sp1. (A) CAT assays were performed on *Drosophila* SL2 cells cotransfected with the p65 promoter (pKBCAT) or the control construct (pCAT Basic) along with the Sp1 expression construct (pPacSp1) or the control construct (pFXSp1) and/or the IE expression constructs (pcDNA3-IE1-72, pcDNA3-IE2-55, pcDNA3-IE2-86 [labeled IE1-72, IE2-55, IE2-86, respectively]). Equal amounts of total plasmid DNA was used for all samples. Fold induction represents the difference between the percent acetylation of the test samples and that of the control samples. (B) EMSA of HEL nuclear extract pretreated with the various GST-IE fusion proteins prior to electrophoresis. The specific Sp1 and nonspecific (NS) bands are marked. A wild-type consensus GC box (CCTTTTTAAGGGGCGGGGCTT) was used as a probe. (C) Input levels of the three in vitro-translated IE gene products (IE1-72, IE2-55, and IE2-86) used for panel D. (D) Examination of the interaction between Sp1 (GST-Sp1 or the control product, GST alone) and the IE gene products (in vitro-translated IE1-72, IE2-55, and IE2-86). All experiments were repeated.

with the nuclear extract, prior to electrophoresis. The data demonstrated (Fig. 6B) that the addition of GST–IE2-86 increased Sp1 DNA binding in both mock and 4-hpi lysates (5- to 10-fold). GST–IE2-55 slightly increased Sp1 binding in a reproducible manner in the 4-hpi lysate (twofold), while GST–IE1-72 and GST alone could not, suggesting that one of the mechanisms involved in the increased Sp1 activity seen during infection could be mediated by the IE2-86 gene product. Additional studies showed that this IE2-86-mediated event was titratable and could occur at all time points tested (data not shown). This idea of a negative regulator of Sp1 function that mediates its effect through a direct protein-protein interaction (8) was supported by experiments in which an increase in Sp1 DNA binding occurred following treatment of extracts with deoxycholate to disrupt protein-protein interactions (in a manner similar to that used for the disruption of the cytosolic NF-κB–IκB complex) (63).

Sp1 physically interacts with the HCMV IE gene products. The data presented in Fig. 6A and B suggested that there might, in fact, be a protein-protein interaction between the IE genes and Sp1; therefore, we examined if the IE gene products could directly interact with Sp1. To address this possibility, GST-Sp1 or the control product, GST alone, was incubated with in vitro-translated IE1-72, IE2-55, and IE2-86 prior to the GST pull-down assay and electrophoresis. The results of these pull-down assays (Fig. 6D) demonstrated that the three IE gene products interacted with GST-Sp1 but not GST alone. Figure 6C shows the input level of the in vitro-translated products that were used in the pull-down assays presented in Fig. 6D.

DISCUSSION

In conclusion, we have demonstrated an induction of the cellular transcription factor Sp1 during infection and showed that Sp1 is a key player in the upregulation of the p65 and p105/p50 NF-κB promoters. The discovery of the transactivation of the p65 and p105/p50 promoters by Sp1 supports our hypothesis of the importance of this factor in NF-κB regulation during HCMV infection. From a cellular and viral standpoint, this finding has important biological consequences because under normal circumstances, Sp1 levels are limiting within the cell (50). If Sp1 levels are usually limiting, then overexpression of Sp1 (such as during HCMV infection) would be expected to dramatically increase transactivation due to the presence of the excess pool of Sp1. Furthermore, because overexpression of Sp1 results in superactivation (13, 45), the HCMV-induced overexpression of Sp1 would definitely significantly affect promoter regulation. With the exception of an increase in Sp1 by simian virus 40 (28, 50), little evidence exists documenting an increase in Sp1 levels during other types of stimulation. This apparent viral restriction on the induction of Sp1 suggests that it must play an essential role during the viral life cycle (at least during simian virus 40 and HCMV infections). This potentially vital role of Sp1 for HCMV is emphasized by our recent studies demonstrating that the addition of Sp1 results in a 5- to 10-fold increase in MIEP activity (63). The importance of Sp1 is further underscored by the possibility that the p65 promoter, which has not been documented to be induced under any stimulus except HCMV infection (38, 65), contains only Sp1 binding sites (60).

