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Small Internal Deletions in the Human Cytomegalovirus IE2 Gene Result in Nonviable Recombinant Viruses with Differential Defects in Viral Gene Expression

Elizabeth A. White, Charles L. Clark, Veronica Sanchez, and Deborah H. Spector*

Molecular Biology Section and Center for Molecular Genetics, University of California—San Diego, La Jolla, California 92093-0366

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The human cytomegalovirus (HCMV) IE2 86-kDa protein is a key viral transactivator and an important regulator of HCMV infections. We used the HCMV genome cloned as a bacterial artificial chromosome (BAC) to construct four HCMV mutants with disruptions in regions of IE2 86 that are predicted to be important for its transactivation and autoregulatory functions. Three of these mutants have mutations that remove amino acids 356 to 359, 427 to 435, and 505 to 511, which disrupts a region of IE2 86 implicated in the activation of HCMV early promoters, a predicted zinc finger domain, and a putative helix-loop-helix motif, respectively, while the fourth carries three arginine-to-alanine substitution mutations in the region of amino acids 356 to 359. The resulting recombinant viruses are not viable, and by using quantitative real-time reverse transcription-PCR and immunofluorescence we have determined the location of the block in their replicative cycles. The IE2 86Δ356-359 mutant is able to support early gene expression, as indicated by the presence of UL112-113 transcripts and UL112-113 and UL44 proteins in cells transfected with the mutant BAC. This mutant does not express late genes and behaves nearly indistinguishably from the IE2 86R356/7/9A substitution mutant. Both exhibit detectable upregulation of major immediate-early transcripts at early times. The IE2 86Δ427-435 and IE2 86Δ505-511 recombinant viruses do not activate the early genes examined and are defective in repression of the major immediate-early promoter. These two mutants also induce the expression of selected delayed early (UL89) and late genes at early times in the infection. We conclude that these three regions of IE2 86 are necessary for productive infections and for differential control of downstream viral gene expression.

Human cytomegalovirus (HCMV), a betaherpesvirus, has a double-stranded DNA genome of approximately 230 kbp that encodes at least 150 open reading frames (17). Infection with HCMV has serious consequences for immunocompromised patients and is the leading viral cause of birth defects (for a review, see reference 48). HCMV gene expression is separated into three temporal categories (for a review, see reference 46). Immediate-early (IE) genes are the first to be activated and do not require de novo host or viral protein synthesis for their expression. Early genes represent a broadly defined class whose transcription is typically regulated by the interaction of IE gene products with cellular factors. Viral DNA replication follows early gene expression. Finally, late viral genes, many of which encode structural proteins, are expressed.

Since IE gene expression begins this cascade of events, the regulation and products of IE genes have been studied extensively. The major immediate-early (MIE) gene, made up of open reading frames UL122 and UL123, is a region of particular interest. It consists of five exons that are transcribed, differentially spliced, and translated to give two predominant products: the IE1 72-kDa protein (exons 1 to 4) and the IE2 86-kDa protein (exons 1 to 3 and 5). The translation of each transcript initiates in exon 2, and the two proteins share 85 amino acids (aa) at their amino termini (66–68; reviewed in reference 20). The IE2 region also encodes an additional product that is expressed later in the infection and a splice variant that is present in infected human monocyte-derived macrophages (32, 36, 53, 64). IE1 72 is the more abundant product at both the mRNA and protein levels and has modest transactivating effects, including the ability to transactivate the MIE promoter (MIEP) (for reviews, see references 20 and 46). Numerous in vitro and in vivo studies have shown that IE2 86 is a strong transactivator and also represses its own promoter (13, 14, 29, 40, 50, 52, 65). Other IE genes include IRS1, TRS1, the UL36 to -38 genes, and US3. Many of these, including US3, UL36, and IRS1, are not required for HCMV replication in cultured cells (8, 10, 33, 49). A virus lacking TRS1 exhibits normal gene expression during IE and early times postinfection but is defective in late stages of replication (8). An IE1 mutant virus is viable but shows growth defects during a low-multiplicity infection (22, 24, 47). In contrast, the failure of a virus lacking most of the IE2 gene to support early gene expression and to replicate indicates that IE2 86 is essential for productive infection (44).

Significant efforts have been directed towards defining the elements that provide IE2 86 with its strong regulatory capabilities, and IE2 86 is thought to transactivate and repress via protein-protein and protein-DNA interactions. IE2 86 binds to the product of the viral UL84 gene and to multiple cellular proteins. These host factors include components of the basal transcription complex TBP and TFIIB, numerous cellular transcription factors, Rb, p53, and others (9, 11, 15, 16, 19, 21, 23, 25, 26, 35, 37, 41, 58–62, 72). In addition, IE2 86 is modified by multiple ubiquitin-like proteins (4, 30). IE2 86 is thought to

* Corresponding author. Mailing address: Molecular Biology Section, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0366. Phone: (858) 534-4584. Fax: (858) 534-6083. E-mail: dspector@ucsd.edu.

bind DNA through interactions with the minor groove, a notable example being its binding to the 14-bp *cis*-repression signal (CRS) between the TATAA box and the transcription start site in the MIEP. It has been shown that this binding is the source of IE2 86's negative regulatory effect on its own transcription (13, 31, 39, 40, 42, 50). In addition, IE2 86 binds to similar 14-bp sites upstream of the TATAA box in early promoters, including the UL112-113 (2.2-kb RNA) and 1.2-kb RNA promoters (5, 12, 59, 60).

