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The Human Cytomegalovirus IE1-72 Protein Interacts with the Cellular p107 Protein and Relieves p107-Mediated Transcriptional Repression of an E2F-Responsive Promoter

ERIC E. POMA,[1,2]* TIMOTHY F. KOWALIK,[3] LIANG ZHU,[4] JOHN H. SINCLAIR,[5] AND ENG-SHANG HUANG[1,2,6]

Lineberger Comprehensive Cancer Center,[1] Department of Microbiology and Immunology,[2] and Department of Medicine,[6] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295; Department of Genetics, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710[3]; Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129[4]; and Department of Medicine, University of Cambridge, Cambridge CB2 2QQ, United Kingdom[5]

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The Rb-related p107 protein has been implicated as an important control element in proper cell cycle progression. The p107 protein is thought to restrict cellular proliferation in part through its interaction with the E2F family of transcription factors and is, therefore, a specific target for regulation by several DNA viruses. Here, we demonstrate that p107 protein levels are induced in a biphasic manner in human fibroblasts during productive infection by the human cytomegalovirus (HCMV). Expression patterns of p107 protein levels during HCMV infection of human embryonic lung cells (HELs) demonstrate a sustained induction from early to late times of infection. We also demonstrate that the HCMV immediate-early protein IE1-72 complexes in vivo with the p107 protein and that this interaction can be reconstituted in an in vitro system by using reticulocyte-translated protein. Our data demonstrate that the interaction between p107 and the IE1-72 protein occurs at times of infection that temporally match the second tier of p107 protein induction and the phosphorylation pattern of the IE1-72 protein. Furthermore, we show here that the ability of p107 to transcriptionally repress E2F-responsive promoters can be overcome by expression of the IE1-72 protein. This effect appears to be specific, since the IE1-72 protein is not capable of relieving Rb-mediated repression of an E2F-responsive promoter. Finally, our data demonstrate that HCMV infection can induce cellular proliferation in quiescent cells and that IE1-72 expression alone can, to a degree, drive a similar progression through the cell cycle. These data suggest that IE1-72-mediated transactivation of E2F-responsive promoters through alleviation of p107 transcriptional repression may play a key role in the cell cycle progression stimulated by HCMV infection.

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus that is rarely associated with disease in healthy adults (42). Severe pathogenesis can occur, however, in immunocompromised individuals, transplant patients, and, in some cases, pregnant women (for a review, see reference 42). Viral gene expression during permissive HCMV infection of fibroblasts is temporally regulated in a strict fashion, with a cascade of gene activation progressing from immediate-early (IE) to early and finally to late gene expression (61). The best-characterized and most abundantly transcribed of the IE genes originate from a series of spliced transcripts from the major IE region of the HCMV genome under the control of the major IE promoter (MIEP) (for reviews, see references 25 and 42). At least five exons are alternatively spliced from the major IE region, resulting in at least three major gene products which appear at IE times. These gene products include the IE1-72 protein (a 72-kDa protein, also referred to as IE1 or IE72), the IE2-86 protein (an 86-kDa protein, also referred to as IE2 or IE84), and the IE2-55 protein (a 55-kDa protein); all of these proteins have the first three exons of the major IE region (exons 1 to 3) in common, with exon 4 being unique to IE1-72 (resulting in a 2.0-kb transcript from the UL123 region), exon 5 being unique to IE2-86 (2.25-kb transcript from the UL122 region), and an alternatively spliced version of exon 5 being unique to IE2-55 (1.7-kb transcript from the UL122 region) (77).

IE1-72 and the IE2-86 are both nuclearly localized proteins that have been demonstrated to have important roles in regulating both viral and cellular gene expression. IE2-86 is a strong repressor of the MIEP (8, 34, 56, 59, 67–69, 78) and has been shown to transactivate a number of viral and cellular promoters, a subset of which are associated with proliferation and DNA replication (4, 14, 26, 29, 35, 51, 58, 62, 69, 72, 80, 83). The IE2-86 protein is a sequence-specific DNA-binding protein with at least five described DNA-binding sites (1, 10, 45, 55, 59, 72); it has also been demonstrated to associate with the TATA-binding protein of the TFIID subunit of the basal transcription complex (6, 24, 29, 45, 46, 58, 74). Direct binding of the IE2-86 protein to specific DNA sequences and interaction of the IE2-86 protein with the TATA-binding protein are thought to be the major mechanisms of IE2-86-mediated transactivation. Transcriptional activation by the IE2-86 protein has been shown to be synergistically enhanced by IE1-72 expression, but the mechanism of this synergy is not fully understood (17, 26, 30, 35, 51, 58, 62, 78, 80, 83).

IE1-72 is a strong activator of the MIEP and can stimulate transcription from several viral and cellular promoters but is, in general, a weaker and less promiscuous transactivator than IE2-86 (2, 4, 9, 18, 26, 30, 51, 78, 82, 83). The IE1-72 protein does not appear to bind DNA specifically and does not interact directly with the TATA-binding protein. Proposed mechanisms for IE1-72-mediated transactivation appear to involve

* Corresponding author. Mailing address: Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599-7295.

both direct and indirect methods. Expression of the IE1-72 protein has been demonstrated to activate the MIEP of HCMV through NF-κB sites in the promoter (70). However, the mechanics of this activation are not clear and do not appear to involve direct protein-protein interaction between the IE1-72 and NF-κB proteins. The IE1-72 protein has been shown to directly bind the CCAAT-box binding protein CTF1, resulting in activation of the TATA-less human DNA polymerase α gene, a component of the host DNA replication machinery (32). Deletion of the CCAAT box motif abolishes transactivation of the promoter by the IE1-72 protein. Recent data have also demonstrated that the IE1-72 protein can directly activate human wild-type dihydrofolate reductase promoter, another gene essential for DNA replication. Activation occurs, at least in part, through the E2F sites in the promoter; the IE1-72 protein was shown to be capable of binding the E2F-1 protein (60).

The p107 protein was first characterized as a host factor associated with the viral transforming proteins E1A of adenovirus and the large T antigen of simian virus 40 (SV40) (20, 31, 88, 92). Regions of E1A and large T required for binding p107 overlap the regions required for viral protein interaction with the tumor suppressor protein pRb, and mutations of the viral proteins in this region have been demonstrated to prevent transformation (7, 15, 16, 21, 47, 49, 84, 87, 88). Amino acid sequences of p107, pRb, and the p130 protein have revealed a strong homology between all of the proteins with a conserved pocket region required for interaction with E2F, a heterodimeric family of transcription factors that play a key role in stimulating cellular proliferation (22, 39, 43, 47, 48, 64). Binding of an E2F family member by one of the pocket proteins creates an active complex which, when bound to an E2F site, results in transcriptional silencing of the promoter (85, 86). In normal cells, the interaction between the pocket proteins and their E2F partners is tightly regulated and is a key to ensuring proper cell cycle progression, with each pocket protein exerting its influence at different stages in the cell cycle. Data from numerous laboratories suggest that proper cell cycle progression requires a tightly regulated release of E2F from various pocket-protein family members at distinct times during the cell cycle (for a review, see reference 19).

The p107 protein, like the pRb protein, can act as a powerful suppressor of cellular proliferation. Overexpression of the p107 protein has been shown to growth arrest certain cell types in G_1 in a manner analogous but not identical to that of pRb (94, 95). The mechanism for p107-mediated suppression is not well understood but appears to be due, at least in part, to the ability of p107 to repress transcription from E2F-responsive promoters. This repression is thought to block the expression of a series of genes associated with progression through the cell cycle, including c-*myc*, c-*myb*, N-*myc*, dihydrofolate reductase, thymidine kinase, DNA polymerase α, cyclin A, cyclin D_1, and *cdc2* (5, 13, 33, 37, 63–66, 81, 91) and resulting in G_1 growth arrest and prevention of DNA replication.

