Development of Cell Lines That Provide Tightly Controlled Temporal Translation of the Human Cytomegalovirus IE2 Proteins for Complementation and Functional Analyses of Growth-Impaired and Nonviable IE2 Mutant Viruses

Rebecca L. Sanders, Charles L. Clark, Christopher S. Morello, and Deborah H. Spector

Department of Cellular and Molecular Medicine and Skaggs School of Pharmacy and Pharmaceutical Sciences, and Division of Biological Sciences, University of California, San Diego, La Jolla, California 92093-0712

Received 26 March 2008/Accepted 1 May 2008

The human cytomegalovirus (HCMV) IE2 86 protein is essential for viral replication. Two other proteins, IE2 60 and IE2 40, which arise from the C-terminal half of IE2 86, are important for later stages of the infection. Functional analyses of IE2 86 in the context of the infection have utilized bacterial artificial chromosomes as vectors to generate mutant viruses. One limitation is that many mutations result in debilitated or nonviable viruses. Here, we describe a novel system that allows tightly controlled temporal expression of the IE2 proteins and provides complementation of both growth-impaired and nonviable IE2 mutant viruses. The strategy involves creation of cell lines with separate lentiviruses expressing a bicistronic RNA with a selectable marker as the first open reading frame (ORF) and IE2 86, IE2 60, or IE2 40 as the second ORF. Induction of expression of the IE2 proteins occurs only following DNA recombination events mediated by Cre and FLP recombinases that delete the first ORF. HCMV encodes Cre and FLP, which are expressed at immediate-early (for IE2 86) and early-late (for IE2 40 and IE2 60) times, respectively. We show that the presence of full-length IE2 86 alone provides some complementation for virus production, but the correct temporal expression of IE2 86 and IE2 40 together has the most beneficial effect for early-late gene expression and synthesis of infectious virus. This approach for inducible protein translation can be used for complementation of other mutations as well as controlled expression of toxic cellular and microbial proteins.

The diseases associated with human cytomegalovirus (HCMV) infections have contributed to a major drive for understanding the regulatory pathways governing its replication and interactions with the host. The DNA genome is approximately 240 kbp and has the potential to encode more than 160 proteins. However, the function of most of these proteins in viral replication and pathogenesis remains unknown. HCMV gene expression is divided into three major phases: immediate-early (IE), early, and late (for a review, see reference 44). The IE genes are transcribed immediately following viral entry and rely on host factors as well as input virion proteins for their expression. The IE proteins have been shown to be important for activating early gene promoters, inhibiting apoptosis, and countering host defenses. Some of the early gene products are directly involved in viral DNA synthesis while others function to create a cellular environment that is optimal for viral gene expression and DNA replication, either by modulating factors involved in the control of cellular DNA synthesis or by altering the host’s immune response to the virus. Late genes, which are transcribed after the onset of viral DNA replication, primarily encode structural proteins that function in assembly and maturation of the virus.

A region of IE transcription that includes the two genetic units IE1 and IE2 has been the focus of many studies (for a review, see reference 42). The IE1 RNA consists of four exons; a single open reading frame (ORF) UL123) initiates in exon 2 and specifies a 72-kDa nuclear protein designated IE1 72. The IE2 gene product, IE2 86 (ORF UL122), is an 86-kDa protein that is encoded by an alternatively spliced RNA that contains the first three exons of IE1 and a different terminal exon. Two other proteins corresponding to the 3’ end of IE2 86, termed IE2 60 and IE2 40, arise later in the infection (29, 54, 55, 69). The IE2 60 protein has an initiator methionine (Met) at amino acid (aa) 170 of IE2 86, with the putative TATAA box for its mRNA upstream of UL122 exon 5. The IE2 40 protein initiates translation at Met 242 of IE2 86. This latter protein is expressed from a 1.5-kb RNA and has a putative TATAA box just upstream of the IE2 60 translation initiation site. Studies from our laboratory and others have demonstrated with mutant viruses that IE1 72 is required at a low but not high multiplicity of infection (MOI), while IE2 86 is essential (15, 19, 21, 25, 41, 43, 57, 58, 78, 87).

Multiple functions have been ascribed to IE2 86. It has been implicated in playing a role in cell cycle control, mutagenesis, countering apoptosis, and limiting expression of host innate antiviral gene products and proinflammatory cytokines (13, 36, 37, 46, 48, 51, 62, 63, 72, 73, 81, 82, 89). The function that has received the most attention, however, is its ability to transactivate a large number of heterologous promoters and HCMV early promoters in transient expression assays, with regions
spanning the length of the protein (between aa 1 to 98 and 170 to 579) appearing to be important (26, 32, 40, 54, 61, 66, 70, 76, 85). IE2 86 can also negatively regulate its own transcription by binding to a 14 bp cis-repression signal between the TATAA box and RNA start site in the major IE promoter (MIEP) (11, 28, 32, 35, 39, 53). Other 14-bp sites of DNA binding are found upstream of the TATAA box in promoters for viral early genes including UL112, UL113, TRL7, and UL4 (4, 10, 28, 60, 61). The region of IE2 86 involved in binding to DNA overlaps the transactivation domain in the carboxy-terminal half of the protein (aa 313 to 579).

Based on the very large number of proteins that IE2 86 has been found to bind to, it is suspected that protein-protein interactions likely underlie most of its functions. IE2 86 binds to itself, to the viral UL84 protein, and to a still expanding list of cellular proteins, including the following: TBP; TFIIB; TBP-associated factors; pRb; p53; p21; mdm2; CHD-1; histone acetylases CBP, p300, and P/CAF; histone deacetylase 1 (HDAC1), HDAC2, and HDAC3; histone methyltransferases G9a and Suvar(3-9)H1; SUMO-1 and Ubc9; PIAS1; Sp1; Tef-1; c-Jun; JunB; ATF-2; NF-κB; Nil-2A; and Egr-1 (3, 6, 8, 65–68, 77, 83, 84, 86, 88). The ability of IE2 86 to interact with the majority of these proteins maps broadly to the region not shared with IE1 72, aa 86 to 542, although binding to the amino-terminal region of IE2 86 has been reported for p53 and the histone acetylase domain of CBP/p300 (aa 737 to 1626) (2, 12, 18, 27, 66, 74). A major concern with the above findings, however, is that most of the interactions were observed either in cells where the proteins were overexpressed or in in vitro binding assays.

To date, there has only been limited evidence that IE2 86 has any of these functions in the context of the infection. Elucidating the role of IE2 86 in viral replication and pathogenesis and understanding its mechanism of action require that mutants be analyzed in the context of the viral infection. One obstacle to accomplishing this goal was the difficulty of generating HCMV recombinants. The advent of bacterial artificial chromosomes (BACs) as vectors for cloning herpesvirus genomes, however, has largely solved this problem (1, 7, 24, 45). The great advantage is that any mutation is easily introduced into the viral genome, and the mutated genome is physically characterized as a BAC. The mutated BAC, along with a plasmid encoding HCMV UL82, is electroporated into cells (5), generating mutant viruses that are free of the wild-type (wt) virus.

With the above strategy, several growth-impaired viruses with mutations in IE2 86 have been studied. For example, using the AD169 BAC, we constructed a recombinant virus with a mutant IE2 86 gene that has an internal deletion of aa 136 to 290 (IE2 86ΔSX) (58). The deletion also removes the promoter and initiator Met for IE2 40 (aa 242) and the initiator Met for IE2 60 (aa 170), and thus these late gene products are not expressed. When cells are infected at a low MOI, there is a marked delay in the production of virus and slower cell-to-cell spread than in a wt infection. By immunofluorescence assay (IFA) and Western blot analysis, it appeared that the expression of viral early genes was comparable to that of the wt virus. However, in mutant-infected cells, there was no longer a block in the expression of cellular HMGA2 and cyclin A (64), and virus-mediated transcriptional induction of cellular antiviral gene products and proinflammatory cytokines was not suppressed (73). The most notable molecular defects were observed at late times. The levels of the IE2 protein, pp65 (UL83) matrix protein, and UL84 protein were greatly reduced, but, surprisingly, only in the case of UL83 was there a corresponding decrease in the mRNA. Subsequently, we showed that the loss of the IE2 60 and IE2 40 early-late proteins played a major role in the observed effects at late times (79).

Another viable mutant virus is IEΔ30–77, which lacks the majority of IE exon 3 (aa 30 to 77) and thus expresses smaller forms of both IE1 72 and IE2 86 (80). This virus is growth impaired at both high and low MOIs and exhibits a kinetic defect that is not rescued by growth in cells expressing IE1 72. The kinetics of mutant IE2 protein accumulation in IE Δ30-77-infected cells is comparable to that in wt virus-infected cells, but the mutant shows delayed expression of early viral genes and only low levels of IE1 72. Its capacity to upregulate the expression of cellular cyclin E has also been reduced. Interestingly, a mutation in exon 5 of IE2 that substitutes arginine for glutamine at aa 548 also alters the inhibitory effects of the virus on cellular DNA synthesis and the cell cycle; but the MIEP is still autoregulated, and viral early genes are activated (51).

