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The UL84 Protein of Human Cytomegalovirus Acts as a Transdominant Inhibitor of Immediate-Early-Mediated Transactivation That Is Able To Prevent Viral Replication

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The 86-kilodalton immediate-early (IE) 2 protein (IE2-p86) of human cytomegalovirus (HCMV) is a multifunctional regulator of HCMV gene expression which appears to be essential for triggering the lytic replicative cycle. IE2-p86 functions as a promiscuous transactivator of both viral and cellular gene expression and can repress transcription from its own promoter. In this study we demonstrate that a viral early protein, termed pUL84, which is able to interact with IE2-p86 both in vivo and in vitro, modulates IE2-p86 in a specific manner. First, pUL84 acts as a transdominant inhibitor of IE2-p86-mediated transactivation of both homologous and heterologous promoters. Second, negative autoregulation by IE2-p86 is augmented in the presence of pUL84. Using two in vivo assays, we obtained evidence that expression of pUL84 during the IE phase of the viral replicative cycle leads to an inhibition of viral early gene expression which prevents replication of HCMV and results in a persistent infection of UL84-positive cell lines. Transdominant inhibition of a viral IE function by a protein expressed during the later phases of replication appears to be a novel principle used by herpesviruses which could account for the slow replication of HCMV and may be useful in the development of new antiviral strategies.

Human cytomegalovirus (HCMV) is a major human pathogen causing severe disease in newborns and immunocompromised patients. It belongs to the beta subgroup of herpesviruses and is characterized by its narrow host range and prolonged replicative cycle in tissue culture cells. HCMV gene expression occurs in a cascade with at least three main phases, commonly referred to as immediate-early (IE), early, and late. During the IE phase, several proteins that are required for the activation of promoters of the next temporal class, the early class, are expressed (6, 46, 48). The best-characterized proteins are the 72-kDa IE-1 protein (also called IE1-p72 or ppUL123) and the 86-kDa IE-2 protein (also called IE2-p86 or ppUL122a), which originate from the major IE gene region of HCMV during initial stages of the replicative cycle (15, 29, 47) (Fig. 1). In particular, IE2-p86 appears to play a master role in triggering the lytic replicative cycle of HCMV (15, 28).

Two main functions of IE2-p86 have been defined. First, it is able to repress transcription of its own promoter (14, 29), the potent major IE enhancer-promoter of HCMV (4), thus antagonizing its own expression. This negative autoregulation is mediated by a direct DNA contact of IE2-p86 with a sequence element located between the TATA box and the transcriptional start site of the enhancer-promoter (21, 23). DNA binding of IE2-p86 at this specific position of the promoter has recently been shown to block the association of RNA polymerase II with the preinitiation complex (22). Second, IE2-p86 is a strong transactivator of viral early promoters and of several heterologous promoters, including the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (12, 18, 24). This function of IE2-p86 is thought to be required for progression of the replicative cycle from the IE to the early phase. The mechanism of transactivation has not been defined entirely. However, since IE2-p86 interacts with the basal transcription factors TATA-binding protein (TBP) (12, 42) and TFIIB (5) and with distinct cellular transcription factors such as CREB or AP1 (20, 40), protein contacts are believed to be essential for transactivation. The demonstration of interactions with the cell cycle regulatory proteins pRB and p53 suggested that IE2-p86 could also have an influence on cellular proliferation (11, 44), thus resembling the large T oncoprotein of simian virus 40. Further evidence for such a function is the detection of IE2-p86 in smooth muscle cell proliferations of restenotic lesions after coronary angioplasty (44), which implies that expression of IE2-p86 alone may be sufficient to induce disease.

In addition to these cellular interaction partners, it has been shown by coimmunoprecipitation analyses that IE2-p86 is associated with a protein of viral origin within permissively infected cells (38). In parallel, a precursor polypeptide of IE2-p86 could be detected, suggesting that this association depends on posttranslational modifications (38). Subsequently, the viral interaction partner of IE2-p86 was identified as the gene product of the open reading frame (ORF) UL84 of HCMV (43), which is expressed as an early-late gene during the viral replicative cycle (13). Recent studies suggested that the UL84 protein may have an essential function in promoting *ori*Lyt-dependent DNA replication in cotransfection assays (39).

In this study we have examined whether the interaction with pUL84 can modulate the functions of IE2-p86. By performing cotransfection experiments, we demonstrated that pUL84 acts as a transdominant inhibitor of IE2-p86-mediated transcriptional activation. We go on to show that expression of pUL84 during the IE phase is able to block the synthesis of viral early proteins and thereby prevents HCMV replication. Regulated expression of factors like pUL84 may be a novel means by

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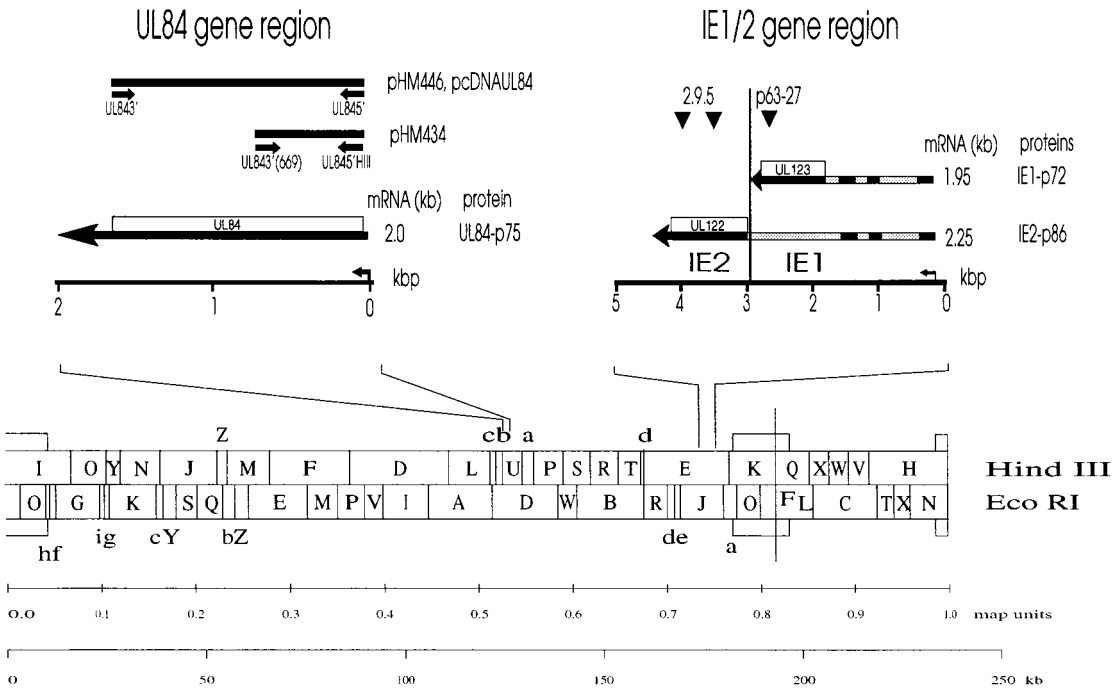

FIG. 1. Prototype arrangement of the HCMV AD169 genome and positions of the IE1/2 and UL84 gene regions. The prototype arrangement of the HCMV AD169 genome is shown in the lower half. In the upper half, a schematic diagram of the UL84 and IE1/2 gene regions, including the respective transcripts and proteins, is shown. PCR amplification products of the UL84 gene region generated in this study are represented as black bars, and the derived vectors are named at the right of the bars. The small arrows indicate the positions and directions of the primers used for amplifications. Solid rectangles on the top of the diagram of the IE1/2 gene region indicate epitopes recognized by the monoclonal antibodies 2.9.5 and p63-27.

which viruses control the function of promiscuous transactivators such as IE2-p86. In the case of betaherpesviruses, this unique principle of transdominant inhibition of a viral IE function could explain their slow replication and may help in the development of peptidomimetic antiviral agents that target the IE2-p86 protein.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). The following oligonucleotides (5′-to-3′ sequences; double-stranded oligonucleotides are indicated by double shills) were used for cloning and PCRs: UL845′, TAAGAATTCATGCCACGCGTCGACCCCAACCT TCGGAAT; UL845′HIII, TGTCCAAGCTTATGCCACGCGTCGACCCCA; UL845′(178), ATATAAGCTTAGGATGGGCGTCGCCAGTACCGA; UL845′(202),ATATGAATTCAGGATGGACAGTCTCCTCCTGAAAAC; UL845′(247), ATATGAATTCAGGATGGGCTCGCCGCAGACCATCCT; UL845′(313), ATATGAATTCAGGATGGGCACCTACCATCTGATCCA; UL845′(329), ATATAAGCTTAGGATGGTCCAGTTGCACCTCGACCTC; UL845′(358), ATATAAGCTTAGGATGGAATTGCGGGATCCCTTCCA; UL843′, TAATCTAGATCCCTAGGTACCTTCGAGATCGCCGCAGACCA TGGCTAAAGTGAC; UL843′Asp, TGCTGGTACCTTCGAGATCGCCGC AGACCATGG; 3′FLAG-Xba, GCCCTCTAGAGCTTGTCATCGTCG; UL843′(669), TGCTGGTACCCATGCGCGTCAGTTGTTGG; UL843′(1539), TGCTGGTACCTGACAGGCAACCCCGATTCA; and FLAG, CGACTACA AGGACGACGATGACAAGCT//CTAGAGCCTGTCATCGTCGTCCTTG TAGTCGGTAC.