Host factors like the p107 and pRb proteins that negatively influence cellular proliferation become obvious targets for regulation by infecting DNA viruses that must activate cellular DNA replication machinery for successful infection. SV40, adenovirus, and the human papillomavirus (HPV) are all small DNA tumor viruses which specifically alter the function of both the p107 and pRb proteins through direct protein-protein interactions with a virally encoded gene product (large T of SV40, E1A of adenovirus, and E7 of HPV) (for a review, see reference 54). Like all DNA viruses, HCMV requires a cellular environment favorable to viral DNA replication, and mounting evidence suggests that HCMV may create such an environment in much the same manner as that of the small DNA tumor viruses. HCMV infection has been demonstrated to initiate several of the hallmark changes brought on with infection by the small DNA tumor viruses. Recent data indicate that HCMV infection induces alterations in E2F-complexes, functional inactivation of the p53 gene product, and accumulation of chromosomal damage in infected cells (23, 76, 82). At a mechanistic level, the IE1-72 and IE2-86 proteins are capable of functionally complementing an E1A deficient adenovirus (35, 75, 80), and the IE2-86 protein has been demonstrated to bind and functionally inactivate the p53 protein in much the same manner as the adenovirus E1B and SV40 large T proteins (76). The IE2-86 protein has also been shown to interact functionally with the pRb protein in a fashion analogous to that of the adenovirus E1A protein (11, 28). These data prompted us to examine regulation of the p107 protein during infection by HCMV.

We demonstrate here that p107 protein levels are induced at early to late times of HCMV infection. Furthermore, the p107 protein is specifically targeted during infection for binding by the IE protein IE1-72, with the result of this interaction being alleviation of p107-mediated repression of E2F-responsive promoters. Finally, our data demonstrate that HCMV infection induces cell cycle progression in quiescent fibroblasts, and expression of the IE1-72 protein alone, to a degree, is also capable of driving cellular proliferation. We believe that these data represent a novel mechanism for IE1-72-mediated transactivation and that this transactivation may play a role in the cell cycle stimulation seen during HCMV infection by activating a series of genes involved in proliferation and DNA replication. We believe that IE1-72 expression is involved in facilitating progression through the cell cycle and subsequent viral DNA replication in infected cells.

MATERIALS AND METHODS

Cell culture and infection. Human embryonic lung fibroblasts (HELs) and human tumor cell line C33A cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, Md.) and penicillin-streptomycin at 37°C in a 5% CO_2 incubator. Infection with the HCMV Towne strain, passages 36 to 39, was performed as described previously (41). A multiplicity of infection of 2 was used for all experiments. Cells and virus were incubated for 90 min at 37°C in a 5% CO_2 incubator. Free virus was washed off, and DMEM with heat-inactivated 4% fetal bovine serum (GIBCO-BRL) was added to the infected cells.

Preparation of cell lysates for Western analysis and immunoprecipitation. HELs were harvested at various hours postinfection (hpi). Cells were pelleted by low-speed centrifugation and were washed twice with phosphate-buffered saline (PBS). Cells were incubated in an EBC buffer (0.05 M Tris-HCl [pH 8.0], 0.12 M NaCl, 0.5% Nonidet P-40, 0.1 M NaF, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride) at 4°C for 1 h, with leupeptin and aprotinin added at 10 μg/ml. Microcentrifugation for 20 min at 4°C was used for clarification. Supernatant was removed, and protein concentrations were determined with the Bio-Rad (Richmond, Calif.) protein assay kit.

Western blot (immunoblot) analysis. Equal protein amounts of lysates per time point (approximately 100 μg) were mixed in Laemmli sodium dodecyl sulfate (SDS) sample buffer and were loaded onto SDS-polyacrylamide gels. A duplicate gel was run and Coomassie stained to ensure equal protein loading. Proteins were separated by electrophoresis and transferred overnight at 18 V to Immobilon-P Transfer Membrane (Millipore, Bedford, Mass.) blots. The blots were blocked for 1 h in 5% (wt/vol). Carnation nonfat dry milk dissolved in PBS. The blots were then probed with primary antibody (the p107 monoclonal antibody was purchased commercially [Santa Cruz Biotechnology, Santa Cruz, Calif.]; the IE protein antibodies were raised to nonconserved domains in the IE1-72 and IE2-86 proteins and have been described previously [76]) for 1 h in PBS (1:200 dilution for p107; 1:1,000 dilution for IE proteins). The blots were washed four times with PBS–0.1% Tween 20. After washing, the blots were probed with secondary antibody (horseradish peroxidase-conjugated anti-mouse immunoglobulin G [Sigma, St. Louis, Mo.]). The blots were again washed extensively in PBS–0.1% Tween and were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, England) according to the manufacturer's specifications.

Immunoprecipitations. Immunoprecipitations were performed at 4°C. Equal amounts of protein lysates (approximately 200 μg of infected cell lysate) were

precleared for 1 h with Pansorbin cells (CalBiochem, La Jolla, Calif.). Monoclonal antibody (1:100 dilution) and 30 μl of protein G beads (Pharmacia, Uppsala, Sweden) were added, and the volume was increased to 0.5 ml with ELB$^+$ buffer (0.25 M NaCl, 0.1% Nonidet P-40, 0.05 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.0], 1 mM phenylmethylsulfonyl fluoride, 5 mM ethylenedinitroltetraacetic acid, 0.5 mM dithiothreitol). The incubation was performed at 4°C for 4 h with constant mixing. Beads were spun down and washed six times with ELB$^+$ buffer. The beads were mixed with Laemmli buffer and boiled. Supernatant was run on SDS-polyacrylamide gel electrophoresis (PAGE) gels. Western analysis (performed as described) was used for detection of proteins in the immunoprecipitated complexes.

Immunoprecipitations of ^{35}S- and ^{32}P-labeled lysates. Infected HELs were pulsed for 1 h with either [^{35}S]methionine or $^{32}P_i$ in DMEM without methionine or phosphate, respectively, before harvesting (24). Lysates were immunoprecipitated with the Dupont monoclonal antibody to the common region of the IE1-72 and IE1-86 proteins. Immunoprecipitation and detection were performed as described.

Plasmids. The IE1-72 and IE2-84 expression vectors (pcDNA3IE1 and pcDNA3IE2) have been previously described (93). The adenovirus E2CAT and the E2CAT mutant were gifts from Steven Bachenheimer and have been described previously (57). The p107 expression vector (p107CMVneo) and the p107 vector used for transcription-translation (pBSKIIp107) were obtained from Liang Zhu (95).

In vitro interactions. All transcription-translation of proteins was done with the Promega TnT-coupled reticulocyte lysate system (Madison, Wis.) for [^{35}S]methionine labeling. The manufacturer's protocol was followed. Simultaneous cotranslation of the IE1-72 and p107 proteins was carried out to demonstrate in vitro interaction. Immunoprecipitations were performed with 5 μl of translated protein, 20 μl of protein G beads, and 0.5 μg of antibody in 0.3 ml of ELB$^+$ buffer. The reactions were allowed to proceed overnight, and the beads were washed extensively (five times with ELB$^+$) before boiling and separation by SDS-PAGE. The gels were dried and exposed to film.