There are many examples of mutations in IE2 86 that do not yield virus when the mutant BACs are electroporated into fibroblasts, including the following: deletion of the entire exon 5; deletion of aa 356 to 359, aa 427 to 435, or aa 505 to 511; substitution of alanine for the arginines at aa 356, 357, and 359; substitution of alanine for the proline at aa 535 and the tyrosine at aa 537; and substitution of alanine for the histidines at aa 446 and aa 452 (50, 78). Studies on these recombinants have been limited to the characterization of viral protein and mRNA expression following electroporation of the BACs into fibroblasts and have required immunostaining with antibodies to representative IE, early, and late proteins and quantitative real-time reverse transcription-PCR (RT-PCR) to determine the block in the replication cycle. Most of the mutant viruses do not activate early genes and are defective in repression of the MIEP. Surprisingly, in cells electroporated with two of the mutant IE2 86 BACs that have a deletion of aa 427 to 435 or aa 505 to 511, there is significant induction of selected delayed early (UL89) and late genes at early times in the infection, suggesting that IE2 86 may play a role in inhibiting some delayed early or late promoters at early times in the infection.

Although the above studies on cells electroporated with BACs have provided some insight into the functions of IE2 86, an understanding of the molecular mechanisms governing its functions throughout the infection requires the generation of recombinant viruses. To this end, our laboratory along with others have attempted to create cell lines that can complement some of these defects and will allow for the production of infectious virus. This has proven to be a very challenging endeavor, as constitutive expression of wt IE2 86 appears to be deleterious to the cell (16). The one fibroblast cell line that was reported to express IE2 86 (6) was later found to have mutations in critical regions of the protein (46). Over the years, our laboratory has tried to generate complementing cell lines with multiple inducible eukaryotic gene expression systems but with no success.
Here, we describe a system that allows complementation of IE2 86 mutant viruses. With a lentivirus-based vector encoding IE2 86, we have devised a strategy in which expression of Cre recombinase allows specific induction of IE2 86 following a DNA recombination event during the IE stages of the infection. In a similar manner, we have created cell lines with lentivirus transductions that can be induced to translate IE2 60 and IE2 40 during the early-late stages of the infection following exogenous expression of FLP recombinase. This system allows appropriate temporal translation and expression of the IE2 proteins. Finally, we have constructed a cell line that can be induced to express both IE2 86 and IE2 40 proteins with appropriate kinetics during the infection. By placing the genes encoding Cre and FLP in the mutant virus under the control of a viral IE and early-late gene promoter, respectively, the temporal induction of the IE2 86 and IE2 40 proteins occurs only in cells that have been infected with the virus.

We have utilized this system to complement the growth of the previously characterized and debilitated IE2 86ΔGSX virus, as well as nonviable viruses that is lacking the entire region encoding exon 5 of IE2 86 (IE2 86ΔExon5 Cre/FLP). Many of the altered expression patterns of the IE2 86ΔGSX virus were restored to various degrees compared to the wt phenotypes when assessed on these cell lines, especially with respect to late gene expression. A greater level of complementation was seen on the cell line that could be induced to express both IE2 86 and IE2 40. Notably, electroporation of the nonviable IE2 86ΔExon5 Cre/FLP BAC into cell lines that can be induced to express IE2 86 alone or both IE2 86 and IE2 40 leads to the production of infectious virus. On both cell lines, this virus exhibits delayed kinetics and grows to reduced titers relative to wt. Plaque size and spread are slightly impaired but less so on the cell line that expresses both IE2 86 and IE2 40, indicating that both proteins are necessary for wt viral growth.

**MATERIALS AND METHODS**

**Molecular cloning of lentiviral vectors.** The lentiviral vectors pLV CMV-BamHI-Swal and p156RRLsinPPTIE2 86-GFP were kind gifts from Ingrid Verma. The pLV CMV-BamHI-Swal vector utilizes the HCMV IE promoter as its internal promoter, followed by unique BamHI and Swal cloning sites. The p156RRLsinPPTIE2 86-GFP vector utilizes the human elongation factor 1-α promoter driving the expression of green fluorescent protein (GFP). The other relevant features of these vectors have been described elsewhere (52).

The lentiviral vectors pLV:CMV-BamHI-SmaI and p156RRLsinPPTIE2 86-BamHI-SmaI were PCR amplified from pcDNA3:IE2 86 and the corresponding fragment (aa 136 to 543) with the corresponding fragment from pHCMV EcoRIJ (71), which is from strain AD169. The corrected cDNA, resulting in an in-frame FLAG tag (DYK-D-D-D-D-K) was introduced at the C terminus of and in frame with the IE2 86 cDNA by Quikchange mutagenesis. The resulting vector was called pcDNA3:IE2 86-FLAG.

Both the 1.2-kb IE2 60 and 1-kb IE2 40 cDNAs were PCR amplified from pcDNA3:IE2 86. The sense primers used in both PCRs were designed to introduce Swal sites just upstream of the AUG for both cDNAs. The antisense primers used for both reactions introduced a 9-aa hemagglutinin (HA) tag (Y-D-V-P-D-Y-A-S-L) in frame with the carboxy terminus of each cDNA, followed by another Swal site. Both PCR products were then cleaved with Swal and cloned as Swal fragments into pcDNA3, forming pcDNA3:IE2 60-HA and pcDNA3:IE2 40-HA, respectively. Finally, the 5’ ends of pcDNA3:IE2 86-FLAG, pcDNA3:IE2 60-HA, and pcDNA3:IE2 40-HA were modified by Quikchange mutagenesis (Stratagene) to introduce 51 bp of IE sequence between the upstream Swal site and the initiator Met in all three vectors. The 51 bp is comprised of 34 bp of the 5’ end of IE exon 1 (nucleotides [nt] 173610 to 173641) plus 17 bp at the 5’ end of IE exon 2 just upstream of the initiator Met located in exon 2 (nt 172766 to 172782). As a result of this insertion, the upstream untranslated regions for each cloned cDNA are identical to what they would be in the HCMV mRNA, putting the AUG for each in a more accurate context for translation. All three vectors were tested for expression by transfection into Cos-7 cells. The Cos-7 cell lysates were analyzed by Western blotting using antibodies to FLAG or HA. The size and expression of each of the three tagged cDNAs were as expected (data not shown). All three tagged cDNAs were then removed from their vectors by digestion with Swal and subcloned into the Smal site of the lentiviral vector pLV:EF1-α-Smal to form pLV:EF1-α-BamHI-IE2 86-FLAG, pLV:EF1-α-BamHI-IE2 60-HA, and pLV:EF1-α-BamHI-IE2 40-HA, respectively.

Several shuttle vectors were constructed in order to introduce selectable marker genes between loxP or FLP recognition target (FRT) sites into our lentiviral vectors. The plasmid pSwarlox-Puro-GFP:loxP-Swarlox was made using successive Quikchange site-directed mutagenesis reactions. The first introduced a Swal and a 34-bp loxP site just downstream of the HCMV IE promoter in the vector pHGF (Stratagene). A second loxP site adjacent to a second Swal was then introduced 89 bp downstream of the GFP stop codon in a subsequent round of third Quikchange reaction. KpnI site insertion was used to introduce the upstream loxP site and the initiation codon for GFP. A KpnI digest was used to remove the GFP ORF, and the plasmid was religated to form pSwarlox-Puro-KpnI-loxP-Swarlox. Next, this vector was digested with Swal, and the insert was ligated to BamHI linkers. Following digestion with BamHI, the cassette was subcloned into the BamHI site of pGEM-1 (Promega) to form pGEM-loxP-KpnI-loxP. The Neo'cycin (Neo') resistance marker, without its promoter, was PCR amplified from pcDNA3. KpnI recognition sites were designed into the 5’ end of each primer. The PCR product was then digested with KpnI and subcloned into pGEM:loxP-KpnI-loxP to form pGEM-loxP-Neo-loxP.

Two successive Quikchange reactions were used to replace the upstream and downstream loxP sites in the vector pGEM:loxP-KpnI-loxP with 48-bp FRT sites. The resulting plasmid was designated as pGEM:FRTKpnI-FRT. The pcDNA3:IE2 60-PURO-ORF and pcDNA3:IE2 86-PURO-ORF vectors were made by the similar method. Again, KpnI recognition sites were designed into the 5’ ends of each primer. Following a KpnI digest of the PCR product, the PURO ORF was subcloned into pGEM:FRTKpnI-FRT to form pGEM:FRTKpnI-PURO-FRT.

The final step in the construction of the lentiviral vectors was the addition of the marker genes. The loxPneo-loxP cassette was isolated from pGEM:loxP-Neo-loxP by digestion with BamHI and subcloned into the BamHI site of pLV:EF1-α-BamHI-IE2 86-FLAG to form pLV:EF1-α-IE2-Neox-Neo-loxP-IE2 86-FLAG. The FRTKpnI-PURO-FRT BamHI cassette was then isolated from pGEM:FRTKpnI-PURO-FRT and subcloned into both pLV:EF1-α-BamHI-IE2 60-HA and pLV:EF1-α-BamHI-IE2 40-HA to form pLV:EF1-α-FRT-PURO-FRT-IE2 60-HA and pLV:EF1-α-FRT-PURO-FRT-IE2 40-HA, respectively.