Antibodies. Monoclonal antibody 2.9.5 recognizes epitopes encoded by exon 5 of the IE2 gene region of HCMV and was used for coimmunoprecipitation analysis of IE2-p86-associated proteins (Fig. 1) (31). Monoclonal antibody anti-FLAG M2, which is directed against the synthetic FLAG octapeptide N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C, was obtained from Integra Biosciences, Tecnomara Deutschland GmbH. The monoclonal antibodies p63-27 (directed against the IE protein IE1-p72 of HCMV), BS510 (directed against the early protein UL44-p52 of HCMV), UL83/28-77 (directed against the early-late protein UL83-pp65 of HCMV), and XP1 (directed against the late protein UL32-pp150 of HCMV) were described previously (1, 17, 30–32). Polyclonal antisera against the IE2-p86 and the UL69 proteins of HCMV were generated by immunizing rabbits with the respective procaryotically expressed proteins (49).

PCR and plasmids. PCR was used to generate specific subfragments of the UL84 gene region of HCMV. Amplification was performed by using Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) and 50 pmol of each primer. Conditions for PCR were as described previously (2). DNA from HCMV strain AD169 served as the template in these reactions. Primers UL845′ and UL843′ (Fig. 1) were used to amplify the entire UL84 ORF. After purification of the amplification product and cleavage with *Eco*RI and *Xba*I, whose recognition sequences were contained within the primers, the respective fragment was cloned into the eucaryotic expression vectors pcDNA3 (Invitrogen Corp., San Diego, Calif.) and pSG424 (37), resulting in plasmids pHM446 and pSG84, respectively. Plasmid pSG84 was modified further by insertion of the FLAG oligonucleotide into the *Kpn*I-*Xba*I-cleaved vector. This fused the UL84 ORF to a sequence encoding a synthetic octapeptide epitope which is recognized by the monoclonal antibody anti-FLAG M2 (Integra Biosciences, Tecnomara Deutschland) and allowed the immunological detection of pUL84 after expression in eucaryotic cells. In the next step, the UL84::FLAG fusion was cloned via *Eco*RI-*Xba*I into the eucaryotic expression vector pcDNA3, which contained a neomycin resistance marker for selection of stably transfected cells. This plasmid was designated pcDNAUL84. For construction of carboxy-terminal deletion mutants of UL84, fragments were amplified by using the primer pairs UL845′HIII-UL843′(669) and UL845′HIII-UL843′(1539) and DNA from plasmid pcDNAUL84 as the template (Fig. 1). After digestion with *Hin*dIII and *Asp*718, the respective amplification products were cloned into the *Hin*dIII-*Asp*718-cleaved vector pcDNAUL84, resulting in plasmids pHM434 and pUL84/1-513, which contained fusions of the carboxy-terminal UL84 deletion to the coding sequence for the FLAG epitope. Similarly, amino-terminal deletion mutants of UL84 were created via PCR with primer pairs UL845′(178)-UL843′Asp, UL845′(202)–3′FLAG-Xba, UL845′(247)–3′FLAG-Xba, UL845′(313)–3′FLAG-Xba, UL845′(329)-UL843′Asp, and UL845′(358)-UL843′Asp. Construction of the eucaryotic IE2-p86 expression plasmids pHM121 and pHM134, the IE1 and -2 expression plasmid pRR47, and the luciferase reporter plasmid pHM142, which contained the UL112 promoter of HCMV upstream of the luciferase ORF, has been described previously (2, 20, 31, 45). An expression plasmid for IE2-p40 was created by inserting the *Sma*I-*Sal*I fragment of the IE2-p86 cDNA into the *Eco*RV-*Sal*I-cleaved vector pcDNA3. The luciferase reporter plasmids pHIVluc (HIV LTR upstream of luciferase) and 5GALluc (five GAL4 binding sites upstream of the adenovirus E1b promoter in front of luciferase) and the Tat expression vector pCT21 were obtained from C. Aepinus (Erlangen, Germany) (26, 49). Plasmids pSCTGal-VP80 (encoding a GAL4 fusion with the activation domain of herpes simplex virus VP16) and GAL4-Oct1Q (encoding a GAL4 fusion with the activation domain of Oct1)

were kindly provided by W. Schaffner (Zürich, Switzerland) (41). Plasmid pHM284 was generated by insertion of a *Bam*HI fragment containing the luciferase gene cassette from plasmid p19luc into the chloramphenicol acetyltransferase reporter vector pRR55, whose chloramphenicol acetyltransferase expression cassette had been removed via digestion with *Bam*HI (7). For in vitro transcription-translation reactions, plasmids pBSIE1 (cDNA for IE1-p72 within the BlueScribe vector BS+), pBSTBP (cDNA for TBP within the BlueScribe vector BS+), and pcDNAUL84 were used. All plasmid constructions were confirmed by DNA sequence analysis with a commercially available T7 sequencing kit (Pharmacia, Freiburg, Germany).

Cell culture, transfections, and luciferase assays. Cell culture conditions for primary human fibroblasts (HFFs) and U373, HeLa, and COS cells were as described previously (2). The protein kinase inhibitor staurosporin was obtained from Boehringer Mannheim and was added to the cells at concentrations of 8.2 and 82 nM and left for 120 min in order to inhibit phosphorylation. For transient-expression experiments, U373 cells were transfected by using the DEAE-dextran procedure exactly as described previously (2). HeLa and COS cells were transfected by the calcium phosphate coprecipitation procedure (3). In cotransfection experiments, 3 μg of luciferase target genes and 5 μg of the cotransfected plasmids were used routinely. The total amount of transfected DNA was kept constant by using the cloning vector pCB6 or pcDNA3 in order to replace the missing transactivator plasmid. About 48 h after transfection, cell extracts were prepared and luciferase assays were performed as previously described (2). Each transfection was performed at least four times.

For the establishment of stably transfected cell lines, U373 cells were transfected by the calcium phosphate coprecipitation procedure with 10 μg of either the UL84 expression vector pcDNAUL84 or the cloning vector pcDNA3. About 48 h after transfection, G418 was added to the cell culture medium at a concentration of 500 μg/ml. After 4 weeks of selection, G418-resistant clones were subcultured in 24-well culture dishes and subsequently tested for UL84 gene expression by indirect immunofluorescence with the monoclonal antibody anti-FLAG M2. Ten UL84-positive and 10 UL84-negative cell clones were selected for further experiments.

Virus infections and determination of viral titers. Virus infections of HFFs and U373 cells were performed as described previously (45). Briefly, cells growing in 100-mm-diameter dishes at about 70% confluence were infected with HCMV strain AD169. The multiplicities of infection (MOIs) ranged from 0.1 to 1. Following adsorption for 1 h at 37°C, fresh medium was added, and the cells were incubated until further analyses were performed. For growth analysis of HCMV in UL84-expressing cell lines, supernatants were collected at various time points after infection and frozen at −80°C. Viral titers were then measured in parallel in all samples by determination of the 50% tissue culture infectious dose.

Metabolic labeling, immunoprecipitation, and immunoblot analysis. Approximately 2×10^6 cells were labeled with 100 μCi of Tran-^{35}S-Label (ICN, Eschwege, Germany) for 2 h or as indicated. Cells were washed in phosphate-buffered saline (PBS) and lysed by incubation at 4°C in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 20 μg of aprotinin per ml. Cell extracts were clarified by centrifugation in an Eppendorf microcentrifuge at 13,000 rpm for 5 min, and 250 μl of the extract, made to 500 μl with lysis buffer, was preadsorbed by incubation for 3 h with Protein A-Sepharose. After centrifugation, the supernatant was immunoprecipitated with a complex of Protein A-Sepharose and either the IE2-specific monoclonal antibody 2.9.5 or a polyclonal rabbit serum against IE2-p86 (31). Protein A-Sepharose complexes were washed three times with SNNTE-buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 100 mM NaCl, 5% sucrose, 1% Nonidet P-40) and one time with lysis buffer. For phosphatase treatment, immunoprecipitated proteins were incubated in a buffer containing 20 mM HEPES (pH 7.8), 20 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 10 U of calf intestinal phosphatase (CIP) for 1 h at 37°C. Competitive inhibition of phosphatase activity was achieved by the addition of *p*-nitrophenyl phosphate (NPP) at a concentration of 10 mM to the reaction mixture. Phosphatase-treated proteins were purified via a second immunoprecipitation reaction. Immunoprecipitated proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), processed by fluorography with Amplify (Amersham), and autoradiographed. Immunoblotting was performed with antibody anti-FLAG M2, and proteins were visualized by using ECL reagents (Amersham).

GST fusion proteins and pull-down assays. Glutathione *S*-transferase (GST) expression plasmids pGEX-IE1 and pGEX-IE2 were kindly provided by J. Sinclair (12). GST fusion proteins were expressed and purified as described previously (20). For pull-down assays, 100 ng of the GST fusion proteins on beads was preincubated for 10 min in 200 μl of ELB buffer (125 mM NaCl, 50 mM HEPES, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA) containing bovine serum albumin (final concentration, 1 mg/ml). After addition of 5 μl of in vitro-translated test protein which had been generated by using the TNT system (Promega, Heidelberg, Germany), the GST beads were incubated overnight at 4°C. The beads were then washed five times in 1 ml of ELB buffer, pelleted, and boiled in 2× SDS-PAGE sample buffer. Bound proteins were resolved in SDS–12.5% polyacrylamide gels. The gels were fixed and rocked in a fluorograph for 30 min prior to drying and autoradiography.