Transfections and CAT assays. C33A cells were transfected by the GIBCO-BRL Lipofectamine kit. C33A cells were grown in 100-mm dishes and transfected at 70% confluency. A total of 5 μg of DNA was used in each transfection mixture with empty pGEX-7 DNA used to normalize total DNA amounts; 1 μg of reporter and 1 μg of each expression vector indicated were used. DNA was suspended in 300 μl of DMEM and was mixed with 300 μl of DMEM containing 6 μl of Lipofectamine reagent. The DNA-Lipofectamine complexes were allowed to form at room temperature for 45 min. The cells were washed with 4 ml of DMEM. The complexes were brought up to 2.4 ml with DMEM-H and added to the cells. After 12 h, 4 ml of DMEM-H with 12% fetal bovine serum (GIBCO) was added to the cells; after 24 h, medium was replaced with fresh DMEM with 10% fetal bovine serum (GIBCO). Cells were harvested, and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (27) with an SV-βGAL (Promega) internal control used to standardize activity.

Induction of cell cycle progression by HCMV infection or IE gene expression. Primary medical research council (MRC5) human fibroblasts were serum starved for 6 days and then mock infected or infected with either HCMV at 10 PFU per cell or UV-irradiated HCMV for 2 h. Virus was washed off with serum-free media, and the cells were analyzed by propidium iodide staining and fluorescence-activated cell sorter (FACS) analysis. COS-1 cells were serum starved for 6 days and transfected by Lipofectin with PHK3 (an SV40 control vector), pSG5IE1 (an SV40-driven IE1-72 expression vector), or HM121 (an SV40-driven IE2-86 expression vector) (30). The cells were analyzed 2 days posttransfection. Transfection efficiencies were 20% for IE1-72 and 5% for IE2-86.

RESULTS

Protein expression of p107 during HCMV infection of HELs is biphasic. HCMV infection has been demonstrated to alter levels of a number of cellular factors involved in regulating proliferation (4, 14, 26, 29, 35, 51, 58, 62, 69, 72, 80, 83). Recent data have shown that HCMV specifically targets the tumor suppressor protein Rb for regulation in much the same manner as do the small DNA tumor viruses (11, 28). Since regulation of the Rb protein during infection by DNA viruses accompanies a similar regulation of the closely related p107 protein, we chose to examine the effect of HCMV infection on p107 expression. To examine protein levels of p107 during HCMV infection, cell lysates were prepared at various times postinfection and Western analysis was performed as described. An initial induction (over mock infection) was seen at 0 hpi (Fig. 1, lanes 1 and 2), but this induction was no longer detectable by 4 hpi (Fig. 1, lane 3); a second tier of protein induction was visible beginning at approximately 16 hpi and persisting through late times of infection (72 hpi), with maximal expression occurring at 24 hpi (Fig. 1, lanes 5 through 8). Identical results were obtained when a polyclonal antibody to p107 (Santa Cruz) and a second monoclonal antibody (a generous gift from David Livingston) to p107 were used (data not shown). The sustained induction seen at early to late times of infection is probably the result of direct viral regulation at the transcriptional, translational, or posttranslational level. These data clearly demonstrate that p107 protein levels are specifically regulated by HCMV infection. It should be noted that temporally, the second tier of p107 protein induction (beginning at 16 hpi and being maximal at 24 hpi) coincides with the first peak of viral DNA replication in fibroblasts (61). A Coomassie stain of the samples is included to demonstrate equivalent protein loading (Fig. 1B).

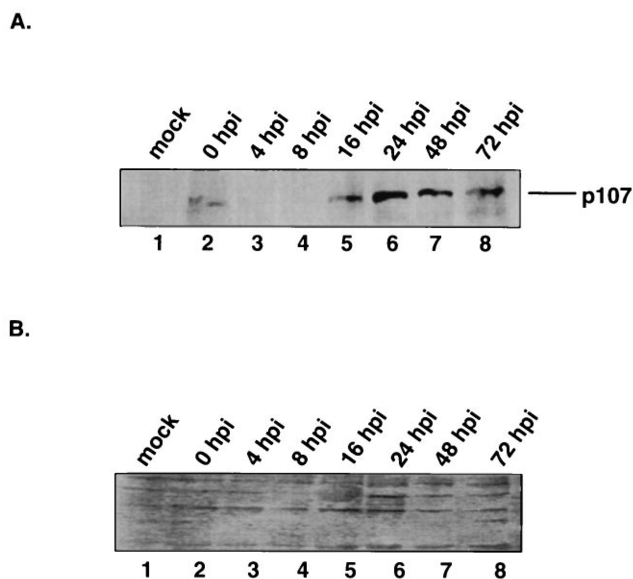


FIG. 1. Protein levels of p107 during HCMV infection of HELs. HELs were infected or mock infected as described. (A) At different hpi cells were harvested, cell lysates were prepared, and equal amounts of lysate (approximately 200 μg) were separated by SDS-PAGE and transferred to an Immobilon membrane. Western blot analysis was performed with an anti-p107 monoclonal antibody (Santa Cruz). (B) A Coomassie stain of samples run was included to demonstrate equal protein loading.

IE1-72 and p107 protein complex formation occurs in HCMV-infected HELs. Regulation of p107 by adenovirus, SV40, and HPV occurs primarily through direct protein-protein interaction between the p107 protein and a virally encoded gene product (54). To establish whether the HCMV IE1-72 protein formed a complex with the p107 protein during HCMV infection, immunoprecipitations were performed from HCMV-infected lysates with a p107-specific monoclonal antibody. We demonstrate that complexes containing p107 immunoprecipitated from HCMV-infected lysates (24 hpi) also contained the IE1-72 protein (Fig. 2A, lane 4) but not the IE2-84 protein (Fig. 2B, lane 3), as determined by Western blot analysis with monoclonal antibodies specific to IE1-72 and IE2-84, respectively. Furthermore, immunoprecipitations of p107-containing complexes from mock-infected cell lysate failed to contain the IE1-72 protein (Fig. 2A, lane 3), confirming that the band detected at 72 kDa in infected lysates is indeed a viral protein. All experiments were repeated with a Santa Cruz polyclonal antibody to p107 and a second monoclonal antibody obtained from David Livingston, with identical results (data not shown). It has been previously demonstrated that the IE2-86 protein interacts with the Rb protein both in vitro and