**IE2 cell line creation via lentiviral transduction.** All three IE2 lentiviral vectors were packaged using the reagents and protocol of the ViraPower Lentiviral Expression System (Invitrogen). Briefly, 3 μg of lentiviral vector was cotransfected with 9 μg of the ViraPower packaging mix into actively growing 293FT cells using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM medium (Gibco). The ViraPower packaging mix consists of three plasmids: pLP1, which provides gag-pol function as well as the Rev-responsive element; pLP2, which provides rev function; and pLP/VSVG, which provides the vesicular stomatitis virus G glycoprotein. The medium was changed at 24 h posttransfection, and lentivirus-containing supernatants were harvested at 48 and 72 h post transfection (p.t.). Fresh supernatants were used to transduce low-passage human foreskin fibroblasts (HFFs) for two consecutive days. Prior to use, each supernatant was clarified by low-speed centrifugation and filtered through 0.45-μm-pore-size polyvinylidene difluoride filters (Millipore). Transduction of HFFs was...
strain harbors a defective lambda prophage that provides the recombination BACs were introduced by electroporation into the recombination strain DY380 SX BAC has been previously described (58). Both /H9004 BAC (pHB5), obtained from M. Messerle (7). The construction of the IE2 86 Cre/FLP viruses was done by electroporating 6.25 and FIGE. This recombination was facilitated in DY380 cells and selected on the basis of resistance to chloramphenicol, Kan, and Amp. Resistant clones were prepared as described (58). These preparations were also analyzed by restriction endonuclease digestion and FIGE. BACs showing the expected restriction pattern and no other deletions or rearrangements were transformed into DH10B cells, and BAC DNAs were amplified and purified as previously described (75) and subcloned into pFastBac1, creating the vector pFB1:1.2. The FLP ORF was then removed from the plasmid pACYC:US11-IE-Cre-US14, yielding the vector pACYC:US11-IE-Cre/Kan/1.2-FLP-US14. Finally, the 1.2-kb early-late RNA promoter was first constructed to serve as a PCR template. To begin, a 4.2-kb PmlI fragment was subcloned from pHCMV EcoRI/BI (71) into the EcoR site of the cloning vector pACYC184 to form pACYC-US11-S11-US14. Quikchange mutagenesis was then used to introduce SpeI and BglII sites just downstream of a unique XbaI site already present within the integrated early-late promoter of the HCMV IE1-4 genes in the HB5 Cre/FLP BAC. The Cre ORF was removed from the vector pTurbo-Cre and subcloned into the vector pFastBac1 (Invitrogen), just downstream of the HCMV IE promoter, resulting in the vector pFB1:IE-Cre. The entire Cre cassette, including the promoter, was then subcloned into the XbaI site of pACYC:US11-US14, forming the vector pACYC:US11-IE-Cre-US14. Next, the kanamycin (Kan) resistance marker with its promoter was PCR amplified from the vector pACYC177 and subcloned into the SpeI site of pACYC:US11-IE-Cre-US14, yielding the vector pACYC:US11-IE-Cre-Kan-US14. Finally, the 1.2-kb early-late RNA promoter was removed from the vector p456-OCAT (75) and subcloned into pFastBac1, creating the vector pFB1:1.2. The FLP ORF was then removed from the plasmid pOG44 (Invitrogen) and subcloned just downstream of the 1.2-kb promoter. The resulting plasmid was referred to as pFB1:1.2-FLP. The entire 1.2-FLP cassette was then subcloned into the BglII site of pACYC:US11-IE-Cre-US14, creating the vector pACYC:US11-IE-Cre-Kan/1.2-FLP-US14. A pair of 21-mer primers, US1/1-Cre (nt 199869 to 199889 of the AD169 virus) and US2/2-Cre (nt 200788 to 200898), were used to amplify a 6.4-kb linear cassette from pACYC:US11-IE-Cre-Kan/1.2-FLP-US14 which included the IE-Cre, Kan, and 1.2-FLP genes flanked by approximately 500-bp homology arms to US1 and US2. This linear fragment was used for recombination into the HBS and IE2 86SX BACs in the DY380 cells as described previously (33), and BACs were selected on the basis of Kan and chloramphenicol resistance. The recombinant BACs were analyzed extensively by restriction endonuclease digestion and field inversion gel electrophoresis (FIGE). BACs showing the expected restriction pattern and no other deletions or rearrangements were transformed into DH10B cells, and BAC DNAs were amplified and purified as previously described (58). These preparations were also analyzed by restriction endonuclease digestion and FIGE to confirm that they were correct (data not shown). The IE2 86 Exon5 Cre/FLP virus was prepared using the HBS Cre/FLP BAC. First, the ampicillin (Amp) gene was removed by PCR from the vector pCmDA3. This cassette contained the promoter, downstream sequences necessary for expression of the Amp gene, and homology arms to the desired recombination site within the IE2 86 coding region (outside of exon 5) in the HBS Cre/FLP BAC. This recombination was facilitated in DY380 cells and selected on the basis of resistance to chloramphenicol, Kan, and Amp, Resistance to Amp was prepared as described earlier and screened extensively by restriction endonuclease digestion and FIGE. Reconstitution of the HBS Cre/FLP, IE2 86SXCre/FLP, and IE2 86 Exon5 Cre/FLP viruses was done by electroporating 6.25 μg of the appropriate BAC along with 3.75 μg of pCDNA7pTtag into HFFs or 86F/40HA cells using a BTX630 generator set at 300 V, 75 μF, and 2,500 μA, as previously described (78). Viral supernatants were harvested when the cultures reached 100% cytopathic effect (CPE); and virus titers were determined by plaque assay, and expression of IE or early proteins was determined by IFA. In the paper, the HBS Cre/FLP, IE2 86SX Cre/FLP, and IE2 86 Exon5 Cre/FLP viruses are referred to as HBS-Cre, IE2 86SX-Cre, and IE2 86 Exon5-Cre, respectively. A rescued virus was derived from the IE2 86 Exon5 Cre/FLP BAC by homologous recombination in HFFs between the mutant BAC with pHCMV EcoRI (71), which contains the MIE region of HCMV strain AD169, followed by the outgrowth of replication-competent virus. To generate the rescued virus, HFFs were electroporated with IE2 86 Exon5 Cre/FLP BAC DNA (6.25 μg), EcoRI-digested pHCMV EcoRI J DNA (5 μg), and pCDNA7pTtag (3.75 μg) as previously described (78). Supernatant was harvested from the infected cells, and a limiting dilution assay was set up. The virus clones from wells that contained a single plaque were further propagated. HCMV-infected cell DNA was isolated (QiAmp DNA Blood Mini Kit; Qiagen) from viral clones, and the IE2 exon 5 region was analyzed by PCR and DNA sequencing to verify that the wt IE2 exon 5 sequence had replaced the Ampr cassette in the mutant. Also, the resulting BAC was obtained from the infected cells using a modified Hirt protocol, retransformed into bacteria, and assays a second time to make sure that no large deletions or rearrangements had occurred during the growth of virus in HFFs. Cell culture and infections. HFFs were obtained from the University of California, San Diego, Medical Center and cultured in Earle’s minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1.5 μg/ml amphotericin B (Invitrogen), 2 mM-l-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO₂ and maintained at confluence for 3 days before infection. HFFs or the cells described above were infected at the MOI indicated in the figures. Mock-infected cells were treated with medium and dimethyl sulfoxide as a control. At various times p.i., cells were washed with phosphate-buffered saline (PBS), trypsinized, and processed for the appropriate assay. 86F cells were kept under constant selection with G418 (Invitrogen) until infected, while HFF and 80HA and 40HA cells were kept under selection with G418 (Mediatech). The 86F/40HA cells were kept under selection with both G418 and PURO. Growth of the viruses was assayed by infecting cells at an approximate MOI of 0.05 to 0.1 PFU/cell and then allowing cells to become confluent or by following the spread of the virus after electroporation. The virus was allowed to spread until all cells in the flask showed CPE. At this time, supernatants were harvested, and titers were determined by both plaque assay and IFA for both IE and early protein expression. Western blotting. Infected or mock-infected cells were harvested at various times p.i. Cells were lysed and processed for Western blot analyses as previously described (79). The following antibodies and dilutions were used: CH16.0 (1:10,000), UL84 (1:5,000 to 1:10,000), monoclonal IE2 86 mAbB8140 (1:10,000), UL50 (1:5,000), UL44 (1:5,000), and UL99 (1:5,000; 1:10,000), β-actin Mab Ac-15 (1:5,000 to 1:10,000), Flag Mab (1:2,500), HA Mab (1:100), IE1 72 Mab (1:2,000 to 10,000) and horse-radish peroxidase-coupled anti-mouse antibody (1:2,500 to 1:10,000), CH16.0, anti-UL99, anti-UL83, anti-UL44, and anti-UL57 were purchased from the Goodwin Institute and Viruses. Anti-UL84 antibodies were kind gifts from G. Puri and E. S. Huang. Anti-IE2 was purchased from Chemicon, while anti-β-actin and anti-Flag were purchased from Sigma-Aldrich. Anti-HA was from Santa Cruz Biotechnology. Anti-IE1 72 was obtained from Viruses and B. Britt (University of Alabama, Birmingham). Horseradish peroxidase-coupled anti-mouse antibody was obtained from Calbiochem. After incubation and washes, proteins were detected with SuperSignal chemiluminescent substrate (Pierce) according to the manufacturer’s instructions. Immunofluorescence. Cells were seeded onto coverslips and fixed at various times p.i. using 2% paraformaldehyde and then processed as previously described (58). Coverslips were then stained with the following primary antibodies: CH16.0 (1:1,000), IE1 72 Mab (1:50 to 1:500), UL44 Mab (1:1,000), UL50 Mab (1:1,000), Flag Mab (1:250), and HA Mab (1:50). All suppliers and manufacturers are described above. After three washes with PBS, cells were stained with a secondary antibody (fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate, 1:1,000 or 1:1,000, respectively, and Hoechst, 1:500). Pictures were taken using a Zeiss 13492812 NPC Pharcotics 63x oil immersion camera mounted on a Nikon Eclipse E800 Microscope (20× and 40× objectives). Quantitative real-time PCR and RT-PCR analyses. For the quantitative real-time PCR analyses, an aliquot of cells used for the Western blot analysis or separately infected cells (for the second experiment) were harvested at 24 h p.i.,
and then DNA was prepared with a Qiagen DNA Blood Mini kit and analyzed for input viral DNA as previously described (80).