Indirect immunofluorescence analysis. For indirect immunofluorescence, cells were fixed with methanol and the respective antibodies were layered over the cells for 30 min at 37°C. Rabbit antisera were diluted 1:200 in PBS, the monoclonal antibody anti-FLAG M2 was used at a dilution of 1:1,000, and the other monoclonal antibodies were used as cell culture supernatants. Cells were then rinsed three times with PBS and incubated with either fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Dako GmbH, Hamburg, Germany) (1:40 dilution) or FITC-conjugated anti-rabbit immunoglobulin (Dako GmbH) (1:40 dilution). For double immunofluorescence analysis, a mixture of FITC-conjugated anti-rabbit immunoglobulin (Dako GmbH) (1:40 dilution) and tetramethyl rhodamine isothiocyanate-conjugated anti-mouse immunoglobulin (Dianova, Hamburg, Germany) was used. After incubation for 30 min at 37°C, the coverslips were washed three times with PBS and mounted for microscopy. Fluorescence micrographs were taken on a Zeiss Axioskop photomicroscope with either a 20× or 60× objective. A Bio-Rad MRC-600 confocal imaging system was utilized for laser-scanning confocal microscopy. Confocal and digital images were processed by using Corel Photopaint and Corel Draw 4.0 (Corel Corporation, Ottawa, Ontario, Canada). Each experiment was repeated at least two times, and multiple fields of view were examined. Micrographs were chosen as representative of typical fields.

RESULTS

Stable interaction of IE2-p86 with pUL84 is independent of its phosphorylation and occurs in the absence of additional viral proteins. Previous studies, which demonstrated that the 86-kDa IE2-protein of HCMV (IE2-p86) is synthesized as a precursor protein of 80 kDa (IE2-p80), suggested that the reported interaction between IE2-p86 and pUL84 depends on posttranslational modification of this protein (38, 43). As IE2-p86 is a known phosphoprotein (15), we used the strong protein kinase inhibitor staurosporin to prevent phosphorylation of IE2-p86. HCMV-infected fibroblast cells were metabolically labeled and incubated in the presence of increasing concentrations of staurosporin. After immunoprecipitation analysis with the IE2-specific monoclonal antibody 2.9.5, only the precursor protein IE2-p80 could be observed at the highest concentration of staurosporin used (Fig. 2A, lane 4). There was no quantitative difference in the amount of coprecipitated pUL84 compared to that for cells without staurosporin (Fig. 2A, lanes 2 and 4). To further confirm that the protein detected in the presence of staurosporin corresponded to the precursor polypeptide of IE2-p86, HCMV-infected fibroblast cells were either pulse-labeled for 30 min prior to cell harvest or metabolically labeled for 3 h in the presence or absence of staurosporin. After immunoprecipitation with monoclonal antibody 2.9.5, the IE2-p80 protein could be detected in extracts from pulse-labeled cells, whereas only the mature IE2-p86 was present in cells that were labeled for 3 h (Fig. 2B, lanes 1 and 2). The IE2 protein that was immunoprecipitated from staurosporin-treated cells comigrated with the IE2-p80 precursor, as observed with extracts from pulse-labeled cells (Fig. 2B, lanes 1 and 3). In order to show that the slower migration of IE2-p86 compared to IE2-p80 in SDS-PAGE is due to phosphorylation, immunoprecipitated IE2-p86 was treated either with CIP or with CIP in the presence of the phosphatase substrate NPP, which served as a competitive inhibitor. After purification of the resulting IE2 proteins by performing a second immunoprecipitation reaction and SDS-PAGE, the IE2-p80 precursor could be observed after treatment with CIP, but not in the presence of NPP (Fig. 2C, lanes 2 and 3). In summary, these experiments indicate that the IE2-p80 precursor is converted into the mature IE2-p86 protein via phosphorylation. Since comparable amounts of pUL84 could be detected with both IE2-p80 and IE2-p86 (Fig. 2A and B), we conclude that the interaction between these two viral proteins does not depend on this specific phosphorylation event.

We next wanted to test whether IE2-p86 could interact with pUL84 in the absence of other viral proteins. For this purpose, a eucaryotic expression vector for pUL84 was constructed (Fig. 1). After coexpression of IE2-p86 together with pUL84 in COS

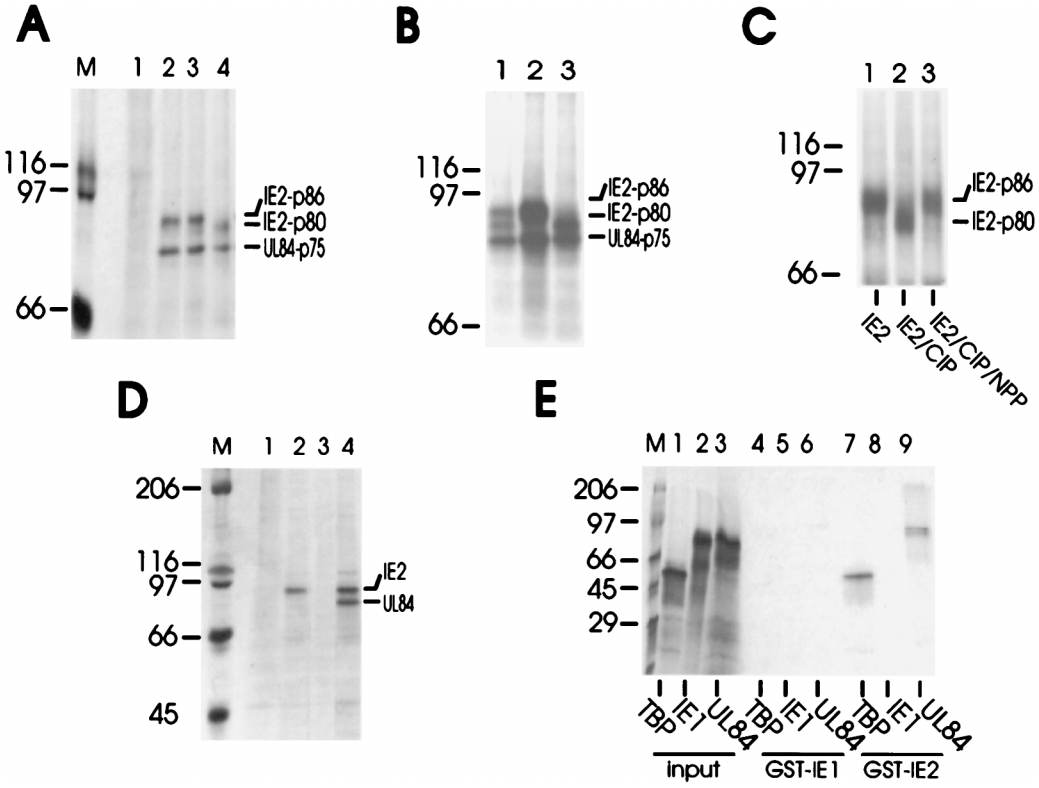

FIG. 2. Stable interaction between the IE2 protein and pUL84 in the absence of posttranslational modifications and in the absence of additional viral proteins. (A) Immunoprecipitation analysis with the IE2-specific monoclonal antibody 2.9.5 and lysates from mock-infected and HCMV-infected HFF cells (48 h postinfection). HFFs were either mock infected or infected with HCMV AD169 and labeled for 2 h with Tran-^{35}S-Label in the absence or presence of the protein kinase inhibitor staurosporin. Cell extracts were immunoprecipitated with the IE2-specific monoclonal antibody 2.9.5. Lane M, molecular mass standards; lane 1, lysate from mock-infected cells; lane 2, lysate from HCMV-infected cells; lane 3, lysate from HCMV-infected cells grown in the presence of 8.2 nM staurosporin; lane 4, lysate from HCMV-infected cells grown in the presence of 82 nM staurosporin. (B) Immunoprecipitation analysis with the IE2-specific monoclonal antibody 2.9.5 and lysates from HCMV-infected HFF cells (48 h postinfection). HFFs were infected with HCMV AD169 and were labeled either for 30 min or for 3 h with Tran-^{35}S-Label in the absence or presence of staurosporin. Lane 1, lysate from HCMV-infected cells labeled for 30 min; lane 2, lysate from HCMV-infected cells labeled for 3 h; lane 3, lysate from HCMV-infected cells labeled for 3 h in the presence of staurosporin. (C) Phosphatase treatment of immunoprecipitated IE2-p86. IE2-p86 was immunoprecipitated from HCMV-infected cells labeled for 2 h with Tran-^{35}S-Label and incubated in the presence or absence of CIP and the phosphatase substrate NPP. After a second immunoprecipitation reaction with monoclonal antibody 2.9.5, proteins were separated by SDS-PAGE and detected via autoradiography. Lane 1, no phosphatase treatment; lane 2, incubation in the presence of 10 U of CIP; lane 3, incubation in the presence of CIP and NPP. (D) Immunoprecipitation analysis with the IE2-specific monoclonal antibody 2.9.5 and lysates from transfected COS cells. COS cells were transfected with various expression vectors and labeled with Tran-^{35}S-Label, and cell lysates were prepared for immunoprecipitation analyses. Lane M, molecular mass standards; lane 1, lysate from mock-transfected COS cells; lane 2, lysate from COS cells transfected with the IE2 expression vector pHM121; lane 3, lysate from COS cells transfected with the UL84 expression vector pcDNAUL84; lane 4, lysate from COS cells cotransfected with pHM121 and pcDNAUL84. (E) Interaction between procaryotically expressed IE2 and pUL84 in a pull-down assay. In vitro-translated ^{35}S-labeled TBP (lanes 1, 4, and 7), IE1 (lanes 2, 5, and 8), and UL84 (lanes 3, 6, and 9) proteins were used for pull-down assays. Lanes 1 to 3, SDS-PAGE of input proteins; lanes 4 to 6, SDS-PAGE of proteins after incubation with a GST-IE1 fusion protein; lanes 7 to 9, SDS-PAGE of proteins after incubation with a GST-IE2 fusion protein. The sizes (in kilodaltons) of molecular mass standards are shown on the left of each panel; positions of immunoprecipitated proteins are indicated on the right.