Because of the potential importance of Sp1 in the regulation of the NF-κB-promoters, we first investigated the regulation of Sp1 itself during HCMV infection. There was an increase in Sp1 mRNA expression, protein levels, and nuclear DNA binding activity (≥10-fold increase in each). The increase in protein levels and DNA binding activity followed a biphasic induction pattern whereas the increase in message expression did not, suggesting different mechanisms of regulation at the transcriptional (and/or perhaps mechanisms of message stability) and

translational levels. The IE induction of Sp1 levels is probably due to a signal transduction event that occurs following the initial virus-ligand interaction or a virion-associated factor (64, 65), while the later peak is probably due to a combination of viral and cellular transactivators (65). Analysis of the Western blot data demonstrated an increase in the slower-migrating band or the phosphorylated protein (28) at the peak times of Sp1 induction, suggesting a change in the posttranslational modification of this product. This finding has important ramifications because Sp1 is phosphorylated only after binding to DNA (by a DNA-dependent protein kinase [28]), suggesting that during these peaks of Sp1 protein levels, there are also increased levels of functionally active Sp1 binding to DNA. This possibility is supported by recent findings that showed that there was an increase in functional Sp1 during infection as measured by increased Sp1-driven promoter activity in a promoter construct driven solely by Sp1-responsive elements (63).

Because the goal of our study was to correlate changes in Sp1 to the upregulation of the NF-κB subunit promoters, we next addressed whether the p65 promoter was responsive to Sp1. The data demonstrated that the p65 promoter strongly responded to Sp1 and that as the levels of Sp1 increased, there was a correlative increase in promoter activity, suggesting that as Sp1 levels rise during infection, as we have documented, there is probably a concomitant increase in p65 promoter activity. To more precisely map the role of Sp1 in NF-κB promoter regulation, we made a series of promoter mutants and examined their activities in response to Sp1 alone (cotransfections in SL2 cells, which lack endogenous Sp1) and to HCMV (transfection-infection in HEL fibroblasts) and showed that as the Sp1 binding sites were eliminated or mutated, p65 promoter activity was significantly reduced. Thus, these results demonstrated that the GC-box elements within the p65 promoter were responsive to Sp1 and, more importantly, that these sites were utilized during activation following HCMV infection. The results document a potential mechanism(s) for the unique upregulation of the usually constitutively expressed p65 gene product by HCMV.

The analysis of the p105/p50 promoter proved to be more complex in SL2 cells following cotransfection of Sp1 and in HEL fibroblasts following viral infection. The p105/p50 promoter was strongly transactivated by Sp1 in a titratable fashion, and when the documented Sp1 binding site (11) was deleted or mutated, activity dropped under both conditions examined. The results also demonstrated that a number of other important factors played a critical role. When the NF-κB sites were deleted, there was a decrease in activity, demonstrating the functional nature of these sites in p105/p50 regulation (12, 56) and/or an interaction between NF-κB and Sp1 (41, 46, 47, 53, 54). Nevertheless, these results, like those from the p65 promoter studies, are consistent with the hypothesis that Sp1 is involved in the regulation of both promoters during infection. Sp1 appears to be essential for the upregulation of p65 promoter activity but only one of numerous factors in the upregulation of p105/p50 promoter activity. The fact that Sp1 is not essential to the regulation of the p105/p50 promoter but rather one of several important documented players is not surprising and appears to arise from the complex regulation of this promoter by a variety stimuli under normal conditions (11, 12, 18, 52, 56, 57). During infection, this complex regulation is further complicated by the large number of cellular transcription factors induced during viral infection, and the induction of the various HCMV IE genes that we have previously shown transactivates the NF-κB promoters (65).

In both promoters, particularly during infection, a low level of transactivation even when upstream sites were deleted or mutated was seen, suggesting that other factors were regulating a certain low level of activity. One possible player could be E2F, because there exists in both of these TATA-less promoters a putative E2F site which sites at the start site of transcription (12, 56, 60). During infection, E2F message and activity and E2F-containing complexes have been documented to increase during HCMV infection (38, 43, 48, 61); thus, E2F presents itself as a potential low-level modulating factor at least during infection for the activation of these promoters. Interestingly, E2F and Sp1 have recently been shown to interact, and this interaction was shown to promote heightened promoter activity (34, 39). The potential role of E2F, however, is clouded by the fact that these promoters are not known to be cell cycle regulated.