For the quantitative real-time RT-PCR analyses, cells were harvested, and RNA was prepared using a Qiagen RNA extraction kit as recommended by manufacturer. RNA samples were then rigorously treated with DNase and diluted to 12.5 ng/μl. A portion of the RNA sample was treated with RNase as a negative control for amplification. Real-time RT-PCR and analysis were performed as previously described (79) using primers and probes directed against the HCMV IE1 72 gene and the cellular housekeeping gene glucose-6-phosphate dehydrogenase.

RESULTS

Generation of IE2 86-Flag, IE2 60-HA, and IE2 40-HA cell lines and construction of HB5 C-F, IE2 86ΔSX C-F, and IE2 86ΔExon5 C-F recombinant BACs. Many attempts to create cell lines that can complement the growth of nonviable and debilitated viruses with mutations in IE2 86 have led to the method presented here. The strategy that was successful utilized lentiviral vectors to create cell lines that can be induced to undergo a DNA recombination event, allowing proper temporal translation of the IE2 proteins. This approach was based on the principle that only the first ORF on a bicistronic eukaryotic mRNA is translated. Thus, by placing the ORF for the IE2 protein as the second ORF on the lentiviral mRNA, it should not be translated until the first ORF is removed.

One lentiviral vector, termed LV/EF1a:loxP-Neo-loxP:IE2 86-FLAG, was constructed such that the ORF for a protein conferring Neo resistance was flanked by loxP sites and placed just upstream of the AUG for IE2 86 (Fig. 1A). A FLAG tag was placed at the C-terminus of the IE2 86 gene in order to discriminate between the IE2 86 provided by the cell line and the IE2 86 provided by the virus. The EF1-α promoter was used to drive expression of the lentiviral mRNA (Fig. 1A) because we had found that it was not affected by the HCMV infection (unpublished results). Low-passage HFFs were transduced with the lentivirus, and the cells were selected for drug resistance. These cells were designated 86F. Since the lentiviral mRNA produced is bicistronic, only the AUG for the Neo ORF could be translated. The expression of Cre recombinase then could be used to initiate recombination events through loxP sites, resulting in the deletion of the Neo ORF. Following the recombination, the AUG for the IE2 gene became the first ORF, allowing translation of the protein from the lentiviral mRNA.

Similarly, cell lines were constructed to express the IE2 60 and IE2 40 early-late proteins. Construction of these cell lines was similar to that of the 86F cell line, except that the resistance marker used was PURO, and the tag placed at the C-terminus of the IE2 proteins was HA instead of FLAG (Fig. 1A). In this case, the PURO ORF was flanked with FRT sites, and placed just upstream of the AUG for the early-late IE2 60 and IE2 40 proteins. These vectors were termed LV/EF1α: FRT-PURO-FRT:IE2 40HA (or IE2 60-HA) (Fig. 1A). Low-passage HFFs were transduced with the either the IE2 40HA or IE2 60HA lentivirus, the cells were selected for resistance to PURO, and these cells were designated 40HA and 60HA, respectively. The expression of FLP recombinase then could be used to initiate recombination events through the FRT sites, resulting in the deletion of the PURO ORF and translation of the IE2 60 or IE2 40 protein from the lentiviral mRNA.

A cell line that could be induced to express both IE2 86F and IE2 40HA was also constructed by cotransducing HFFs with the two lentiviruses encoding these proteins and selecting for cells that were resistant to both drugs. This cell line was designated 86F/40HA.

Since prolonged expression of IE2 86 is toxic for the cell and propagation of the recombinant virus would require multiple rounds of replication following electroporation of the BAC into the cells, it was necessary to devise a means for induction of the IE2 proteins only when the cell became infected. We also wanted to maintain the normal temporal pattern of expression of the IE2 proteins, with IE2 86 expressed at IE times and the IE2 60 and IE2 40 proteins expressed at early-late times. This was accomplished by placing the Cre coding sequence under the direction of the HCMV major IE promoter between the US12 and US11 ORFs in BACs that encoded wt IE2 86 or mutant IE2 86 (Fig. 1B). Thus, after the electroporation of the BAC into the appropriate cell line, the Cre expressed from the IE promoter would allow the deletion of the first ORF from the lentivirus and expression of IE2 86. This process would be repeated each time the virus infected a new cell. In order for the IE2 60 and IE2 40 proteins to be expressed later in the infection, we placed the FLP recombinase under the control of the viral 1.2 kb promoter (75), which is not expressed until the early-late stages of the infection. This construct was placed in the BACs adjacent to the cassette containing the Cre driven by the HCMV IE promoter. This BAC, as well as the virus propagated from this BAC, is referred to as HB5 C-F.

Figure 1B shows the recombinant BACs (expressing Cre and FLP) that were used in the studies reported here. All recombinant BACs were prepared from the wt HCMV strain AD169 BAC (pHB5). One mutant recombinant, termed IE2 86ΔSX C-F, was derived from the previously described IE2 86ΔSX BAC that contains an in-frame deletion of aa 136 to 290 of IE2 86 (58). The second mutant is a newly constructed BAC that is missing all of exon 5 of the IE2 86 coding region, termed IE2 86ΔExon5 C-F. The IE2 86ΔExon5 C-F BAC contains an Amp resistance marker in place of exon 5 of IE2 86. A rescued BAC was derived for the IE2 86ΔExon5 C-F BAC to ensure that all phenotypes observed in the mutant were due to the loss of exon 5 and not due to any extraneous mutations throughout the rest of the BAC coding sequence. This virus was termed IE2 86ΔExon5 Res C-F. Rescued forms of the wt HB5 C-F and IE2 86ΔSX C-F viruses were not constructed, since infection with these viruses lacking the Cre/FLP cassette has already been described (58), and as described below, there were no notable differences in the replication of the Cre/FLP versions of these viruses. All of the recombinant BACs were analyzed by digestion with restriction endonucleases and FIGE to ensure that no major rearrangements of the genome had occurred during mutagenesis (data not shown). The BACs were reconstituted as viruses following electroporation into HFFs or the designated cell line.

Growth of the wt HB5 virus is not affected by the presence of the Cre/FLP cassette. To ensure that the addition of the Cre/FLP cassette did not have any deleterious effects on the viral life cycle or virus production, HFFs were infected with either the HB5 virus or the HB5 C-F virus at an MOI of 1 PFU/cell. At various times p.i., cells were harvested, and protein expression was analyzed by Western blotting.
FIG. 1. IE2 cell lines and recombinant BACs. (A) Lentiviral vectors used to express IE2 86F, IE2 60HA, or IE2 40HA are shown. Sequences of one loxP site, the FLAG tag, and a portion of the IE2 coding region are displayed in the first vector. Coding regions are indicated as capital letters, and noncoding regions are represented in lowercase letters. The initiator Met for IE2 86 (underlined) is shown immediately after the exon 1 and 2 sequences. The end of the IE2 coding sequences are also underlined, as well as the stop codons following the FLAG and HA tags. The lentiviral vectors used to create the 60HA and 40HA cell lines have FRT sites that are targeted by the FLP recombinase. The IE2 60 and IE2 40 proteins are tagged with HA. Sequences of one FRT site, the HA tag, and a portion of the IE2 coding region are shown in the second vector. Important regions within the vectors are noted, including the long terminal repeats (LTR), the gag gene, the promoter used to drive expression of the selectable marker (P\text{EF1a}), and the gene to be expressed (IE2 86-FLAG, IE2 40-HA, or IE2 60-HA). (B) Schematics of the BACs used to create the HB5 C-F, IE2 86\DeltaSX C-F, IE2 86\DeltaExon5 C-F, and IE2 86\DeltaExon5 Res C-F viruses expressing Cre and FLP recombinases are shown. The Cre and FLP coding regions, the 1.2-kb (1.2) and MIE promoters, the selectable marker (Kan), and the HCMV ORFs in this region of the BAC (US12 and US11-7) are noted. The region of the viral IE2 gene that is present in HB5 C-F, IE2 86\DeltaSX C-F, IE2 86\DeltaExon5 C-F, and IE2 86\DeltaExon5 Res C-F are illustrated. The IE2 86 coding regions in exons 2, 3, and 5 and the deletions within exon 5 are indicated. In the case of the IE2 86\DeltaExon5 C-F BAC, the entire exon 5 coding region has been replaced by an Amp resistance gene.
IE gene expression was detected with both the CH16.0 antibody, which is directed against a sequence common to IE1 72 and IE2 86, and the monoclonal IE2 86 antibody (mIE2 86), which is specific for IE2 86, IE2 60, and IE2 40. The levels of IE1 72, IE2 86, IE2 60, and IE2 40 were comparable at all time points in cells infected with the two viruses. Similarly, the two viruses showed no differences in early, early-late, and late gene expression, as indicated by the kinetics of appearance and levels of UL44, UL84, and UL99, respectively.