cells, both proteins could be coimmunoprecipitated by using a monoclonal antibody directed against the IE2 protein (Fig. 2D, lane 4). This demonstrates that other viral proteins are not necessary for the formation of a stable complex. In order to confirm these results, GST fusion pull-down assays were performed. The UL84 protein, the HCMV transactivator IE1-p72, and TBP were expressed and labeled in reticulocyte lysates (Fig. 2E, lanes 1 to 3). These proteins were then incubated with a bacterially expressed GST-IE1 or GST-IE2 fusion protein in a pull-down assay (Fig. 2E, lanes 4 to 9). As previously reported, IE2 could interact with TBP (Fig. 2E, lane 7). In addition, we could observe an interaction between IE2 and pUL84 (Fig. 2E, lane 9). In contrast, no binding was detectable when the radiolabeled IE1-p72 was used together with GST-IE2 or when the GST-IE1 protein was incubated together with the three radiolabeled proteins (Fig. 2E, lanes 4 to 6 and lane 8). This finally shows that IE2-p86 and pUL84 are able to interact in the absence of other viral factors and in the absence of posttranslational modification.

pUL84 is a transdominant inhibitor of IE2-p86-mediated transactivation. IE2-p86 is known to be a strong transactivator of both homologous and heterologous promoters. To investigate whether pUL84 could modulate IE2-p86-mediated promoter activation, cotransfection experiments with permissive U373 cells and nonpermissive HeLa cells were performed. First, a reporter plasmid containing the HIV-1 promoter upstream of luciferase was cotransfected with the pUL84 expression plasmid alone. Neither activation nor repression of the HIV-1 promoter in the presence of pUL84 was noted, although there was a clearly detectable constitutive activity of this promoter (Fig. 3A, bars 1 and 2). As reported previously, cotransfection of an IE2-p86 expression vector resulted in strong stimulation of promoter activities (Fig. 3A, bar 3). Surprisingly, however, when both the IE2-p86 and the pUL84

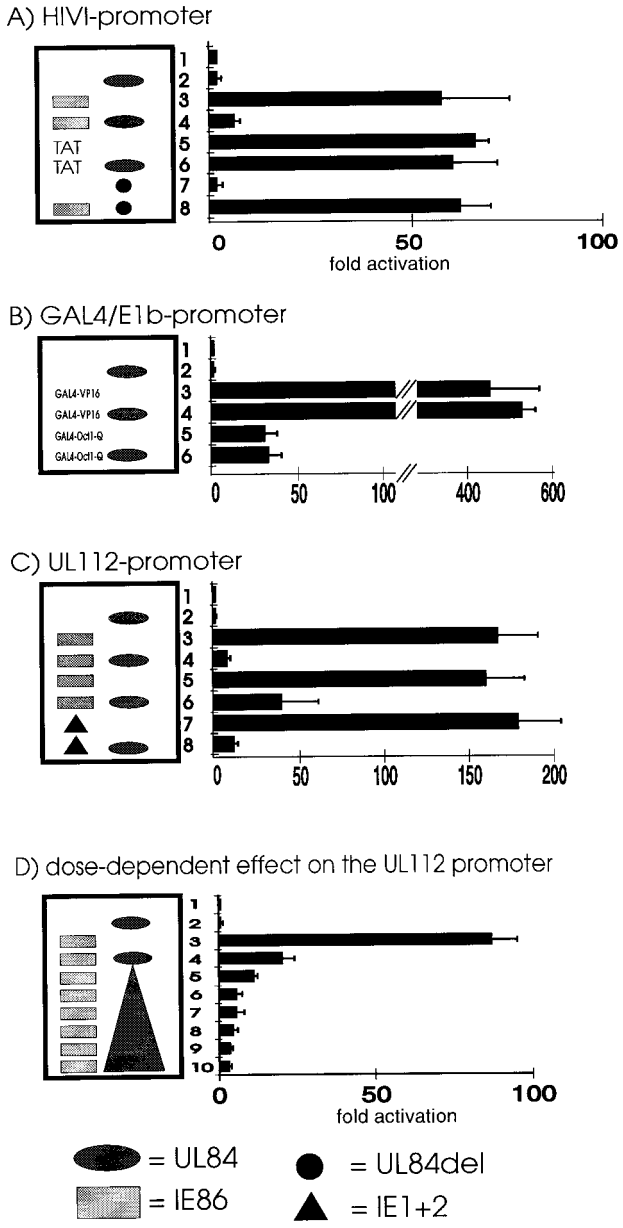

FIG. 3. Effect of pUL84 on IE2-p86-mediated transactivation of various promoter constructs. U373 cells or HFF cells were cotransfected with reporter plasmids containing either the HIV-1 LTR (A), the adenovirus E1b promoter with five GAL4 binding sites (B), or the early UL112 promoter of HCMV (C and D) upstream of luciferase and a combination of various expression vectors. (A) Bars: 1, cotransfection with the cloning vector pcDNA3; 2, cotransfection with the UL84 expression plasmid pHM446; 3, cotransfection with the IE2-p86 expression plasmid pHM134; 4, cotransfection with a combination of pHM446 and pHM134; 5, cotransfection with the Tat expression plasmid pCT21; 6, cotransfection with a combination of pCT21 and pHM446; 7, cotransfection with plasmid pHM434, expressing a truncated pUL84; 8, cotransfection with a combination of pHM434 and pHM134. (B) Bars: 1, cotransfection with the cloning vector pcDNA3; 2, cotransfection with the UL84 expression plasmid pHM446; 3, cotransfection with expression vector pSCTGal-VP80, encoding a GAL4-VP16 fusion protein; 4, cotransfection with a combination of pSCTGal-VP80 and pHM446; 5, cotransfection with expression vector GAL4-Oct1Q, encoding a fusion of the GAL4 DNA-binding domain to the activation domain of Oct1; 6, cotransfection with a combination of GAL4-Oct1Q and pHM446. (C) Bars: 1, cotransfection of U373 cells with the cloning vector pcDNA3; 2, cotransfection of U373 cells with the UL84 expression plasmid pHM446; 3, cotransfection of U373 cells with the IE2-p86 expression plasmid pHM134; 4, cotransfection of U373 cells with a combination of pHM446 and pHM134; 5, cotransfection of HFF cells with the IE2-p86 expression plasmid pHM134; 6, cotransfection of HFF cells with a combination of pHM446 and pHM134; 7, cotransfection of U373 cells with the IE1 and -2 expression vector pRR47; 8, cotransfection of U373 cells with a combination of pHM446 and pRR47. (D) Bars: 1, cotransfection with 10 μg of pcDNAUL84; 2, cotransfection with 10 μg of pHM446; 3 to 10, cotransfection with 3 μg of the IE2-p86 expression plasmid pHM134; 4 to 10, cotransfection performed with 1, 2, 3, 4, 5, 6, and 7 μg of the UL84 expression vector pHM446, respectively. The left of each panel shows a schematic diagram of expression vectors used for cotransfection. On the right the relative luciferase activity, expressed as fold activation relative to the activity of the respective reporter construct in the absence of effector plasmids, is indicated; error bars indicate standard deviations.

expression plasmid were used together, IE2-p86-mediated transactivation of the HIV-1 promoter was substantially diminished. This was specific for IE2-p86, since pUL84 had no significant effect when cotransfection was performed with an HIV-1 tat expression plasmid (Fig. 3A, bars 5 and 6). Similarly, no influence of pUL84 on NF-κB-mediated transactivation of the HIV-1 promoter could be detected (data not shown). The intact pUL84 was required for this effect, since a deletion of 363 amino acids from the carboxy terminus of pUL84 abolished negative regulation (Fig. 3A, bars 7 and 8).

To further confirm that pUL84 repression is IE2-p86 specific, we tested the effect of pUL84 on transcriptional activation domains that are known to contact cellular proteins. For this purpose, the luciferase reporter plasmid 5GAL-luc, containing five binding sites of the *Saccharomyces cerevisiae* factor GAL4 upstream of the adenovirus E1b promoter, was cotransfected with GAL4 fusions in either the absence or presence of a pUL84 expression vector. As shown in Fig. 3B, neither activation mediated by a GAL4-VP16 fusion nor activation mediated by a fusion containing the Q domain of the Oct1 factor was affected in the presence of pUL84. This argues against a nonspecific inhibition of transactivation by pUL84.

Transdominant inhibition of IE2-p86-mediated activation in the presence of pUL84 could also be observed when a viral early promoter which drives expression of an abundant class of early transcripts originating from the UL112/113 gene region of HCMV was used. Again, cotransfection of the IE2-p86 expression plasmid alone resulted in a strong stimulation of the UL112 promoter, whereas pUL84 inhibited IE2-p86-mediated transactivation (Fig. 3C, bars 3 and 4). This was also detectable after transfection of primary HFF cells (Fig. 3C, bars 5 and 6). The use of an IE1 and -2 expression plasmid instead of the IE2-p86 expression plasmid had no influence on inhibition by pUL84 (Fig. 3C, bars 7 and 8).

As shown in Fig. 3D, this negative effect of pUL84 occurred in a dose-dependent manner: increasing amounts of the pUL84 expression plasmid led to an increase of this negative regulation. Identical results were obtained when HeLa cells were used for cotransfection experiments, which excludes a cell-type-specific function of pUL84 (data not shown). In summary, these experiments identify pUL84 as a transdominant inhibitor of IE2-p86-mediated transactivation.