Presently, the mechanism for the regulation of Sp1 during infection is unclear. Our preliminary results, however, show that the viral IE proteins increased steady-state Sp1 mRNA (data not shown). Unfortunately, until the Sp1 promoter is isolated and cloned, studies about the role of the IE genes in Sp1 promoter regulation are not possible. Our present data demonstrate that in the presence of Sp1, the IE genes can increase the levels of promoter transactivation, suggesting a cooperative interaction between the viral IE products and Sp1, at least in regard to promoter activation. These findings are consistent with our previous study in which we showed that the IE genes could, with a similar pattern of activity detected (IE2-55>IE2-86>IE1-72), act as transactivators of the p65 promoter in HEL fibroblasts (65). In addition, in a recent study showing that the HCMV IE gene products transactivate promoters with upstream Sp1 sites, it was also observed that in the absence of these upstream Sp1 activators, little activity is seen (40), supporting our observation of little or no activity in the absence of Sp1.

Interestingly, a recent report has documented a potential role for pRB as a positive regulator of Sp1 by freeing functional Sp1 from its negative inhibitor termed, Sp1-I (8), indicating a mechanism where by Sp1 through an interaction with another product can be relieved of its negative regulation. Because the IE genes are expressed during the time frame in which we saw an increase in Sp1 levels and Lukac et al. have demonstrated an interaction between IE2-86 and Sp1 (40), the IE products could act similarly to pRB by releasing free Sp1 from its putative negative regulator. Our results support this hypothesis as IE2-86 increased the levels of Sp1 EMSA binding at all time points tested, suggesting a potential mechanism by which the virus increases transcriptionally active Sp1. Although pRB might be involved and is induced during infection (30), we did not at this time address the role of this product in the regulation of Sp1. The other IE gene products did not significantly increase Sp1 binding, although all three interacted with Sp1 and could cooperate with Sp1 in the CAT assays, implying that all three of these IE gene products play some sort of a role in the regulation of the NF-κB promoters. Because little is known about the mechanisms of IE1-72 coactivator function, its specific role in the regulation of Sp1 activity is currently unknown. The same argument could be used for IE2-55, although because of its similarity to IE2-86 and its significant ability to cooperate with Sp1 in the CAT assays, one might have predicted that it, like IE2-86, would have increased Sp1 DNA binding levels. However, because IE2-55 is a splice variant of IE2-86, it is possible that the spliced-out region contains the active site for its ability to disrupt the Sp1 inhibitor, while contained in the common regions is the ability to act as a promoter transactivator or to interact with Sp1 in a protein-protein complex. This hypothesis is consistent with the findings that IE2-55 can antagonize IE2-86 function (4, 37, 40)

by potentially acting as a sink for various transcription factors (40). Thus, one might predict then that IE2-55 would bind Sp1 but not free up additional active Sp1 as the IE2-86 product does. Nevertheless, the conflicting results as to the true role IE2-55 plays during infection (transactivator versus negative modulator) remain. Additional studies are under way to better define the role that the IE genes play in Sp1-mediated regulation.

Overall, our current data along with our previously reported work clearly demonstrate that there are multiple pathways utilized by HCMV for the regulation of the vital NF-κB activity necessary for the maintenance and regulation of the viral life cycle. We have shown that there are at least two tiers to the regulation of NF-κB (65): (i) an initial induction via a receptor/ligand-mediated signaling event in which preformed stores are probably released (38, 62–65) and (ii) the increase in new p50 and p65 molecules through the de novo synthesis of new message (38, 65). The increase in new NF-κB molecules itself occurs through multiple regulated mechanisms: (i) autoregulation by NF-κB (p105/p50 promoter), (ii) increase in Sp1-directed activity (p65 and p105/p50 promoters), (iii) transactivation of the cellular promoters by the viral IE proteins (p65 and p105/p50 promoters (65), and (iv) interactions between these various players (34, 39–41, 46, 47, 53, 54).

ACKNOWLEDGMENTS

We thank Shu-Mei Huong for valuable technical assistance and K. Ueberla and W. A. Haseltine for the generous gift of the p65 promoter. We thank T. D. Gilmore for helpful suggestions and the generous gift of the GST-Sp1 construct. We also thank R. Scott, P. Wicked, and B. Wing for helpful suggestions and critical reading of the manuscript.

The work was supported by grants AI-12717 and CA-10914 from NIH (E.S.H.). A.D.Y. is an Hulda Irene Duggan Arthritis Investigator (AF#5-40974), M.W.M. is an NRSA postdoctoral fellowship recipient (1F32-CA69790-01), and E.E.P. is a recipient of a graduate student virology training grant 5T32-AI07419-04.

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