Many stocks of the HB5 C-F and HB5 virus were prepared and analyzed on each of the different cell lines (including HFFs, 86F, 60HA, 40HA, and 86F/40HA) during the course of our studies. Based on plaque size, it appeared that there were no significant differences in spread of either virus on any of the cell lines. Both viruses also appeared to replicate with similar kinetics at both high and low MOIs, and comparable levels of infectious virus were harvested from the cell lines after they showed full CPE. The only effect that we noticed was that when titers were determined for the same stock of HB5 C-F virus on either the 86F or 86F/40HA cell lines, the number of plaques observed at the higher dilutions was consistently slightly lower (less than twofold) than those observed on the HFFs (data not shown). When all factors are considered, however, there were no significant differences in the replication of the HB5 and HB5 C-F viruses.

The kinetics of induction of the IE2 86, IE2 60, and IE2 40 proteins from the cell lines parallels their expression from the virus. To assess the pattern of induction of the tagged IE2 proteins in the 86F, 60HA, 40HA, and 86F/40HA cell lines, the cells were infected with the HB5 C-F virus and then harvested at 24 and 72 h p.i. (Fig. 3). Expression of IE2 86F could be detected with an antibody to the FLAG tag at both 24 and 72 h p.i (FLAG/HA). This indicated that IE2 86F was being expressed during all stages of the infection. To ensure that the protein being made by the cell line was migrating at the appropriate size, samples were run on the same gel, and parallel Western blots were performed with the mIE2 86 antibody. As can be seen in Fig. 3, at 24 h p.i., both the FLAG-tagged version of IE2 86 and the protein being expressed from the virus migrated at the same size. In this experiment, the only difference between the cell lines that we noted was that the viral IE2 86 was present at lower levels in the 40HA cells at 72 h p.i. Also, expression of the IE2 86F was slightly higher than expression of IE2 86 from the HB5 C-F virus. However, this difference was minimal and did not cause any deleterious effects on the infection process. Similarly, expression of the IE2 60HA and IE2 40HA proteins...
was assessed at both times p.i. Expression of these proteins could not be detected at 24 h p.i. using an antibody directed against the HA tag (Fig. 3, FLAG/HA blot at 24 h p.i.). However, by 72 h p.i., IE2 60HA and IE2 40HA were readily detected, and both migrated at the appropriate size (Fig. 3, compare FLAG/HA blot with mIE2 86 blot at 72 h p.i.). This was in accord with the known expression pattern of early-late genes.

As a complement to the above analysis, we used IFA with antibodies to both FLAG and HA to confirm that the majority of the cells could be induced to express the tagged IE2 proteins. Cells were infected at an MOI of 1 PFU/cell with the HB5 C-F virus and then fixed onto coverslips at either 24 or 72 h p.i. As expected at both 24 and 72 h p.i., expression of IE2 86F from the 86F and 86F/40HA cell lines could be detected with antibody to the FLAG tag although only the IFAs from 72 h p.i. are shown (Fig. 4A). This was congruent with the expression pattern seen in the Western blot analyses. In contrast, the expression of IE2 60HA and IE2 40HA was only detected at 72 h p.i. by IFA with an antibody to the HA tag, confirming that the 1.2-kb viral promoter was directing expression of these proteins with the kinetics of an early-late gene (Fig. 4A, HA column). This was in accord with the known expression pattern of early-late genes.

Expression of IE2 86F and IE2 40HA aids the IE2 86ΔSX C-F virus in cell-to-cell spread and increases titer. The replication of the pH5 and IE2 86ΔSX viruses has been extensively analyzed (58, 79). In the following studies, we evaluated the growth of the mutant virus containing the Cre/FLP cassette in the different cell lines with respect to the ability of the IE2 family of proteins to aid in viral gene expression, production of infectious virus, and cell-to-cell spread. Since we did not observe any significant complementation by the cells that expressed only IE2 40 (40HA) or IE2 60 (60HA), the remainder of the studies in this paper show the experiments with the HFFs, 86F, and 86F/40HA.

To confirm that the IE2 86ΔSX C-F virus could induce the synthesis of the exogenously expressed IE2 proteins in the same manner as HB5 C-F virus, we first used IFA to assess their expression in the 86F and 86F/40HA cells following infection at an MOI of 1 PFU/cell. At 24 and 72 h p.i., cells infected with the IE2 86ΔSX C-F virus were fixed onto coverslips and costained for FLAG and HA. As a representative example of the induction of the FLAG- and HA-tagged pro-
teins by this virus. Fig. 4B shows that IE2 86F and IE2 40HA could be detected at 72 h p.i. in the 86F/40HA cell line. The 86F and 86F/40HA cells also showed appropriate expression of the IE2 86F protein at 24 h p.i. (data not shown). Analogous to what was observed in the HB5 C-F virus-infected cells, some cells expressed either IE2 86F or IE2 40HA to a greater degree in the double cell line.

The IE2 86ΔSX virus has been previously shown to grow with slower kinetics and to reduced titers in HFFs than HB5 virus (58; also R. L. Sanders and D. H. Spector, unpublished data). As expected, this was also true for the IE2 86ΔSX C-F virus. When the IE2 86ΔSX C-F Bac was electroporated into HFFs, plaque formation was slow, and it took an average of 60 days to grow until all cells exhibited substantial CPE (Table 1).

The titer of the extracellular virus generated was also very low following electroporation of the Bac into HFFs (Table 1). This is in contrast to observed growth kinetics of the HB5 virus, which often produces titers of 10^6 to 10^7 PFU/ml and spreads throughout the culture in approximately 9 to 11 days (unpublished results). Table 2 shows that a similar pattern of viral replication was observed following infection of the HFFs with the IE2 86ΔSX C-F virus at an MOI of 0.05 PFU/cell. It took an average of 44 days for the HFFs to reach full CPE, and the titers of extracellular virus were only 2.2 x 10^2 PFU/ml. This is consistent with previous observations regarding the growth of the IE2 86ΔSX virus, indicating that the addition of the Cre/FLP cassette did not change the growth of the mutant virus in HFFs (58).

In contrast, when the IE2 86ΔSX C-F virus was grown in 86F cells, cell-to-cell spread as assessed by plaque formation was significantly enhanced compared to the virus grown in HFFs (data not shown). This was also reflected in the reduced number of days for all cells to exhibit CPE (Tables 1 and 2). However, the titers of the extracellular IE2 86ΔSX C-F virus that was released from the 86F cells after reaching full CPE following electroporation were only slightly increased compared to those obtained on the HFFs (Table 1). Likewise, there was only a modest increase in titer (approximately sixfold) when 86F cells were infected at a low MOI (Table 2).

To further elucidate the role of IE2 60 and IE2 40 in restoring the spread and titer of the IE2 86ΔSX C-F virus, replication in the 60HA, 40HA, and 86F/40HA cells was also assessed. The 60HA and 40HA cell lines alone were comparable to HFFs and did not facilitate the growth of the virus (data not shown). However, when the combination of IE2 86F and IE2 40HA was expressed (86F/40HA cell line), both the spread and the titer of the mutant virus increased substantially. The more rapid cell-to-cell spread was illustrated by the significant reduction in the days p.i. required to reach full CPE. Moreover, following the initial electroporation, the IE2 86ΔSX C-F progressed nearly twice as fast in the 86F/40HA cell line and the average titer obtained from these harvests of virus was increased by more than 10^3-fold (Table 1). These cells infected with the IE2 86ΔSX C-F virus at a low MOI also showed full CPE earlier and produced greatly increased titers (Table 2). Taken together, these results show that both IE2 86 and IE2 40 are important for cell-to-cell spread as well as overall progression of the viral infection.

### Table 1. Growth and titer of the IE2 86ΔSX C-F virus following the initial electroporation of the Bac in HFF, 86F, or 86F/40HA cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of days to harvest</th>
<th>Titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF</td>
<td>60</td>
<td>2.5 x 10^2</td>
</tr>
<tr>
<td>86F</td>
<td>42</td>
<td>2.7 x 10^2</td>
</tr>
<tr>
<td>86F/40HA</td>
<td>33</td>
<td>3.0 x 10^5</td>
</tr>
</tbody>
</table>

*a Number of days until all cells exhibited CPE.

*b Titer of extracellular virus by plaque assay.

### Table 2. Growth and titer of the IE2 86ΔSX C-F virus following infection of HFF, 86F, or 86F/40HA cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No. of days to harvest</th>
<th>Titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF</td>
<td>44.0 ± 1.4</td>
<td>2.2 x 10^2 ± 2.1 x 10^2</td>
</tr>
<tr>
<td>86F</td>
<td>26.0 ± 1.8</td>
<td>1.3 x 10^3 ± 1.8 x 10^2</td>
</tr>
<tr>
<td>86F/40HA</td>
<td>13.8 ± 1.0</td>
<td>2.7 x 10^2 ± 2.8 x 10^2</td>
</tr>
</tbody>
</table>

*a Cells were infected at an approximate MOI of 0.05 PFU/cell. Standard deviations are shown and are representative of at least four experiments, with the exception of the virus propagated on the HFFs, for which only two experiments were performed.

*b Number of days until all cells exhibited CPE.