Domains of pUL84 involved in negative regulation of IE2-p86-mediated transactivation. To delineate the regions of pUL84 that are required for inhibition of transactivation by IE2-p86, a series of N- and C-terminal deletion mutants was constructed within the eucaryotic expression vector pcDNA3 (Fig. 4A). Permissive U373 cells were then cotransfected with a reporter plasmid containing the UL112/113 promoter upstream of luciferase together with either the respective pUL84 expression vector alone or a combination of pUL84 and IE2-p86 plasmids. Figure 4B summarizes the results of three independent cotransfection experiments that were performed for each pUL84 deletion mutant. It shows that deletions within the 83 N-terminal amino acids led to a slight and gradual reduction

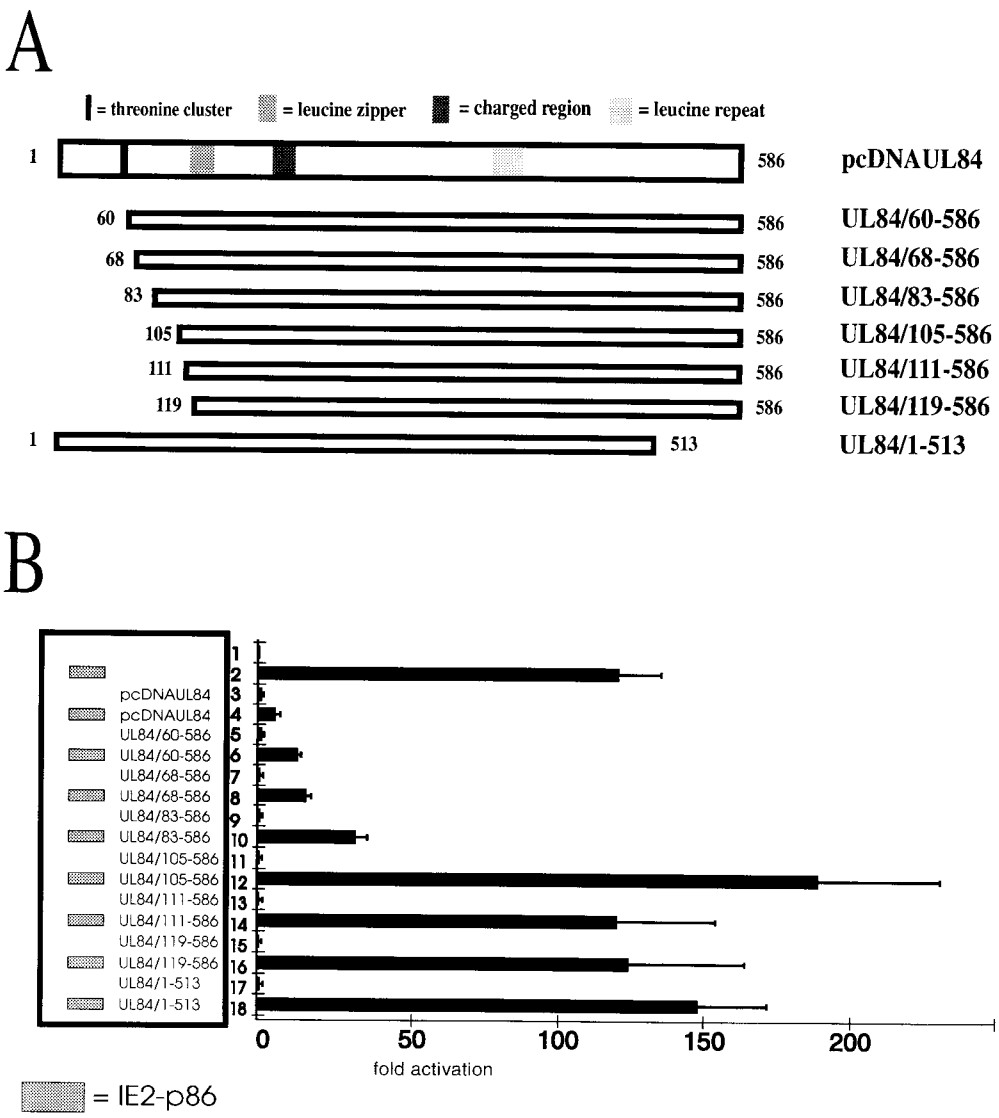

FIG. 4. Delineation of functional domains within pUL84. (A) Schematic drawing of regions contained within each pUL84 deletion mutant. Amino acid sequences of potential importance are indicated in the upper bar, which represents the full-length pUL84. Numbering refers to the positions of these regions in the amino acid sequence of pUL84. Names of the expression vectors containing deletions of pUL84 are on the right. (B) U373 cells were cotransfected with a reporter plasmid containing the early UL112 promoter of HCMV and various effector plasmids. Bars: 1, cotransfection with the cloning vector pcDNA3; 2, cotransfection with the IE2-p86 expression vector pHM134; 3, cotransfection with the UL84 expression plasmid pcDNAUL84; 4, cotransfection with a combination of pHM134 and pcDNAUL84; 5, cotransfection with UL84 deletion mutant UL84/60-586; 6, cotransfection with a combination of pHM134 and UL84/60-586; 7, cotransfection with UL84 deletion mutant UL84/68-586; 8, cotransfection with a combination of pHM134 and UL84/68-586; 9, cotransfection with UL84 deletion mutant UL84/83-586; 10, cotransfection with a combination of pHM134 and UL84/83-586; 11, cotransfection with UL84 deletion mutant UL84/105-586; 12, cotransfection with a combination of pHM134 and UL84/105-586; 13, cotransfection with UL84 deletion mutant UL84/111-586; 14, cotransfection with a combination of pHM134 and UL84/111-586; 15, cotransfection with UL84 deletion mutant UL84/119-586; 16, cotransfection with a combination of pHM134 and UL84/119-586; 17, cotransfection with UL84 deletion mutant UL84/1-513; 18, cotransfection with a combination of pHM134 and UL84/1-513. On the left is a schematic diagram of vectors used for cotransfection. On the right the relative luciferase activity, expressed as fold activation relative to the activity of the reporter construct in the absence of effector plasmids, is indicated. Results are from at least three independent experiments; error bars indicate standard deviations.

in pUL84-mediated negative regulation (Fig. 4B, bars 1 to 10). A further deletion up to amino acid 105 of pUL84, however, resulted in a complete loss of inhibitory activity (Fig. 4B, lanes 11 and 12). Deletion of 73 amino acids from the carboxy terminus also destroyed negative regulation by pUL84 (Fig. 4B, lanes 17 and 18).

Next we examined whether negative regulation of IE2-p86 transactivation correlates with the ability of pUL84 mutants to interact with IE2-p86. This was investigated by cotransfection of COS cells with various pUL84 expression plasmids together with the IE2-p86 vector followed by immunoprecipitation analysis with a polyclonal serum against IE2-p86. All mutant proteins that exerted an inhibitory activity were coimmunoprecipitated together with IE2-p86, whereas no interaction could be observed with mutants defective in negative regulation (Fig. 5A). Interestingely, the gradual reduction in negative regulation by pUL84 mutants UL84/68-586, and UL84/83-586 as observed in cotransfection experiments was reflected by a decrease of binding affinity, since mutant UL84/83-586 interacted only weakly with IE2-p86 (Fig. 5A, lanes 7 and 9). After trans-

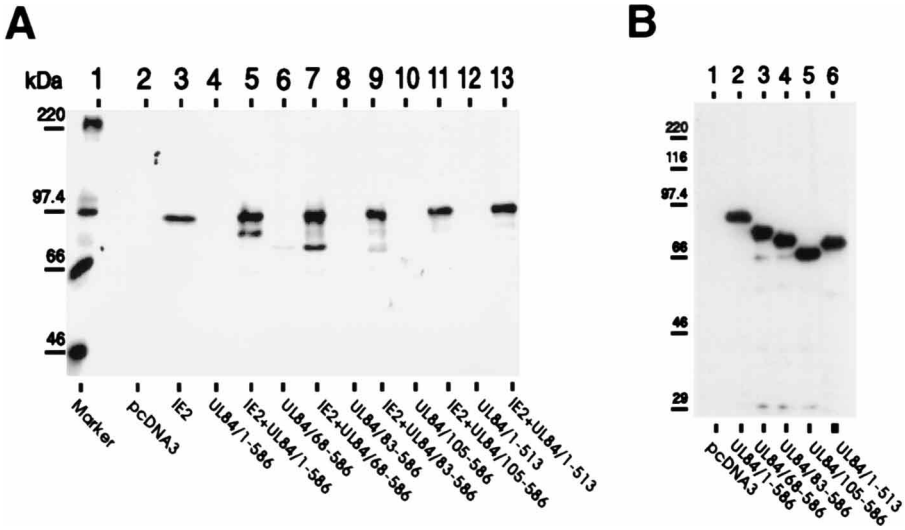

FIG. 5. Interaction of IE2-p86 with deletion mutants of pUL84. (A) Immunoprecipitation analysis with a polyclonal antiserum against IE2-p86 and lysates from transfected COS cells. COS cells were transfected with various expression vectors and labeled with Tran-^{35}S-Label, and cell lysates were prepared for immunoprecipitation analyses. Lanes: 1, molecular mass standards; 2, lysate from COS cells transfected with the cloning vector pcDNA3; 3, lysate from COS cells transfected with the IE2 expression vector pHM121; 4, lysate from COS cells transfected with plasmid pcDNAUL84 expressing the full-length pUL84/1-586; 5, lysate from COS cells cotransfected with pHM121 and pcDNAUL84; 6, lysate from COS cells transfected with UL84 deletion mutant UL84/68-586; 7, lysate from COS cells cotransfected with pHM121 and UL84/68-586; 8, lysate from COS cells transfected with UL84 deletion mutant UL84/83-586; 9, lysate from COS cells cotransfected with pHM121 and UL84/83-586; 10, lysate from COS cells transfected with UL84 deletion mutant UL84/105-586; 11, lysate from COS cells cotransfected with pHM121 and UL84/105-586; 12, lysate from COS cells transfected with UL84 deletion mutant UL84/1-513; 13, lysate from COS cells cotransfected with pHM121 and UL84/1-513. (B) Western blot analysis of UL84 deletion mutants after expression in COS cells. Monoclonal antibody anti-FLAG M2 was used to detect UL84-proteins. Lanes: 1, lysate from COS cells transfected with pcDNA3; 2, lysate from COS cells transfected with plasmid pcDNAUL84, expressing the full-length pUL84/1-586; 3, lysate from COS cells transfected with UL84 deletion mutant UL84/68-586; 4, lysate from COS cells transfected with UL84 deletion mutant UL84/83-586; 5, lysate from COS cells transfected with UL84 deletion mutant UL84/105-586; 6, lysate from COS cells transfected with UL84 deletion mutant UL84/1-513.