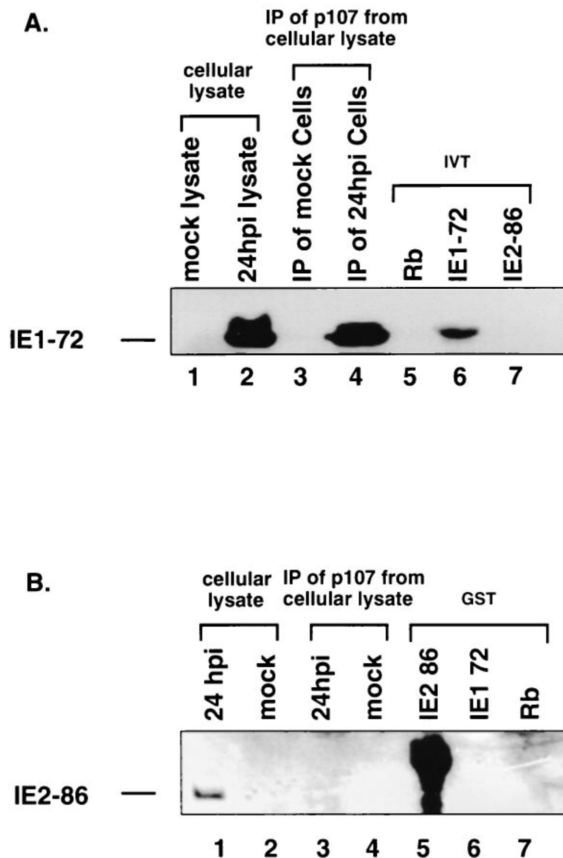


FIG. 2. In vivo interaction between the HCMV IE1-72 protein and the cellular p107 protein. p107 protein was immunoprecipitated from mock- and HCMV-infected cellular lysate (approximately 200 μg) by mixing protein A beads and anti-p107 monoclonal antibody (Santa Cruz) with lysate for 4 h at 4°C. The beads were washed extensively with ELB$^+$ buffer, and bound protein was boiled off and separated on SDS-PAGE and transferred to Immobilon. The blots were analyzed by Western blotting for IE1-72 or IE2-86 protein to determine coimmunoprecipitation between the p107 protein and the IE proteins of HCMV. (A) Immunoprecipitated (IP) complexes were analyzed by Western blotting for IE1-72 protein. Lanes: 1 and 2, mock-infected and 24-hpi-infected lysate (20 μg) as negative and positive controls for IE1-72 protein, respectively; 3 and 4, p107 protein immunoprecipitated from mock- or HCMV-infected (24 hpi) cells, respectively; 5 and 7, negative controls with in vitro-translated pRb and IE2-86 protein; 6, a positive control of in vitro-translated (IVT) IE1-72 protein. The blot was analyzed by Western analysis for the IE1-72 protein. (B) Identical immunoprecipitated (IP) complexes were analyzed by Western analysis for the IE2-86 protein. Lanes: 1 and 2, 24-hpi- and mock-infected lysate as positive and negative controls for IE2-86 protein, respectively; 3 and 4, immunoprecipitations of the p107 protein from HCMV-infected (24 hpi) and mock-infected cells, respectively; 6 and 7, glutathione *S*-transferase (GST) fusion proteins of the IE1-72 protein and the Rb protein as negative controls; 5, a GST–IE2-86 protein run as a positive control.

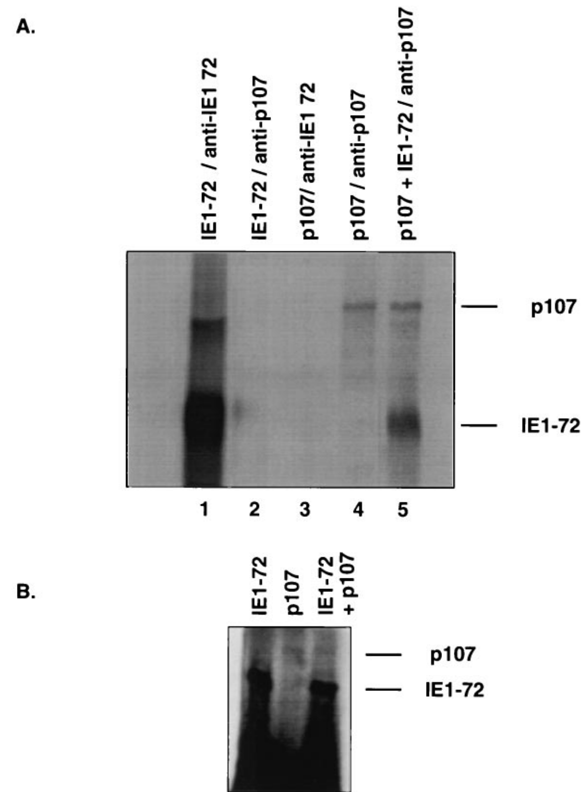


FIG. 3. In vitro interaction between the IE1-72 protein and the p107 protein. The Promega TnT system ([^{35}S]methionine labeled) was used for in vitro translation; the p107 and IE1-72 proteins were cotranslated to test for interaction. (A) The IE1-72 protein was immunoprecipitated with monoclonal antibody to the IE1-72 protein (lane 1) but not with monoclonal antibody to the p107 protein (lane 2). The p107 protein was immunoprecipitated with monoclonal antibody to the p107 protein (lane 4) but not with monoclonal antibody to the IE1-72 protein. Monoclonal antibody to the p107 antibody was used to coimmunoprecipitate both the IE1-72 and p107 proteins (lane 5). (B) One-half of total input levels of translated proteins used.

in vivo but does not appear to bind the p107 protein (10, 26). To our knowledge, this represents the first system described in which two distinct viral proteins interact exclusively with two separate members of the pocket protein family (IE1-72 with p107 and IE2-86 with pRb). The p107 and pRb proteins appear to exert their effects at different points during the cell cycle; distinct targeting of the proteins by viral factors could be construed as a mechanism evolved by the virus to temporally control its functional inactivation of the pocket protein family, thereby allowing a more regulated progression through the cell cycle.

The IE1-72 and p107 proteins interact in vitro. We wished to confirm the in vivo data obtained on the complex formation between the p107 and IE1-72 proteins by reconstituting the interaction in an in vitro assay. We used a rabbit reticulocyte protein expression system to in vitro translate and methionine label the IE1-72 and p107 proteins and then assayed for interaction. The antibodies used for immunoprecipitation of the translated proteins (monoclonal antibodies to the IE1-72 and the p107 proteins, respectively) recognize their specific in vitro-translated product and fail to cross-react with the other proteins (Fig. 3A, lanes 1 to 4). Here, we demonstrate that when IE1-72 and p107 genes are cotranslated, immunoprecipitation of p107 coprecipitates the IE1-72 protein (Fig. 3A, lane 5). We included a control demonstrating half of the total input protein used in the immunoprecipitations; it should be noted that the levels of translation for p107 were very low compared with the translation levels for IE1-72 (Fig. 3B). We have been unable to coimmunoprecipitate the IE1-72 and p107 proteins using a monoclonal antibody to the IE1-72 protein but have been able to do so using a polyclonal antibody (data not shown), suggesting that the epitope of the IE1-72 recognized by the monoclonal antibody may be masked by interaction with p107. These data demonstrate that the interaction can be recreated in vitro and that the interaction does not require the presence of additional viral proteins.

IE1-72 and p107 complex formation occurs at early to late times of HCMV infection in HELs. We wished to determine

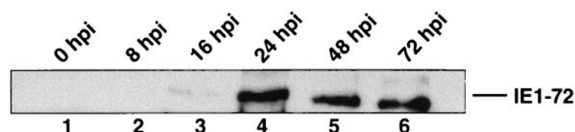


FIG. 4. Interaction between the IE1-72 and p107 proteins occurs at early to late times of infection. Coimmunoprecipitation of the p107 and IE1-72 proteins was performed (as described in the legend to Fig. 2) at various times postinfection.

the temporal kinetics of the interaction occurring between the p107 and IE1-72 proteins. Immunoprecipitations against the p107 protein were done over a time course of HCMV infection to determine the kinetics of this complex formation during infection. Complexes containing both the IE1-72 and the p107 proteins are not observed until 16 hpi, become maximal at 24 hpi, and remain detectable until late times of infection (Fig. 4, lanes 3 through 6). The temporal kinetics of IE1-72 and p107 interaction matches almost identically with the second tier of p107 protein induction seen during HCMV infection (Fig. 1). It is possible that the high levels of p107 protein detected are a consequence of the interaction between the IE1-72 and the p107 proteins. The IE1-72 protein may trigger induction of p107 protein levels by acting at the transcriptional or translational level. Again, it should be noted that the time of maximal interaction between the p107 and IE1-72 proteins (24 hpi) coincides temporally with the initial peak of viral DNA replication seen during HEL infection.