*c Titer of extracellular virus by plaque assay.

The expression of IE and early proteins was also assessed. As seen in Fig. 5, with an antibody that detects both IE2 86F and IE2 40HA, the level of total IE2 86 detected at both 48 and 96 h p.i. was slightly higher in the 86F and 86F/40HA cells, which was due to the combined expression of FLAG-tagged IE2 86 and viral-encoded IE2 86. This could also be seen using an antibody that...
FIG. 5. Expression of viral proteins in the HB5 C-F and IE2 86ΔSX C-F virus-infected cells. Cells were infected or mock infected at an MOI of 3 PFU/cell with either the HB5 C-F or IE2 86ΔSX C-F (ΔSX C-F) virus and then analyzed at 48 and 96 h p.i. by Western blotting for expression of the indicated viral proteins. Equal amounts of protein were loaded in each lane. Expression of IE2 86, IE2 86F, and IE1 72 was analyzed with the CH16.0 antibody. Expression of IE2 86, IE2 86F, IE2 60, IE2 40, and IE2 40HA was analyzed with mIE2 86. The levels of an early protein (UL57), early-late proteins (UL84 and UL83), and a late protein (UL99) were also assessed with their corresponding antibodies. Induction of IE2 86F and IE2 40HA in the 86F and 86F/40HA cell lines was visualized with antibodies to FLAG and HA. Actin served as a loading control. Mock-infected samples are also shown.

recognizes all forms of the IE2 proteins (mIE2 86), including IE2 86, IE2 60, and IE2 40. In addition, it appeared that both the IE2 60 and IE2 40 levels were higher at 48 h p.i. in these cells. This may be due in part to increased IE2 40 in the 86F/40HA cells and slightly faster progression of the infection in both cell lines, as the levels were comparable at 96 h p.i. Early gene expression remained unaffected in the HB5 C-F virus-infected cells as judged by expression of UL57.

The same proteins were assessed by Western blotting in the cells infected with the IE2 86ΔSX C-F virus. Expression of IE2 86 from both the IE2 86ΔSX C-F virus and the cell lines was assessed with the CH16.0 (IE1 72 and IE2 86) and mIE2 86 antibodies (IE2 86, IE2 60, and IE2 40) (Fig. 5). As previously described (58), the IE2 86ΔSX C-F virus showed a delay in the expression of mutant IE2 86 (IE2 ΔSX at 48 h.p.i.), while IE1 72 remained generally unaffected, although the level was slightly lower in the HFFs at 96 h p.i. As expected, no IE2 60 was detected in any of the infected cells, full-length IE2 86 was only seen in the infected 86F and 86F/40HA cells, and IE2 40 was present only in the infected 86F/40HA cells. It should be noted that although the level of full-length IE2 86 in the IE2 86ΔSX C-F-infected 86F and 86F/40HA cells (which contained only the induced protein) was slightly lower than in the same cells infected with the wt HB5 C-F virus (which contained both induced and virus-encoded IE2 86), the 86F and 86F/40HA cells infected with the mutant virus expressed comparable levels of IE2 86F as the HFFs infected with the wt virus. However, a greater amount of IE2 40 was present in all of the cells infected with the wt virus than in the 86F/40HA cells infected with the mutant. As expected, there was no significant difference in the expression of the early protein UL57 in cell lines infected with IE2 86ΔSX C-F virus. This confirmed that the infections with the HB5 C-F and IE2 86ΔSX C-F viruses were properly matched and showed that the presence of the induced IE2 86F, IE2 60HA, or IE2 40HA proteins had no significant effect on the expression of UL57.

Since we previously found that the levels of many early-late and late proteins, particularly UL83, UL84, and UL99, were substantially lower in IE2 86ΔSX virus-infected cells (58, 79), we were interested in whether any of the IE2 family of proteins could aid in recovery of expression of these proteins. In general, the kinetics of appearance and levels of UL84 and UL83 (early-late proteins) and UL99 (a late protein) were comparable in the three HB5 C-F virus-infected cell lines (Fig. 5). However, differences were detected in the recovery of expression of UL84, UL83, and UL99 in the cell lines infected with the IE2 86ΔSX C-F virus. Interestingly, the induction of IE2 86F alone was sufficient for a partial increase in the levels of UL84 and UL83 by 48 h.p.i. compared to their respective levels in HFFs, and the levels increased further by 96 h.p.i. over those in HFFs. Enhancement of the UL84 and UL83 levels, however, was most notable in the IE2 86ΔSX C-F-infected 86F/40HA cells that expressed both IE2 86F and IE2 40HA. The late protein UL99 also exhibited slightly reduced levels of the pro-
tein in HFFs at 96 h p.i. when infected with the mutant virus. However, in either the 86F or 86F/40HA cells, the levels of the protein were comparable to the level of the HB5 C-F virus at 96 h p.i.

Expression of IE2 86 alone or in combination with IE2 40 complements a nonviable IE2 mutant virus. Since a major obstacle in studying IE2 86 function in the context of the infection has been the lack of a means to propagate a nonviable mutant virus, an important test of our system was whether it would complement the growth of a virus with a null mutation in the IE2 gene. To this end, we used a mutant virus that is missing exon 5 of IE2 86, IE2 86ΔExon5 C-F (Fig. 1). HFFs, 86F, and 86F/40HA cells were electroporated simultaneously and initially analyzed for viral expression and plaque formation (data not shown). By day 6 postelectroporation, IE gene expression was detectable, and early gene expression (UL44) was visible in a few of the 86F/40HA cells. By day 9 postelectroporation, early gene expression had progressed to a great degree, and some small plaques were visible. This was also seen in the 86F cell line. In contrast, expression of only IE1 72 could be detected with HFFs infected with this virus, and no plaques were visualized at either time postelectroporation. In general, the progression of the plaques on the 86F cells following electroporation with IE2 86ΔExon5 C-F BAC was slower than that on the 86F/40HA cells, although some plaques on the 86F cells were comparable in size to those on the 86F/40HA cells at the same time postelectroporation. Overall, more plaques were observed on the 86F/40HA cell line following electroporation and infection, and many of these plaques spread more rapidly relative to those on the 86F cells. Virus grown in 86F cells following electroporation took approximately 57 days to spread completely through the culture, while the 86F/40HA cell line reached 100% CPE in approximately 48 days. This is in contrast to the known growth characteristics of the wt HB5 virus following electroporation, which forms plaques much more quickly and spreads throughout the culture by day 9 postelectroporation (data not shown).

The titer of the IE2 86ΔExon5 C-F virus that was grown in the 86F/40HA cell line (approximately 3.5 × 10⁵ PFU/ml) was also consistently higher than that of virus grown in the 86F cell line, although it was still significantly lower than the titer of wt HB5 C-F virus grown in these cell lines (data not shown). For these reasons, only the 86F/40HA cells were used to prepare stocks of the mutant virus. Taken together, these data indicate that IE2 86 alone is necessary for formation of productive virus and that expression of IE2 40 provides additional benefits for complementing a nonviable virus.

To verify that the IE2 86ΔExon5 C-F virus (as well as the rescued version of the mutant virus) was able to induce proper expression of the IE2 86F and IE2 40HA proteins, the 86F/40HA cells were infected at an MOI of 1 PFU/cell with either the IE2 86ΔExon5 C-F or IE2 86ΔExon5 Res C-F virus and were seeded onto coverslips. At 72 h p.i., cells were fixed with 2% paraformaldehyde and then analyzed for expression of FLAG and HA by IFA. The nucleus is indicated by Hoechst staining, and the merged panels show all three stains combined.

FIG. 6. Expression of IE2 86F and IE2 40HA in 86F/40HA cells infected with either the IE2 86ΔExon5 C-F (ΔExon5 C-F) or IE2 86ΔExon5 Res C-F (Res C-F) virus. 86F/40HA cells were infected at an MOI of 1 PFU/cell with either the IE2 86ΔExon5 C-F or IE2 86ΔExon5 Res C-F virus and were seeded onto coverslips. At 72 h p.i., cells were fixed with 2% paraformaldehyde and then analyzed for expression of FLAG and HA by IFA. The nucleus is indicated by Hoechst staining, and the merged panels show all three stains combined.
early gene expression produced infectious virus that spread to adjacent cells. Panel 9C shows that in the plaques, almost all of the cells that expressed IE1 72 also expressed the early-late protein UL83 by this time, although there was a small percentage that expressed only IE1 72. We could not determine, however, whether these were newly infected cells or abortively infected cells that were not able to progress to early-late gene expression. Based on these results, it is likely that at low MOIs,

FIG. 7. IE, early, and early-late gene expression following infection with the IE2 86ΔExon5 C-F virus at a low MOI. HFFs, 86F, and 86F/40HA cells were infected at a low MOI (0.01 PFU/cell) and seeded onto coverslips. At day 4 p.i. (A and B) coverslips were fixed and then stained with antibodies directed against IE1 72 and UL44. (A) Pictures were taken of cells observed with a 40× objective. The field in the figure was specifically selected to show cells that expressed both proteins. (B) Pictures were taken of cells on the same coverslips as in panel A, observed with a 20× objective to show that a significant number of the cells expressed only IE1 72 and had not begun to form plaques. (C) At day 8 p.i., cells were fixed, and coverslips were stained with an antibody directed against IE1 72 and UL83. The nucleus is indicated by Hoechst staining.
viable IE2 86ΔExon5 C-F virus can be produced on the complementing cells, but the block to early gene expression is only partially overcome. Furthermore, early-late gene expression seems to progress in the same percentage of cells that overcome the block to early gene expression, indicating that once viral replication commences, early-late gene expression is efficient.