fection into COS cells, all pUL84 mutants used for cotransfection were found to be expressed at comparable levels (Fig. 5B) and showed a distinct nuclear localization as judged from indirect-immunofluorescence experiments (data not shown). This demonstrates a clear correlation between binding of pUL84 mutants to IE2-p86 and inhibition of transactivation.

pUL84 augments IE2-p86-mediated repression of the IE-1/2 enhancer-promoter. As an additional function IE2-p86 is able to repress transcription from its own promoter, the IE-1/2 enhancer-promoter. To assess whether pUL84 could also modulate this negative autoregulation of IE2 gene expression, we performed cotransfection experiments using plasmid pHM284 as a reporter. This vector contained the IE-1/2 enhancer-promoter upstream of the coding sequence for luciferase. Cotransfection of the UL84 expression plasmid alone had no effect on the activity of this strong enhancer-promoter (Fig. 6, bars 1 and 2). In agreement with previous data, expression of IE2-p86 led to a downregulation of the promoter (Fig. 6, bars 3). When pUL84 was used in combination with IE2-p86, however, an even stronger reduction in the activities of the IE-1/2 enhancer-promoter, which occurred in a dose-dependent manner, was noted (Fig. 6, bars 4 to 6). The same result was obtained when IE2-p40 was expressed in combination with pUL84, demonstrating that the carboxy terminus of IE2 is sufficient to obtain an increased negative regulation (Fig. 6, bars 7 and 8).

This indicated that pUL84 is able to specifically abrogate the transactivating function of IE2-p86, whereas the repressor function is augmented, suggesting a negative regulatory effect of this protein on progression of the viral replicative cycle. As transactivation of viral early promoters by IE2-p86 appears to be essential for the switch to the early phase of the replicative cycle, we hypothesized that expression of pUL84 during the IE phase should be able to neutralize IE2-p86-mediated transactivation; this should therefore result in an inhibition of viral replication.

Inhibition of HCMV replication after transient expression of pUL84. In order to test the hypothesis that pUL84 could prevent viral replication when expressed aberrantly during initial stages of the viral replicative cycle, we first performed transient-expression experiments. U373 cells were transfected with the pUL84 expression vector pcDNAUL84 and then superinfected with HCMV. About 48 h after infection, cells were fixed and analyzed by double immunofluorescence staining. The transfected pUL84 protein could specifically be detected by using a monoclonal mouse antibody against the synthetic FLAG epitope, which has been attached to the carboxy terminus of the UL84 protein as expressed from plasmid pcDNAUL84. Viral early gene expression was assessed by using a polyclonal rabbit serum against the early UL69 protein of HCMV. As shown in Fig. 7A, panels 1 and 2, cells that stained positive for the transfected pUL84 were negative for pUL69 expression at the same time. This could also be demonstrated for a larger number of cells after analysis by using confocal microscopy (Fig. 7B). To demonstrate that pUL84-expressing cells were infected with HCMV, double staining was performed with the monoclonal mouse antibody against the synthetic FLAG epitope together with a rabbit serum against the IE2-p86 protein (Fig. 7A, panels 5 and 6). This revealed abundant IE gene expression in cells that were positive for pUL84. In contrast, when plasmid pHM434, expressing a carboxy-terminal deletion mutant of pUL84 defective in negative regulation, was used for transfection, both IE and early antigens could be observed in transfected cells (Fig. 7A, panels 3, 4, 7, and 8). In summary, these experiments suggest that the intact UL84 protein is able to prevent the activation of early

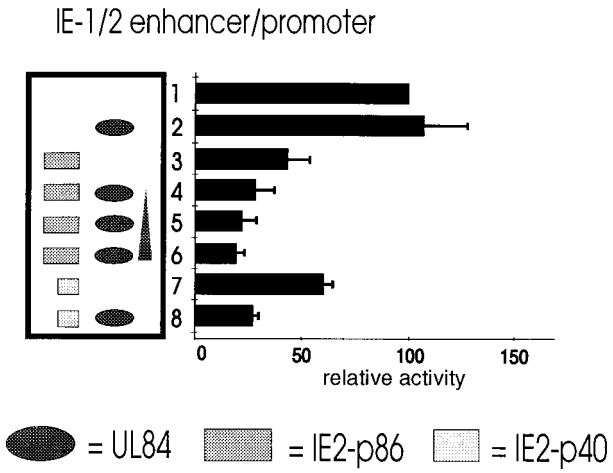

FIG. 6. Effect of pUL84 on IE2-mediated repression of the IE-1/2 enhancer-promoter of HCMV. U373 cells were cotransfected with a reporter plasmid containing the IE-1/2 enhancer-promoter of HCMV upstream of the luciferase gene and various effector plasmids. Bars: 1, cotransfection with the cloning vector pcDNA3; 2, cotransfection with the UL84 expression plasmid pHM446; 3, cotransfection with the IE2-p86 expression plasmid pHM134; 4, cotransfection with a combination of pHM134 and 1 μg of pHM446; 5, cotransfection with a combination of pHM134 and 2 μg of pHM446; 6, cotransfection with a combination of pHM134 and 5 μg of pHM446; 7, cotransfection with the IE2-p40 expression vector; 8, cotransfection with a combination of the IE2-p40 expression vector and 5 μg of pHM446. On the left is a schematic diagram of vectors used for cotransfection. Bars on the right indicate the luciferase activity after cotransfection with effector plasmids relative to the activity of the reporter plasmid without effector, which was set 100%. Results are from five independent experiments; error bars indicate standard deviations.

promoters in the context of viral infection of U373 cells via neutralizing IE2-p86-mediated transactivation.

Inhibition of HCMV replication after stable expression of pUL84. To further confirm these results, we decided to establish cell lines which stably expressed the UL84 protein. For this reason, U373 cells were transfected with the UL84 expression plasmid pcDNAUL84, which contained the UL84 ORF fused to the coding sequence for the FLAG epitope and a neomycin resistance marker. Ten G418-resistant cell clones which showed strong pUL84 expression after immunofluorescence staining with the anti-FLAG antibody were obtained. In parallel, G418-resistant cell lines were established with the cloning vector pcDNA3 and served as negative controls. After infection of pUL84 expressing cell lines with HCMV, a clear inhibition of viral replication, which was not observed with pUL84-negative cells, was evident. This was tested by immunofluorescence staining with antibodies that were specific for IE, early, or late antigens of HCMV. In Fig. 8A, panels 9 and 11, a cell line which expressed pUL84 as detected by the anti-FLAG antibody is shown, whereas the cell line in panels 1 and 5 was pUL84 negative. Both cell lines were either mock infected or infected with HCMV. About 60 h after infection, the cells were analyzed for the expression of various HCMV proteins. When a monoclonal antibody against the IE protein IE1-p72 was used, both the UL84-positive and -negative cell lines showed abundant protein expression after HCMV infection (Fig. 8A, panels 6 and 12). Thus, both cell lines could be infected with HCMV and synthesized proteins of the IE phase. However, when the cells were stained with antibodies against the early UL69 or the late UL32 protein, signals could be observed only for the UL84-negative cell line (Fig. 8A, panels 7 and 8). This was even more pronounced when cells were infected for a longer period of time (Fig. 8B). When cells were analyzed 132 h after infection, again both cell lines expressed abundant amounts of the IE1-p72 protein. In contrast, early and late gene expression were extremely diminished in the UL84-positive line, whereas UL84-negative cells produced viral proteins of all temporal phases as detected with antibodies against the early proteins UL44 and UL69 and the late UL32 protein. Interestingly, HCMV gene expression persisted at the level of IE gene expression even after extensive subculture of pUL84-expressing cells that had been infected with HCMV (data not shown).

Inhibition of HCMV replication not only was evident from immunofluorescence staining experiments but could easily be observed by monitoring the cytopathic effect (CPE) after HCMV infection. As shown in Fig. 9A and B, a strong CPE was detectable 5 days after infection of the UL84-negative cell line. At day 23 after infection, nearly all cells were lysed (Fig. 9C), whereas no CPE was recognizable after infection of UL84-positive lines (Fig. 9D to F).

Consistent with this, there was also a strong reduction in viral titers after infection of UL84-expressing cells. In the experiment shown in Fig. 10, one UL84-negative and two UL84-expressing cell lines were infected with HCMV. Cell culture supernatant was removed at the indicated time points, and the virus titer was determined. High viral titers were observed after infection of UL84-negative cells, but nearly no virus was detectable with the UL84-expressing cell lines (Fig. 10). In summary, we conclude from these experiments that expression of pUL84 at IE times is able to prevent viral early gene expression, which leads to a block of HCMV replication.

DISCUSSION

In the present study, we have identified the UL84 protein of HCMV as a direct and stable interaction partner of the IE regulator IE2-p86, which is a dual-function activator-repressor polypeptide thought to be essential for the switch from IE to early gene expression during HCMV replication (15, 29). Moreover, we were able to show that the interaction between these two proteins results in a differential modulation of IE gene function. First, we could demonstrate in cotransfection experiments that pUL84 is able to inhibit IE2-p86-mediated transactivation of both homologous and heterologous promoters in a transdominant manner. This effect of pUL84 occurred in a dose-dependent manner and was specific for IE2-p86, since no inhibition of transactivation mediated by either the Tat protein of HIV-1 or the transactivation domains of herpes simplex virus type 1 VP16 and the Oct1 transcription factor could be observed. Second, the negative autoregulatory function of IE2-p86 was not inhibited but was even augmented in the presence of pUL84, indicating a very specific modulation of particular functions of IE2-p86.