IE1-72 protein expression patterns during HCMV infection of HELs. The IE1-72 protein is in abundance at IE times of infection, and protein levels are stable through late times of infection (Fig. 5C, lanes 3 through 8). Immunoprecipitation of IE1-72 from [^{35}S]methionine-labeled infected cell lysates shows IE1-72 synthesis occurring by as early as 4 hpi, but new synthesis is absent at later times of infection (Fig. 5A, lane 2). By comparison, IE2-84 protein expression and synthesis are detectable at 4 hpi and continue throughout infection (Fig. 5A). Therefore, it is interesting that the interaction between the IE1-72 protein and the p107 protein is minimal at 16 hpi and does not become maximal until 24 hpi (Fig. 4, lanes 3 and 4), even though IE1-72 protein levels are high at earlier times of infection (Fig. 5C, lanes 3 to 5). These results suggest that elevated protein levels of IE1-72 and p107 do not alone appear to be sufficient to drive the interaction. Phosphorylation patterns for the IE1-72 protein in HELs show low levels of phosphorylation at IE times of infection, maximal phosphorylation at 24 hpi, and gradual decrease at later times of infection (Fig. 5B, lanes 2 through 6). The temporal kinetics for IE1-72 phosphorylation closely match the times when the IE1-72 and p107 protein interaction is detectable. Even though new phosphorylation of IE1-72 decreases after 24 hpi (Fig. 5B, lane 4), stable phosphorylated IE1-72 protein could account for the continued interaction with p107. These data suggest that the phosphorylation status of the IE1-72 protein may be a key regulatory element in determining complex formation with the p107 protein.

IE1-72 protein expression is sufficient to alleviate p107 suppression of an E2F-responsive CAT reporter construct. An E2F-responsive promoter derived from the adenovirus early gene 2 promoter with the ATF sites mutated (Fig. 6A) was used as the reporter construct for all CAT assays. In a C33A cell line, constitutive levels of transcription from the reporter were easily detectable; the mutant E2CAT reporter (mutations in the promoter inactivated the E2F sites) had no activity even when cotransfected with the IE1-72 expression vector (Fig. 6B,

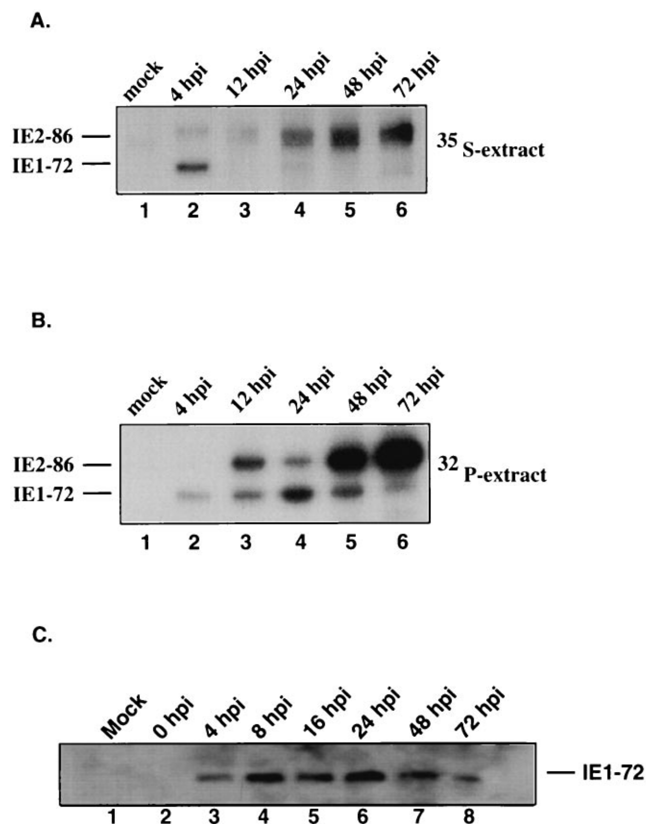
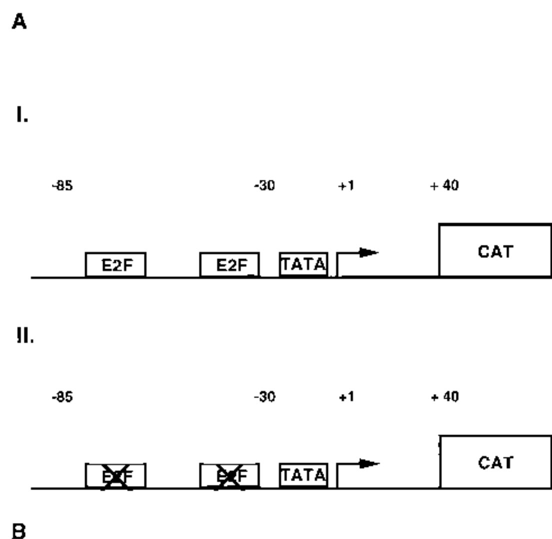


FIG. 5. Temporal posttranslational modification of the IE1-72 and IE2-86 proteins in HELs. HCMV-infected HELs were pulse-labeled with [^{35}S]methionine or $^{32}P_i$ 1 h prior to harvesting. The IE1-72 and IE2-86 proteins were immunoprecipitated with a monoclonal antibody to a conserved domain in both proteins (Dupont IE monoclonal antibody). Immunoprecipitated complexes were analyzed by SDS-PAGE; [^{35}S]methionine immunoprecipitates were enhanced with 1 M sodium salicylate. Gels were exposed to X-ray film and developed. (A) [^{35}S]methionine pulse-labeling of infected cells was used to detect de novo synthesis of the IE1-72 and IE2-86 proteins. (B) $^{32}P_i$ pulse-labeling of infected cells was used to detect phosphorylation of the IE1-72 and IE2-86 proteins. (C) Protein levels of the IE1-72 protein during HCMV infection, as determined by Western analysis. Western analysis was performed as described in the legend to Fig. 1 with a monoclonal antibody to the IE1-72 protein.

lanes 2, 3, and 4). E2CAT reporter activity was not significantly altered when the IE1-72 expression vector was cotransfected with the E2CAT reporter, suggesting that IE1-72 expression was not capable of further transactivating this promoter in the C33A cell line (Fig. 6B, lane 5).

The p107 protein has been demonstrated to repress transcription off an E2F-responsive promoter (71, 94). This effect is presumably due to active transcriptional repression by the p107-E2F complex bound to the E2F sites in the promoter. We demonstrate here that p107 transcriptional repression is reversible by coexpression of the IE1-72 protein. Activity dropped approximately sixfold when the E2CAT reporter was cotransfected with a p107 mammalian expression vector (Fig. 6B, lane 6). When 1 μg of both the IE1-72 and the p107 expression vectors was cotransfected with the E2CAT promoter, activity was restored to approximately two-thirds of constitutive levels (Fig. 6B, lane 7). When the amount of the IE1-72 expression vector cotransfected was increased to 2 μg, full promoter activity was restored (Fig. 6B, lane 8). These data demonstrate that even though in C33A cells the IE1-72 protein is not capable of further activating an E2F promoter, it is capable of relieving p107-mediated transcriptional repression.