**Kinetics of viral gene expression following infection of 86F and 86F/40HA cells at high MOIs with IE2 86ΔExon5 C-F.** To more carefully characterize the pattern of viral gene expression in the IE2 86ΔExon5 C-F virus-infected cells and to determine the patterns of expression at a higher MOI, Western blot analyses were used. HFF, 86F, and 86F/40HA cells were infected or mock infected at an MOI of 1 PFU/cell with the IE2 86ΔExon5 C-F or the HB5 C-F virus and then harvested at 24, 72, and 120 h p.i. (Fig. 8). Levels of representative viral proteins from each stage of the IE2 86ΔExon5 C-F virus infection were assessed and compared to those in the three cell lines infected with HB5 C-F virus.

At 24 h p.i., there were slightly higher levels of IE2 86 in the HB5 C-F virus-infected 86F and 86F/40HA cells due to the presence of the induced IE2 86F from the lentivirus. All of the detectable IE2 86 from the IE2 86ΔExon5 C-F virus-infected cells was expressed from the induced protein, and the levels were comparable to levels present in the HB5 C-F virus-infected cells. As expected, no IE2 86 could be detected in the HFFs infected with the IE2 deletion virus. Interestingly, all three cell lines infected with the IE2 86ΔExon5 C-F virus exhibited greatly increased levels of IE1 72 expression by 24 h p.i., and this expression continued throughout the time course (Fig. 8). Expression of the IE2 86F provided by the cell lines was also assessed with the FLAG antibody. At 24 h p.i., an exposure comparable to that at the later times does not indicate any expression of IE2 86F; however, upon longer exposure, expression of IE2 86F can be detected to a comparable degree in the IE2 86F and IE2 86F/IE2 40HA cell lines (Fig. 8A, only the longer exposure of the FLAG blot is shown). As expected, no
expression of IE2 40HA could be detected at 24 h p.i., even upon long exposure (Fig. 8A).

In examining representative early genes, we analyzed the expression of the viral replication proteins UL44 and UL57. At 24 h p.i., expression of both UL44 and UL57 in the IE2 86ΔExon5 C-F virus-infected cells was greater than in the HB5 C-F virus-infected cells, most likely due to the entry of a larger number of IE2 86ΔExon5 C-F virions into the cells, relative to the measured PFU. This is also reflected in the higher levels of early-late and late virion matrix proteins UL83 and UL99 in the IE2 86ΔExon5 C-F virus-infected cells at 24 h p.i. in all cell lines tested. In particular, the presence of these virion proteins in the HFFs at this time p.i. indicated that they were input proteins, since in these cells the IE2 86ΔExon5 C-F infection does not proceed beyond the IE phase. Although we cannot exclude the possibility that there was some late gene expression from the mutant viral genomes at early times, the experiments described below support the conclusion that the input particle-to-PFU ratio of the IE2 86ΔExon5 C-F virus was significantly higher than that of the HB5 C-F virus. Furthermore, given that the expression of these proteins did not continue throughout the time course (Fig. 8B and C), this further supported the idea that the expression seen at 24 h p.i. was due to the increased number of input virions.

At 72 h p.i., expression of IE2 86, IE2 60, and IE2 40 could be detected in the HB5 C-F virus-infected cells (Fig. 8B). IE2 86F was still present in the IE2 86ΔExon5 C-F virus-infected cells, although no IE2 60 or IE2 40 could be seen with the mIE2 86 antibody. However, staining with the HA antibody indicated that some IE2 40HA was present at this time in the 86F/40HA cells infected with either virus. The levels of IE2 86F, as detected with the FLAG antibody, were higher in the 86F cells infected with HB5 C-F than with IE2 86ΔExon5 C-F, but there was comparable expression in the 86F/40HA cell line infected with either virus. Similar to what was observed at 24 h p.i., the amount of IE1 72 continued to be much higher in the mutant-virus-infected cells than the HB5 C-F virus-infected cells. This could be seen with both the CH16.0 and the IE1 72-specific antibodies. At this time, early gene expression was comparable for the two viruses, as indicated by UL44 and UL57. No expression of these proteins could be detected in the HFFs infected with IE2 86ΔExon5 C-F, providing additional evidence that the virus cannot progress to the early stages of the infection without IE2 86.

Early-late and late gene expression was complemented to various degrees in the 86F and 86F/40HA cell lines at 72 h p.i., as indicated by the levels of UL84, UL83, and UL99. Interestingly, although the levels of UL99 were comparable for both viruses, the levels of UL84 and UL83 were lower in the cells infected with the IE2 86ΔExon5 C-F virus than with the HB5 C-F virus. Our previous studies with other mutant viruses have indicated that a very dynamic relationship between these two proteins and IE2 86 and IE2 40 exists, which might account for the lack of full complementation. As can be seen with the mIE2 86 antibody in Fig. 8B and C, the amount of the IE2 40HA protein induced in the 86F/40HA cell lines was significantly lower than the levels that are present during the normal wt infection at the later times. Given these results, it is likely that more IE2 40 protein would be needed to see full complementation of this virus.

As shown in Fig. 8C, IE1 72 levels in all three cell lines infected with IE2 86ΔExon5 C-F virus remained very high at 120 h p.i. IE2 86F and IE2 40HA could be detected with the FLAG and HA antibodies in the 86F and 86F/40HA cells, and the levels of the viral early proteins in these cells remained comparable between the two viruses. Interestingly, at this point the amount of UL99 was slightly higher in the IE2 86ΔExon5 C-F virus-infected cells than in the HB5 C-F virus-infected cells. UL84 and UL83 levels, however, were still lower in the mutant-infected 86F and 86F/40HA cells than in HB5 C-F virus-infected cells. The levels of UL83, however, seemed to be higher in the IE2 86ΔExon5 C-F-infected 86F/40HA cell line than in the 86F cell line, indicating that UL83 expression is dependent on the presence of IE2 40. This confirms previous data that the loss of IE2 60 and IE2 40 results in decreased expression of UL83 protein and RNA (79).

As noted above, there were very high levels of IE1 72 throughout the infection and increased levels of the virion matrix proteins UL83 and UL99 at early times in all three cell lines infected with the IE2 86ΔExon5 C-F virus. In these infections, the multiplicity was based on the number of PFU in the viral inoculum, and thus it was possible that the actual number of input virions (that did not progress to form plaques) was significantly higher for the mutant virus. To determine if the increase in input viral proteins was due to an increase in input viral genomes, quantitative PCR was performed to analyze the comparative amount of input genomes when normalized to the same number of PFU. Cells were infected with the same number of PFU/cell, and at 24 h p.i., the levels of intracellular viral DNA were measured by quantitative real-time PCR (Fig. 9A). Interestingly, the IE2 86ΔExon5 C-F virus-infected cells showed approximately 40-fold higher levels of viral DNA, indicating that many more genomes were entering the cell than were forming plaques. Furthermore, in order to determine if IE gene expression was affected at the RNA level, quantitative real-time RT-PCR assays were performed at 24 h p.i. (Fig. 9B). Analogous to what was observed for the IE1 72 protein, the levels of IE1 72 RNA were markedly higher (~100-fold) in the IE2 86ΔExon5 C-F virus-infected cells than in the HB5 C-F virus-infected cells.

In view of the very high levels of IE1 72 produced in cells infected with the IE2 86ΔExon5 C-F virus and the large particle-to-PFU ratio, it was important to ensure that all defects seen in the IE2 86ΔExon5 C-F virus were due to the loss of exon 5. A rescued virus, IE2 86 ΔExon5 Res C-F, was created, and the patterns of protein expression were compared to expression of the HB5 C-F. HFFs, 86F, and 86F/40HA cells were infected at an MOI of 1 PFU/cell and then analyzed by Western blotting at 24 and 72 h p.i. (Fig. 10, RES C-F). At 24 h p.i., expression of IE2 86 and IE1 72 could be detected, and the levels were comparable for the two viruses. Furthermore, similar levels of the UL44 early gene were present in the 86F and 86F/40HA cell lines infected with either virus, and induction of the FLAG-tagged IE2 86 could be detected. At 72 h p.i., there also were no differences in the expression of the viral proteins for the two viruses in any of the cell lines. Likewise, the particle-to-PFU ratio was comparable to that of the HB5 C-F virus, indicating that this was also a result of the loss of exon 5 (data not shown). These results showed that all of the defects
The deletion of aa 136 to 290 from the C-terminal exon of IE2 86 results in a viable virus, IE2 86ΔSX, that expresses a smaller IE2 86 protein but is unable to express the early-late IE2 60 and IE2 40 proteins (58, 79). Prior studies with this mutant showed that early gene expression remains comparable to the wt HB5 virus, as evidenced by the levels of the UL44 and UL57 proteins (58). In contrast, there is delayed and decreased expression of the mutant IE2 86 throughout the infection. In addition, the accumulation of the early-late proteins UL83 and UL84 is significantly inhibited. However, the mechanisms governing these effects appear to differ, as RNA expression remains normal for the mutant IE2 86 and UL84 but is greatly reduced for UL83 (58, 79).