Since both effects of pUL84 on IE2-p86 protein function are predicted to inhibit progression of the viral replicative cycle, we hypothesized that pUL84, when expressed in large amounts during the IE phase, might be able to prevent viral replication. Experimental evidence demonstrating that this is the case could be obtained by two alternative approaches: (i) after transient expression of pUL84 and superinfection with HCMV, no early antigens could be detected in pUL84-positive cells, although IE proteins were synthesized, and (ii) cell lines which stably expressed pUL84 showed a block of the viral replicative cycle at the level of early gene expression. This could be observed even after extensive subculture of infected cell lines, demonstrating that HCMV could establish a persistent infection in pUL84-expressing cells. We consider it rather unlikely that this effect is due to the selection of variant U373 cells that are more resistant to HCMV infection, since we isolated and

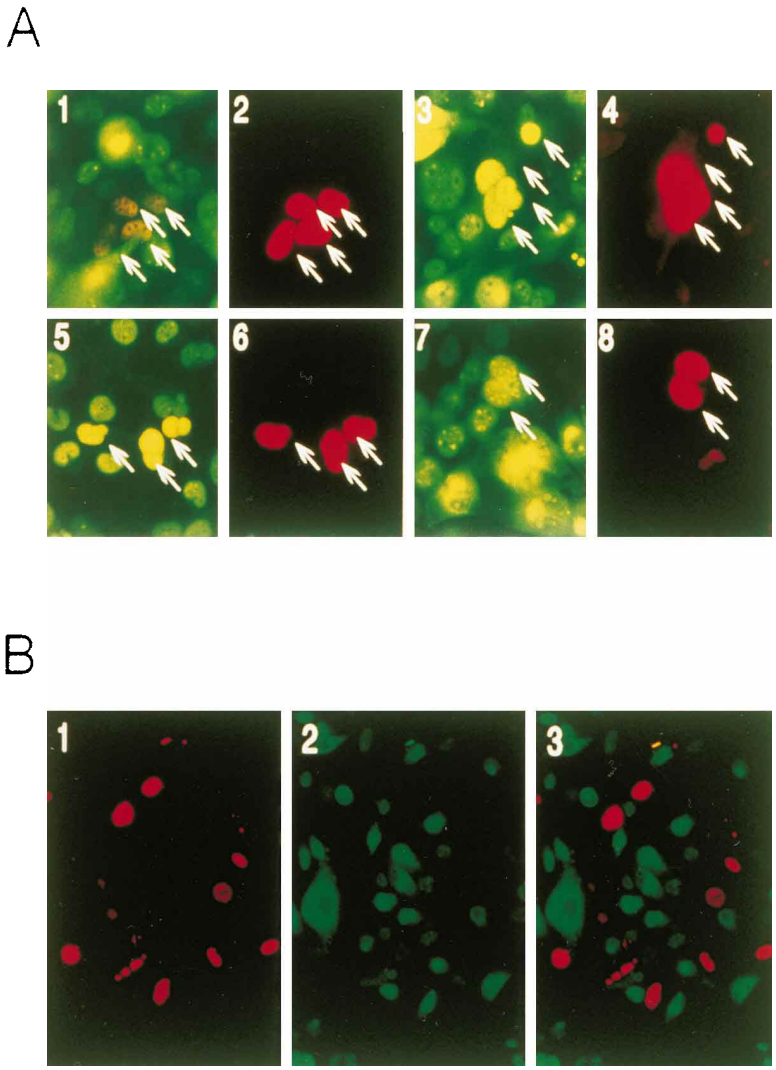

FIG. 7. Inhibition of viral early gene expression in the context of HCMV infection after transient expression of pUL84. U373 cells were transfected either with plasmid pcDNAUL84, expressing a full-length UL84 protein, or with plasmid pHM434, expressing a carboxy-terminal deletion mutant of pUL84. Cells were then superinfected with HCMV AD169. Protein expression was investigated by double immunofluorescence analysis with the monoclonal mouse antibody anti-FLAG M2 against the synthetic FLAG epitope (which had been attached to the carboxy termini of UL84 proteins as expressed from pcDNAUL84 and pHM434) in combination with either a polyclonal rabbit serum against the viral early protein pUL69 or a polyclonal rabbit serum against the IE protein IE2-p86 of HCMV. (A) Panels: 1, 2, 5, and 6, transfection performed with plasmid pcDNAUL84 (full-length pUL84); 3, 4, 7, and 8, transfection performed with plasmid pHM434 (deletion mutant of pUL84); 1 and 3, immunofluorescence staining with anti-UL69 serum; 2 and 4, immunofluorescence staining with monoclonal antibody anti-FLAG M2; 5 and 7, immunofluorescence staining with anti-IE2-p86 serum; 6 and 8, immunofluorescence staining with anti-FLAG M2. (B) Transfection was performed with plasmid pcDNAUL84. Confocal microscopy was used for analysis. Panels: 1, immunofluorescence staining with monoclonal antibody anti-FLAG M2 (red pseudocolor); 2, immunofluorescence staining with anti-UL69 serum (green pseudocolor); 3, green and red channels for the image shown in panels 1 and 2.

tested 10 independent UL84-positive and -negative clones: with all UL84-positive clones we could observe inhibition of HCMV replication; however, the degree of inhibition was dependent on the amount of expressed UL84 protein and the MOI, since high-multiplicity infection of cell lines with low UL84 gene expression resulted in lytic replication. This again demonstrates the dose dependence of UL84-mediated inhibition, which was also observed in transient-expression assays. In contrast, no HCMV resistance was observed with any of the 10 UL84-negative lines.

Therefore, we assume that pUL84 is able to exert the described negative regulatory function on IE2-p86 transactivation also during HCMV replication. The UL84 gene is expressed under control of a viral early promoter during lytic replication, and the respective protein is first detectable at 6 h after infection (13). Consistent with this, coimmunoprecipitation experiments demonstrated that IE2-p86 exists in a free form during the IE phase and then associates with pUL84, with a considerable part of IE2-p86 being complexed at 24 h postinfection (38). Clearly, the amounts of pUL84 expressed during lytic replication do not suffice to neutralize the entire transactivation capacity of IE2-p86, as observed after overexpression of pUL84 at IE times. However, down-regulation of IE2-p86-mediated transcriptional activation by pUL84 during the replicative cycle may be required in order to limit potential toxicity of this promiscuous transactivator. Alternatively, pUL84 may recruit IE2-p86 molecules for an additional function in the control of DNA replication which does not require transactivation by IE2-p86. This is suggested by a recent study reporting that both pUL84 and IE2-p86 when expressed under control of