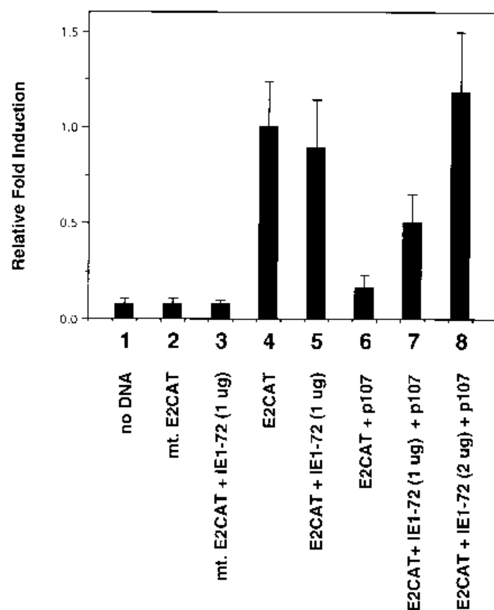


FIG. 6. Alleviation of p107-mediated repression of the E2CAT reporter by IE1-72 expression. Transfections were done with the Lipofectamine kit from GIBCO-BRL by using C33A cells with an internal β-galactosidase control used to standardize for transfection efficiency. The reporter and indicated expression vectors were all used at 1 μg per transfection. All expression vectors were driven by the CMV MIEP. (A) Schematic representation of the E2CAT reporter with the ATF sites mutated and the E2CAT E2F$^-$ reporter with the E2F sites mutated. (B) CAT assay of C33A cells transfected with the E2CAT reporter construct and various expression vectors. Constructs used are defined as follows: no DNA, cells alone; mt E2CAT, the E2CAT promoter minus the ATF sites with the E2F sites mutated; E2CAT, the E2CAT promoter minus the ATF sites; IE1-72, a CMV-driven IE1-72 eukaryotic expression vector; and p107, a CMV-driven p107 eukaryotic expression vector. Unless otherwise noted, 1 μg was used for each construct. All CAT assays were done at least three times.

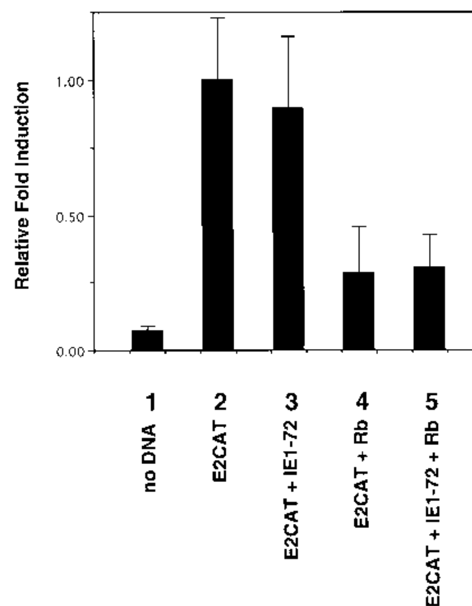


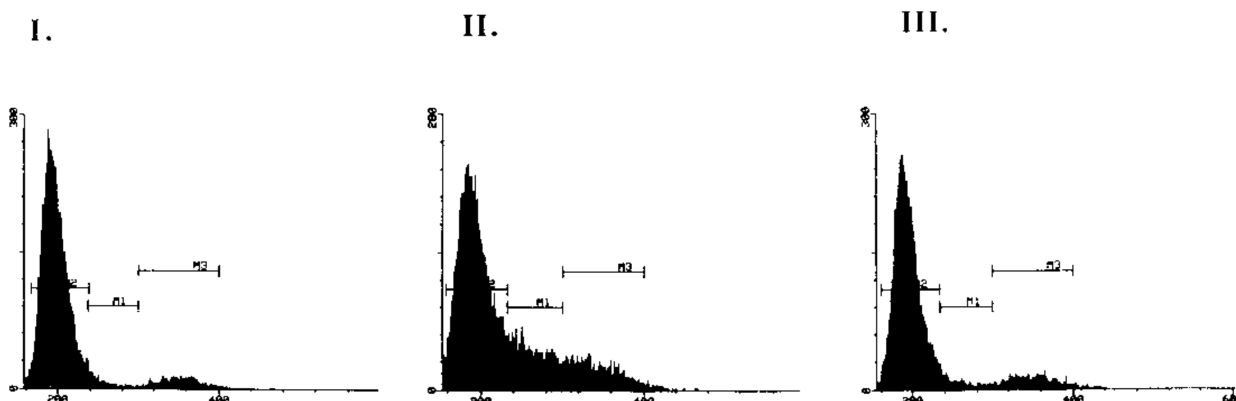
FIG. 7. IE1-72 expression does not alleviate Rb-mediated repression of the E2CAT promoter. Transfections were done as described in the legend to Fig. 6, with identical amounts of DNA used. The constructs used were also identical to those in Fig. 6, with the exception of Rb, a CMV-driven Rb eukaryotic expression vector. All CAT assays were done at least three times.

We have obtained similar data in a DG75 cell line, a non-Epstein-Barr virus Burkitt's lymphoma cell line (data not shown).

Expression of the IE1-72 protein in human glioblastoma cells has been demonstrated to transactivate an E2CAT reporter (60), while our data demonstrate no transactivation in C33A cells. We have reasoned that this may be due to the lack of functionally active pocket-protein complexes in C33A cells. In normal cycling cells, E2F-responsive promoters are maintained in a transcriptionally silent form through E2F-pocket protein complexes bound to the E2F sites in the promoter. Since C33A cells lack a functional Rb protein, high levels of E2F transcriptional activity persist. This would account for the high levels of constitutive reporter activity detected (constitutive E2CAT promoter activity was close to 50% acetylation). In this type of system, an indirect form of activation, such as the alleviation of repression by the IE1-72 protein which we see, might be masked by the constitutively high level of activity off the E2CAT reporter construct. In a cell line in which E2F complexes were transcriptionally repressed at most times during the cell cycle, such as glioblastoma cells, alleviation of suppression of even just a subset of those complexes could have a dramatic effect on activation.

The IE1-72 protein is incapable of alleviating Rb-mediated repression of an E2F-responsive CAT reporter construct. The pRb and p107 proteins appear to be functionally identical in their ability to suppress transcription from E2F-responsive promoters. We wished to determine whether alleviation of repression of an E2F-responsive promoter by the IE1-72 protein extended to Rb-mediated repression or was specific only to p107 suppression. The constitutive activity of the E2CAT promoter was reduced by approximately fourfold when the promoter was transfected with a pRb expression construct (Fig. 7, lane 4). Cotransfection of the E2CAT promoter with both the pRb expression vector and the IE1-72-kDa expression vector failed to restore promoter activity (Fig. 7, lane 5). These data indicate that the HCMV IE1-72 protein is capable of relieving p107-mediated suppression of an E2F-responsive promoter but not Rb-mediated repression of the same promoter. Since the IE1-72 protein fails to interact physically with pRb (Fig. 2B), we reason that protein-protein interactions are