To determine which of the IE2 proteins was responsible for the defects observed in the IE2 86ΔSX-infected cells, each of the cell lines was infected with the mutant IE2 86ΔSX C-F virus, and gene expression as well as virus production was seen in the mutant virus-infected cells were likely due to the deletion of exon 5 of IE2 86.

DISCUSSION

The IE2 86 protein is multifunctional and is essential for productive HCMV infection. Most studies on this protein, however, have been in an isolated context due to the difficulty of constructing and propagating recombinant viruses with selected mutations in its coding region. A major goal of our laboratory for many years has been to use mutant viruses to elucidate the functional domains of this protein and the underlying mechanisms governing its activities in the context of the infection, with the long-term objective of applying this information toward understanding the role of the protein in vivo pathogenesis and developing strategies for treatment or prevention of disease.

The use of the BAC recombination system has greatly facilitated the introduction of mutations into the viral genome. However, the lack of a complementing cell line for IE2 86 has made it impossible to propagate a mutant virus that is nonviable and to study the consequences of the mutation at a more mechanistic level. Moreover, even in the case of debilitated but viable mutant viruses, there is the concern that propagation of the virus in the absence of complementation will lead to the selection of viruses that have sustained second-site mutations that enhance the replication of the mutant virus.

Many attempts have been made to create a system that has the ability to complement nonviable viruses with mutations in the MIE coding region. Although a cell line has been constructed to complement mutations in the IE1 region (43), there have been no reports of a successful strategy for complementing mutations in the IE2 region. The advantages to the complementation system that we have developed include tightly controlled expression using an inducible recombination system, the ability to provide exogenous expression of the IE2 family of proteins in the same temporal pattern as the infection, and the decreased likelihood of second-site mutations or rearrangements occurring during the propagation of the virus. This system provides added benefits in that the IE2 86 is expressed in the cell line only when necessary for infection, and therefore IE2 86-mediated cell toxicity in uninfected cells is circumvented. The synthesis of the IE2 86 protein in the cell line is induced with a protein (Cre recombinase) that is expressed from the virus under the control of the MIEP. Thus, the IE2 86 is expressed with the same kinetics as the protein normally expressed by a wt virus. Similarly, the IE2 60 and IE2 40 proteins in the cell lines are induced with a protein (FLP recombinase) that is expressed from the virus under the control of the 1.2-kb viral promoter (early-late promoter), which allows proper temporal expression of these proteins as well. To this end, we have created cell lines that allow the assessment of IE2 86, IE2 60, and IE2 40 alone, as well as a double cell line expressing IE2 86 and IE2 40. These studies have helped define the individual roles of these proteins and have provided a means to propagate both a growth impaired virus, as well as a completely nonviable virus.

FIG. 9. Input viral DNA and expression of IE1 72 RNA during the IE stages of the infection. At 24 h p.i., 86F/40HA cells that were infected with the HB5 C-F and IE2 86ΔExon5 C-F (ΔExon 5 C-F) viruses (from the same infection shown in Fig. 8) were harvested for analysis of viral DNA (A) or RNA (B). (A) Quantitative real-time PCR was used with the primers and probe for the viral UL77 gene to measure the amount of total input viral DNA. A separate experiment was also set up with another preparation of the IE2 86ΔExon5 C-F virus in order to ensure that these data were not specific to a single viral preparation. The increase relative to the HB5 C-F infected cells (value set to 1) is shown and is an average of these two experiments (represented by error bars). Duplicate samples were assayed for each experiment. (B) The RNA from the 86F/40HA cells infected with both viruses was analyzed using quantitative real-time RT-PCR analyses with the primers and probe for the viral UL77 gene to measure the amount of total input viral DNA. A separate experiment was also set up with another preparation of the IE2 86ΔExon5 C-F virus in order to ensure that these data were not specific to a single viral preparation. The increase relative to the HB5 C-F infected cells (value set to 1) is shown and is an average of these two experiments (represented by error bars). Duplicate samples were assayed for each experiment. For both panels, a standard curve was generated in order to assess the relative quantities of either DNA or RNA in each sample.
assessed. The cells that could be induced to express either the IE2 60 protein (60HA) or IE2 40 protein (40HA) alone were unable to provide any notable complementation. Interestingly, expression of full-length IE2 86 alone was able to counter some of the defects. However, the presence of IE2 86 and IE2 40 in combination appeared to have the most beneficial effect on expression of UL83 and UL84 and virus production. It seems likely that IE2 86 plays an important role in regulation of both of these gene products and that IE2 40 is involved in much of this regulation at the later stages of the infection.

It was notable that even in the 86F/40HA cells, the levels of UL83 and UL84 in the IE2 86ASX-infected cells were still significantly lower than the levels in the wt virus-infected cells. It seems unlikely that there was insufficient full-length IE2 86 induced in the complementing cells, as the levels in the mutant-infected cells were comparable to levels observed in HFFs infected with wt virus. Rather, we suspect that the problem is due, at least in part, to the relative amount of the IE2 40 protein induced in the complementing cells, which was significantly lower than the levels of the IE2 40 protein expressed by the wt virus at late times in the infection. In accord with this, we previously found that the levels of UL83 and UL84 were also substantially less in HFFs infected with an IE2 mutant virus (IE2 Δ40+60) that expressed full-length wt IE2 86 but no IE2 40 or IE2 60 (79). We hypothesize that this may be due to a very tight regulatory system by which proper levels of IE2 86 and IE2 40 are necessary for UL84 protein expression. To date, the mechanism underlying this phenomenon is unclear, but studies are under way to address this. The defect in UL83 expression appears to be at the level of RNA synthesis, which more clearly fits into the known roles of the IE2 proteins. Based on our observation that the combination of IE2 86 and IE2 40 is required for optimal cell-to-cell spread and production of the mutant virus, it is tempting to consider that IE2 40 may also play a role in packaging and release of the viral particle or in regulation of other genes important for this aspect of the infection.

Assessment of the replication of the IE2 86ΔExon5 C-F virus has led to several interesting findings. By IFA and Western blot analysis, it appears that both the 86F and the 86F/40HA cell lines allow expression of all temporal classes of viral proteins, but only IE expression can commence on HFFs. However, despite the complementation of the nonviable IE2 86ΔExon5 C-F virus, the growth and titers of the virus obtained were still impaired relative to the wt HB5 C-F virus. When cells were infected at a relatively high MOI (MOI of 1 PFU/cell), the expression of the early proteins in the complementing cells infected with the mutant virus was comparable to that observed with the wt virus at a comparable MOI, but the levels of the newly synthesized UL83 and UL84 proteins were lower. A striking result was that expression of IE1 72 is significantly upregulated in the complementing cell lines when in-
fected at this apparent MOI based on the number of PFU. This appears to be due to a requirement for a large number of input virions for the infection to progress beyond the IE phase even in the complementing cells and is consistent with the observation that many mutant viruses replicate at high MOIs but show a significant growth defect at low MOIs. In accord with this, we found by IFA that at low MOIs only 10 to 15% of the complementing cells that expressed viral IE proteins also expressed early proteins and went on to form plaques. At this point, we do not know whether this is due to some defect in the viral DNA or the composition of the input virions. Although these defective viral genomes likely contributed to the observation that many more cells expressed IE1 72 than UL44 following infection at a low MOI (Fig. 7), it is not likely that this is the major reason for the lack of early gene expression in these mutant virus-infected cells.

One question raised by these results is what would be required to achieve full complementation. The temporal expression and levels of full-length IE2 86 induced in the complementing cells are comparable to the endogenous IE2 86 produced by the wt virus in HFFs, but the amount of induced IE2 40 is still much lower than the endogenous IE2 40 produced by the wt virus at late times. Yet if insufficient levels of IE2 40 were solely responsible for the growth defect of the progeny IE2 86ΔExon5 C-F virus during the next round of infection at a low MOI, we might expect that IE2 86ΔSX C-F progeny virus would show a similar phenotype with inefficient progression from IE to early gene expression. As previously reported and shown here, early gene expression and viral DNA replication appear to be normal in IE2 86ΔSX virus infected cells, and the primary effects of the mutation appear to be at late stages of the infection. The major difference between IE2 86ΔExon5 and IE2 86ΔSX viruses is that the mutant IE2 86 produced by the IE2 86ΔSX still includes much of exon 5 (aa 85 to 135 and 291 to 579). It is possible that in the cells infected with IE2 86ΔSX virus there are alternative gene products that contain sequences from exon 5. We also cannot exclude the possibility that the absence of exon 5 has indirect effects on the expression of other genes that are not related to the IE2 protein itself. Studies to address these questions are currently in progress.

The approach for deriving complementing cell lines that is described here can be applied to other HCMV genes, particularly in the case where even very low levels of the viral protein may be toxic for the cell and where correct temporal expression is critical for the replication of the virus. There is a slight delay in production of the induced protein using this system, which may provide some restrictions for its use; however, the tightly controlled expression clearly provides an advantage over many other expression systems. Our experience with multiple approaches that are inducible at the transcriptional level has been that it is difficult to completely eliminate expression of the gene. In contrast, translation in eukaryotic cells of the second ORF on a multistrand RNA, in the absence of an internal ribosome entry site, is very uncommon, and control at this level is therefore much tighter. Thus, this strategy could also be generally useful for controlled expression of other highly toxic cellular or microbial proteins.
inhibitor of immediate-early-mediated transcription that is able to prevent viral replication. J. Virol. 71:7048–7060.