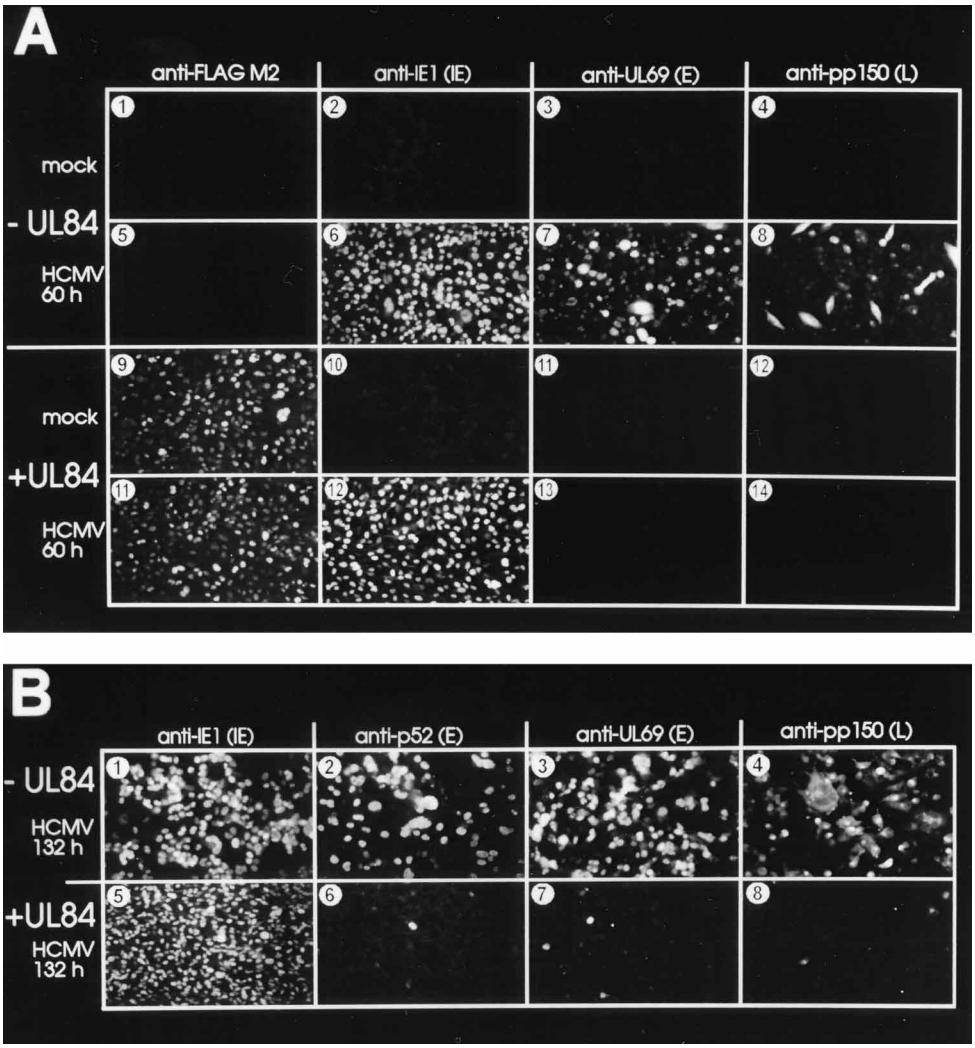

FIG. 8. Inhibition of HCMV replication after infection of stably transfected U373 cells expressing pUL84. U373 cells were stably transfected either with the UL84 expression plasmid pcDNAUL84 or with the cloning vector pcDNA3, which served as a negative control. The stably transfected cells were infected with HCMV AD169 (MOI of 1), and processed for immunofluorescence at 60 h postinfection (A) or at 132 h postinfection (B). (A) Panels: 1 to 8, cell line stably transfected with the cloning vector pcDNA3; 9 to 14, cell line stably transfected with the UL84 expression vector pcDNAUL84; 1 to 4 and 9 to 12, mock infection; 5 to 8 and 11 to 14, infection with HCMV AD169; 1, 5, 9, and 11, staining performed with monoclonal antibody anti-FLAG M2; 2, 6, 10, and 12, staining performed with monoclonal antibody p63-27 against the IE protein IE1-p72; 3, 7, 11, and 13, staining performed with anti-UL69 serum against the early pUL69 protein of HCMV; 4, 8, 12, and 14, staining performed with monoclonal antibody XP1 against the late UL32-pp150 protein of HCMV. (B) Panels: 1 to 4, cell line stably transfected with the cloning vector pcDNA3; 5 to 6, cell line stably transfected with the UL84 expression vector pcDNAUL84; 1 and 5, staining performed with monoclonal antibody p63-27 against the IE protein IE1-p72; 2 and 6, staining performed with monoclonal antibody BS510 against the early UL44-p52 protein; 3 and 7, staining performed with anti-UL69 serum; 4 and 8, staining performed with monoclonal antibody XP1.

a strong constitutive promoter are required for efficient *ori*Lyt-dependent DNA replication in a transient-cotransfection assay in primary HFF cells (39). Consistent with this, IE2 proteins are necessary for the formation of replication compartments and can also be detected in replication compartments at late times after infection of HFFs with HCMV (33, 39). In contrast, a publication by Iskenderian and collegues proposed that the requirement for IE2-p86 in transient-replication assays is due to the fact that this protein plays a critical role for the transactivation of viral early promoters driving the expression of replication fork proteins and is thus necessary for efficient expression of the replication gene products (16). In that study, no significant effect of pUL84 on the regulation of viral early promoters could be detected, which differs from results presented here. A plausible explanation for this may be that we expressed the UL84 gene under control of a strong constitutive promoter, whereas Iskenderian and collegues used the native UL84 promoter, the activity of which depends on viral transactivators. In addition, a transient-cooperativity assay has been used in which the candidate effectors IE1 and -2, UL36-38, UL112-113, IRS1/TRS1, and UL84 were subtracted individually from a transfection mixture containing all five loci (16). Since we assume that pUL84 expression is tightly regulated during lytic infection, the presence of additional regulatory proteins of HCMV and the use of genes in the context of their genuine promoters could explain why the omission of UL84 from a mixture of five expression plasmids was not able to reveal a significant effect, although a slightly increased transactivation in the absence of pUL84 was noted (16).

In conclusion, HCMV encodes a protein expressed during the early phase of replication that is able to inhibit an IE gene function essential for triggering the lytic replicative cycle, a

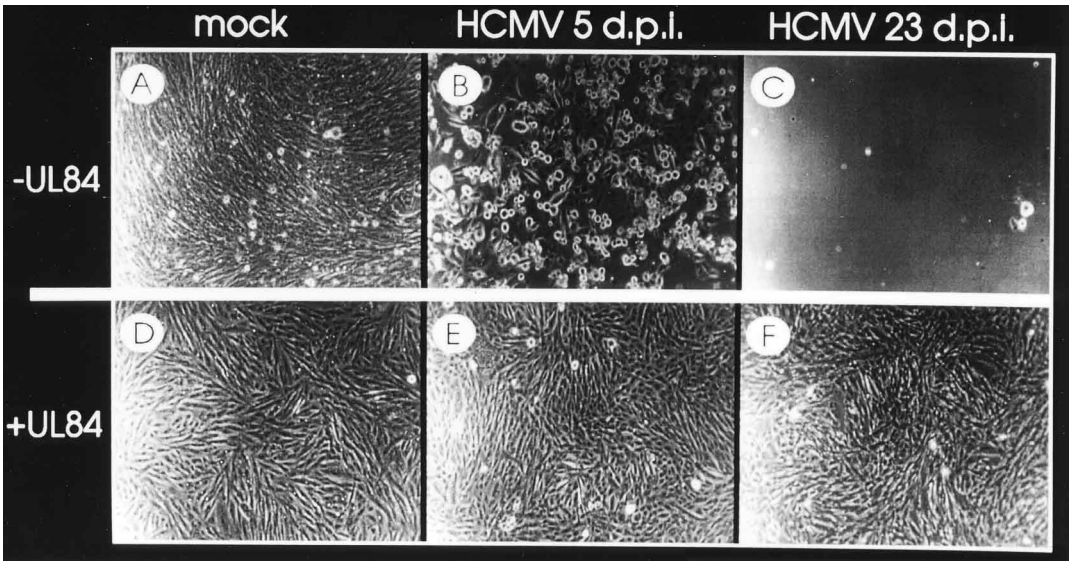

FIG. 9. CPE after infection of UL84-expressing cell lines. Cell lines stably transfected with the cloning vector pcDNA3 (A, B, and C) or the UL84 expression vector pcDNAUL84 (D, E, and F) were either mock infected or infected with HCMV AD169 (MOI of 1) and monitored for the development of a CPE. (A and D) After mock infection; (B and E) 5 days postinfection (d.p.i.); (C and F) 23 days postinfection.

principle that has not been observed in other viruses. UL84 could therefore serve as a model for transcriptional control unique among viruses and distinct from other viral repressor proteins.

For instance, a transdominant repressor of transcriptional activation, termed RAZ, has been described for Epstein-Barr virus (EBV) (9). RAZ is able to form heterodimeric complexes with the EBV transactivator protein Z, thereby preventing its binding to specific DNA response elements. However, in contrast to the UL84 protein of HCMV, which is entirely distinct from its interaction partner IE2-p86, RAZ shares domains with the inhibited transactivator Z, since it is the result of alternative splicing between ORFs encoding the R and Z polypeptides of EBV. Also differing from RAZ, pUL84 does not inhibit DNA binding of IE2-p86, which is consistent with the observation that the negative autoregulatory activity of IE2-p86 is not abolished (8).

Down-regulation of IE gene functions has also been demonstrated for herpes simplex virus: the virion host shutoff function of herpes simplex virus down-regulates IE and early gene expression by destabilizing mRNA (19, 35), and the IE protein ICP4 negatively autoregulates its own synthesis by blocking transcription initiation (36), a mechanism also used by the IE2-p86 protein of HCMV (14, 27). Remarkably, however, the down-regulation exerted by pUL84 appears to be extremely efficient, since the stable expression of pUL84 in cell lines leads to a complete block of viral early gene expression after infection with HCMV. This differs from other described mechanisms, which reduce the synthesis of IE proteins but are not able to result in a total shutoff.

HCMV is the prototype of a specialized subgroup of herpesviruses, the so-called betaherpesviruses, which includes as additional members the human herpesviruses 6 and 7 and several animal cytomegaloviruses. The betaherpesviruses have two main characteristics: first, they are extremely species specific; second, their replication is very slow compared to that of other herpesviruses such as herpes simplex virus (reviewed in reference 25). This is mainly due to a prolonged early phase of viral gene expression, although the regulatory IE proteins are expressed very rapidly after infection. Since homologs of the UL84 protein can be detected only within other members of the betaherpesviruses, such as the murine cytomegalovirus and the human herpesvirus 6 (10, 34), it is tempting to speculate that this protein might be responsible for the slow replication

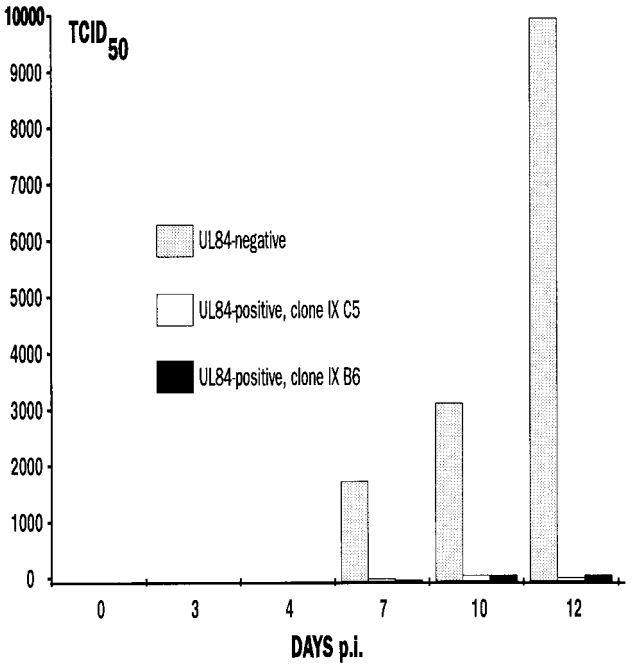

FIG. 10. Viral titers after infection of UL84-positive and -negative cell lines. Two UL84-expressing and one UL84-negative cell line were infected with HCMV AD169 (MOI of 0.1). Cell culture supernatant was collected at the indicated time points (0, 3, 4, 7, 10, and 12 days postinfection [p.i.]), and virus was titrated by determination of the 50% tissue culture infectious dose ($TCID_{50}$).

of betaherpesviruses. Viral knockout mutants will be required in order to test this hypothesis.

In summary, we were able to identify a virally encoded, transdominant inhibitor of the master regulator IE2-p86 of HCMV gene expression. Transdominant inhibition of a viral IE function by a protein expressed during the later phases of replication appears to be a novel principle used by herpesviruses, offering the potential to develop new antiviral agents that target IE2-p86.

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