A



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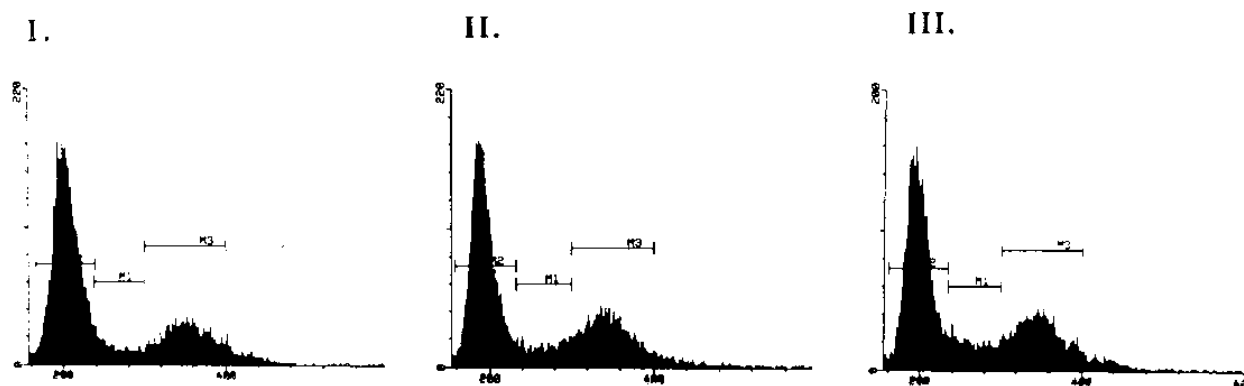

FIG. 8. HCMV infection and IE1-72 and IE2-86 expression induce cell cycle changes in quiescent cells. (A) MRC5 fibroblasts were serum starved for 6 days and then mock infected (I), infected with HCMV (10 PFU per cell) (II), or infected with UV-inactivated HCMV (III). Infections were performed for 2 h, and then the cells were washed with serum-free medium. After 60 h, the cells were propidium iodide stained and analyzed by FACS analysis. (B) COS-1 cells were serum starved for 6 days and transfected with either an empty expression vector (I), an IE1-72 expression vector (II), or an IE2-86 expression vector (III). Staining and analysis was performed as described for the MRC5 cells. Transfection efficiencies for the IE1-72 and IE2-86 expression vectors were 20 and 5%, respectively.

involved in the ability of the IE1-72 protein to relieve suppression of these types of promoters.

HCMV infection induces cell cycle progression in quiescent cells. DNA viruses require host cellular machinery in order to replicate their own viral DNA for successful infection. For this to occur, viruses have had to evolve a means of activating their typically quiescent target cells to stimulate exit of G_0/G_1 and entry into the cell cycle. Infected cells would be pushed from G_0/G_1 into the cell cycle, allowing for activation of the cellular DNA replication machinery in the ensuing S phase and subsequent viral DNA replication. HCMV, which encodes many of its own DNA replication-associated genes, is, nevertheless, dependent on a series of host factors to allow for viral DNA replication; therefore, we wished to determine whether HCMV infection did indeed result in cell cycle progression. Serum-starved fibroblasts (arrested in G_0/G_1) were mock infected, infected with HCMV, or infected with irradiated (inactivated) HCMV. Cells were harvested and examined for entry into S phase by propidium iodide staining and flow cytometry. All infections were done in the absence of serum. At 60 hpi, a dramatic shift from G_0/G_1 to S phase and G_2/M was observed in the HCMV-infected cells, with the percentage of fibroblasts in S phase increasing from 3.2% in the serum-starved fibroblasts to 17.8% in the HCMV-infected cells and the percentage of cells in G_2/M increasing to 14.2% in the HCMV-infected cells from 6.0% in the serum-starved cells (Fig. 8A, I and II). The infection with UV-irradiated virus, however, showed little shift into G_0/G_1, with only 4.7% of the cells in S phase and 8.6% of the cells in G_2/M demonstrating that serum or binding and entry of virus alone is not sufficient to induce cell cycle progression (Fig. 8A, I and III). These data are consistent with other observations of HCMV driving cell cycle progression in infected cells (44). Clearly, HCMV infection is capable of overcoming a serum starvation-induced G_0/G_1 block to stimulate cellular proliferation.

To determine whether expression of the IE proteins IE1-72 and IE2-86 was capable of recreating this effect, expression vectors of the IE proteins were transfected into G_0/G_1-arrested COS-1 cells (cells were arrested by 6 days of serum starvation). At 2 days posttransfection, cells transfected with an expression vector for the IE1-72 protein showed a modest increase in the number of cells in S phase (with 7.8% in the cells transfected

with vector alone compared with 10.0% in the cells transfected with an IE1-72 expression vector [an induction in the number of cells in S phase of almost 25%]) and a more dramatic increase in the number of cells in G_2/M (from 21.8% of cells transfected with vector alone compared with 26.4% in IE1-72-transfected cells) (Fig. 8B, I and II). IE2-86 expression resulted in a progression into S phase more dramatic than that seen with IE1-72 expression (from 7.8% in the cells transfected with vector alone to 11.9% in the IE2-86-transfected cells); however, a similar increase in the number of cells in G_2/M was observed (from 21.8% in the cells transfected with vector alone to 25.9% in the IE2-86-transfected cells) (Fig. 8B, III). All of the increases seen with the transfected IE expression vectors were highly reproducible. Expression of the IE1-72 and IE2-86 proteins can induce shifts in the cell cycle state of quiescent cells; however, the effect does not seem as pronounced as that seen with complete HCMV infection. These data suggest that both the IE1-72 and IE2-86 proteins play a role in inducing cellular proliferation during infection but that other factors employed by HCMV are also necessary.

DISCUSSION

Creation of a cellular environment favorable to DNA replication is a common motif which appears to occur through similar mechanisms among different small DNA tumor viruses (SV40, HPV, and adenovirus). Data increasingly indicate that HCMV functions in a manner analogous to these viruses with respect to stimulating host cell DNA replication; however, the mechanisms that HCMV employs to activate cellular replication machinery have not yet been fully elucidated. The p107 protein has been shown to have a key role in preventing cellular proliferation and, therefore, becomes a focus for regulation by the small DNA viruses. We wished to determine whether HCMV infection resulted in a similar targeting of the p107 protein. Western blot analysis of p107 in HCMV-infected fibroblasts reveals an induction of protein levels during a time course of infection, with sustained elevation of protein detected from early to late times of infection. We demonstrate that the IE1-72 protein can complex with the p107 protein in HCMV-infected HELs and that this interaction coincides with the p107 protein induction seen at early to late times of infection. The interaction also coincides with maximal levels of IE1-72 phosphorylation, leading us to speculate that phosphorylation of IE1-72 might be necessary for the interaction to occur, an avenue of research we are currently investigating.

The IE1-72 protein is capable of relieving the p107-mediated transcriptional repression of an E2F-responsive promoter. We believe that this effect is dependent on complex formation between the HCMV IE1-72 protein and the p107 repressor gene product. We base this assertion on the fact that pRb, which we and others have demonstrated does not complex with the IE1-72 protein, represses the E2CAT reporter in a manner similar to that of p107 but is resistant to alleviation of repression by IE1-72 expression. We believe that these data represent a novel mechanism for transcriptional activation by the IE1-72 protein, involving the overcoming of p107-mediated repression; however, details of this mechanism need to be further elucidated. We hypothesize that p107 is released from its E2F binding partner (presumably E2F4) upon complex formation with the phosphorylated form of the IE1-72 protein, thereby allowing transcriptional activation by E2F in a manner analogous to phosphorylation of p107 (3, 79) (Fig. 9, III); however, data from our laboratory supporting this model are still preliminary. It should be noted that earlier work demonstrated an initial induction of a complex with E2F activity

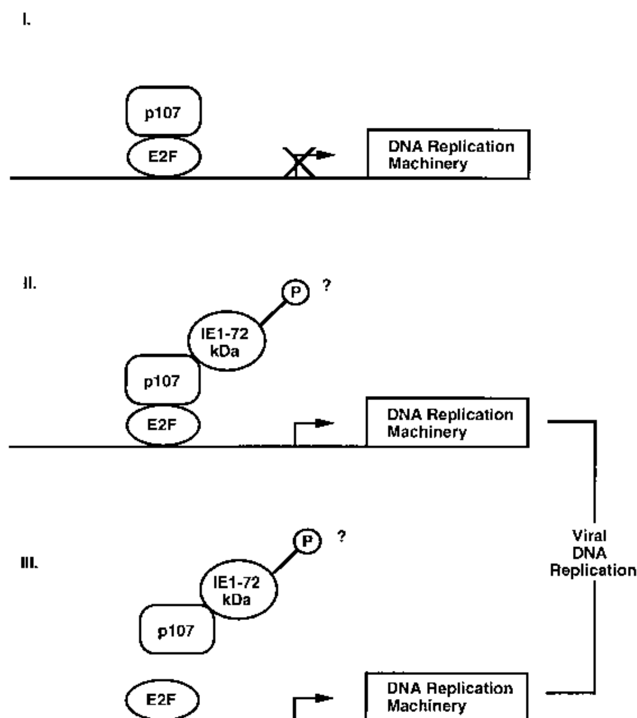

FIG. 9. A model of HCMV IE1-72 and p107 protein-protein interaction. Active transcriptional repression is mediated by p107 (I). Binding of the IE1-72 protein (which may be based on the phosphorylation state of the IE1-72 protein) relieves transcriptional repression of an E2F-responsive promoter by the p107 protein (II). Alleviation of repression may involve release of p107 from its E2F binding partner (III), but data supporting this model are still preliminary. Transcriptional activation of the E2F-responsive genes would result in activation of host cellular DNA machinery and allow for viral DNA replication.

containing p107 at IE times of infection, but the IE1-72 protein could not be detected in the complex (82). We suspect that this complex may be disrupted by the IE1-72 protein at early to late times of infection, but it is also possible that the IE1-72 protein stabilizes the complex and alleviates its repressive function to allow for transcription (Fig. 9, II).

A series of HCMV early promoters contain E2F sites (50, 52, 90), suggesting that E2F plays an important role in activation of viral genes, but we speculate that the role of E2F during HCMV infection is not limited to activation of viral promoters only. The first round of viral DNA replication occurs at a time during infection when the interaction between the p107 and IE1-72 proteins is maximal. Because this interaction is maximal, concomitant with viral replication, and because a series of genes that contain E2F-responsive elements in their promoters are associated with the cellular DNA replication machinery, a potential function of IE1-72 and p107 protein complex formation beyond activation of viral promoters can be hypothesized. The p107 protein is known to repress E2F-responsive promoters, a subset of which are involved in DNA replication and turned on during HCMV infection (dihydrofolate reductase, thymidine kinase, and DNA polymerase α) (4, 14, 26, 29, 35, 51, 58, 62, 69, 72, 80, 83), and our data demonstrate that IE1-72 expression is capable of overcoming p107-mediated repression. It is, therefore, possible that the IE1-72 protein promotes transcriptional activation of these promoters, thereby activating cellular DNA machinery and allowing viral DNA replication to occur. Interestingly, the p107 promoter itself contains E2F sites which can be repressed by p107 (96). The induction of

p107 protein levels seen during infection may, therefore, be due, at least in part, to activation of the p107 promoter through alleviation of repression by IE1-72 protein expression.

Growing evidence has suggested a common mechanism for activating the host DNA replication machinery by small DNA tumor viruses. These viruses all seem to induce cellular proliferation and movement into S phase through direct protein-protein interactions between virally encoded proteins and a subset of host proteins. Cellular targets for regulation by viral proteins expressed by the small DNA tumor viruses include the E2F-regulating family of pocket proteins, pRb, p107, and p130. In the case of these viruses, large T, E7, and E1A all interact with both p107 and pRb. Interestingly, the IE protein IE2-86 of HCMV has been demonstrated to bind the Rb protein (93), while our data indicate that the IE1-72 protein (but not the IE2-86 protein) interacts specifically with the p107 protein. Therefore, HCMV represents a novel system in which two distinct viral proteins target the p107 and pRb proteins separately for regulation through direct protein-protein interaction. The outcome of HCMV viral protein interaction with both pRb and p107 seems to be an activation of transcription from promoters normally repressed by the pocket proteins. Since both the IE1-72 and the IE2-86 proteins are expressed in a temporally distinct fashion during infection, it seems reasonable to speculate that alleviation of Rb-mediated or p107-mediated transcriptional repression would be similarly regulated. HCMV therefore appears to have a level of control in activating E2F-responsive promoters at various stages of infection finer than that of the small DNA tumor viruses which encode a single gene product to bind all members of the pocket protein family.

The biological ramifications of this extra level of control are not completely clear, but the high levels of transformation associated with infection by the small DNA tumor viruses that are absent from HCMV infection may be a function of this. Transformation by the small DNA tumor viruses seems to occur through two pathways: in one, a viral protein binds all of the pocket protein family members (pRb, p107, and p130), resulting in activation of E2F-responsive promoters. The E2F-1 promoter is itself activated, creating an amplification of E2F-1 protein levels. Increased levels of E2F-1 promote cellular proliferation but have also been demonstrated to activate p53-mediated apoptosis (38, 53, 73). Therefore, in the second pathway, a viral protein targets and functionally inactivates p53, preventing the apoptotic pathway from being successfully triggered. In most infections, proliferation allows for replication of the viral DNA and successful infection, but a minority of infections are aberrant, resulting in unchecked proliferation and eventual transformation. It has been shown that HCMV infection induces the p53 gene product in a manner analogous to adenovirus and SV40 infection, that the IE2-86 protein can directly bind the p53 protein and prevent p53-mediated transcription (76), and that HCMV proteins (the IE72 and IE2-86 proteins) can protect against apoptosis (40). Our data also demonstrate that HCMV can induce cellular proliferation and that the IE1-72 and IE2-86 proteins seem to be functionally similar to the adenovirus E1A, the SV40 large T, and the HPV E7 viral proteins in their ability to bind pRb and p107 and, by extension, in their ability to stimulate cellular proliferation. It is, therefore, interesting that a strong association between HCMV infection and human cancer in infected patients has never been demonstrated, even though both the small DNA tumor viruses and HCMV target cellular pathways which can lead to transformation. Our data point to what may be a crucial difference: HCMV encodes at least two IE proteins to specifically interact with different pocket proteins. Since these two proteins are distinctly regulated, sequential activation of E2F-responsive promoters and, potentially, the specificity as to which E2F-responsive promoters are activated might be more tightly regulated during HCMV infection. This additional level of control could help favor productive infection over the aberrant outcome of transformation.

Interestingly, data in our laboratory suggest that neither the IE1-72 nor the IE2-86 protein interacts with the p130 member of the pocket protein family (our unpublished data). The p130 protein is thought to be one of the first checkpoints for allowing entry into the cell cycle from quiescence (12, 36, 89) and is, most likely, an essential and initial target in HCMV infection. It is possible that regulation of the p130 protein occurs through a signal transduction pathway activated by virus binding to the cell or that a third protein is encoded by HCMV that directly binds p130. HCMV infection may coordinate a sequential disruption of normal pocket protein function beginning with the p130 protein and then moving to the pRb and p107 proteins. These directions are all being actively pursued in our laboratory.

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