Multiple studies have aimed to define motifs and domains in IE2 86 that allow these protein-protein and protein-DNA interactions and the locations of residues that are likely to be posttranslationally modified. The ability of IE2 86 to interact with other proteins maps broadly to the majority of the region not shared with IE1 72 (aa 86 to 542) (15). It should be noted to avoid confusion that the amino acid numbers in the text correspond to IE2 86 in the Towne strain. AD169 has an additional serine after amino acid 264. A subset of this region, aa 388 to 542, is required for IE2 86 to dimerize (3, 15, 21). The DNA binding capability of IE2, which allows regulation of early promoters as well as autoregulation, is also the result of sequences present in the C-terminal half of the protein, between residues 290 and 579 (15, 38, 59). Various structures and functional regions have been more finely mapped. There are three potential sites for phosphorylation by casein kinase II located between aa 203 and 277 (51). Harel and Alwine (27) demonstrated that both in vitro and in vivo, IE2 86 is phosphorylated on multiple residues. They performed site-directed mutagenesis of consensus mitogen-activated protein (MAP) kinase motifs at aa 27, 144, 233 to 234, and 555 to alanine and found that in transient expression assays, some of these changes resulted in a protein with a stronger capacity to transactivate than the wild type. IE2 86 has at least two nuclear localization signals, at aa 145 to 151 and 321 to 328; a leucine-rich region predicted to form a helix-loop-helix between aa 463 and 573; and a putative zinc finger domain at aa 428 to 452 (21, 43, 51, 65). Although IE2 mutants with changes in the zinc finger domain fail to bind to DNA or to autorepress their own transcription, in transient expression assays they are able to drive transcription from the MIEP (73). The insertion of four amino acids at residue 356 or 540 reduces at least 6-fold the capacity of the protein to stimulate the viral DNA polymerase (UL54) promoter; however, these mutations have the opposite effect on the MIEP and result in a 10-fold increase in its activation (65).

Until recently, these mapping studies were conducted primarily with expression vectors in transient expression assays or with bacterially expressed, purified IE2 86 mutant proteins in in vitro assays due to the difficulty of examining the mutations in the context of the viral genome. While our group and others have attempted to construct an IE2 86-expressing cell line capable of complementing *ie2* mutants, to date none has been isolated. In the absence of such a tool, it is difficult to propagate recombinant viruses with mutations in essential genes such as IE2 86; however, the advent of bacterial artificial chromosomes (BACs) as vectors for the cloning of herpesvirus genomes has largely allowed this problem to be circumvented. Since the majority of the viral genome is present in the BAC, mutations can be made and characterized entirely in bacteria, regardless of the viability of the resulting virus. The altered genome is then transfected into cells that are permissive for HCMV infection along with a construct expressing pp71 (ppUL82), allowing reconstitution of the virus from the clone. pp71 expression has been shown to increase the infectivity of transfected HCMV DNA (6). Murine cytomegalovirus was the first herpesvirus to be cloned as a BAC, and the technique has been successfully extended to include HCMV, herpes simplex virus type 1, Epstein-Barr virus, and others (10, 18, 45, 55; reviewed in references 2 and 70). Several groups have since used this approach to construct HCMV IE2 86 mutants. Marchini et al. showed that a recombinant virus with most of the IE2 gene (open reading frame UL122) deleted is defective in early gene expression and does not produce infectious progeny (44). Members of our group generated a viable mutant with a deletion of IE2 86 residues 136 to 290 and showed that this virus expresses IE and early genes and replicates its DNA comparably to the wild type but has delayed expression of selected late genes (57). Heider and colleagues used BAC cloning to create a temperature-sensitive IE2 86 mutant virus containing the point mutation C509G (C510G in AD169) and showed that it is able to transactivate the UL112-113 promoter at 32.5°C, but not at 39.5°C. Additionally, it exhibits increased transcription from IE loci (28).

For this work, we have constructed and characterized four HCMV recombinant viruses with the following mutations in the IE2 86 gene: internal deletions of aa 356 to 359, 427 to 435, or 505 to 511 or substitutions of alanine for arginine at positions 356, 357, and 359. These mutations were selected on the basis of the IE2 86 domain mapping and functional studies discussed above and are all located in the C-terminal region that is important for protein-protein interactions and DNA binding. The IE2 86Δ356-359 and IE2 86R356/7/9A mutations remove or disrupt amino acids implicated in the activation of the UL112-113 and UL54 promoters, while the zinc finger and helix-loop-helix motifs are disrupted by the IE2 86Δ427-435 and IE2 86Δ505-511 mutations, respectively. Although none of the mutants are viable, they display differential phenotypes. We found that these mutants have varying degrees of increased IE1 72 and IE2 86 expression compared to the wild-type virus at early times postinfection. The IE2 86Δ356-359 mutant shows wild-type levels of IE1 72, IE2 86, and early gene products, but it is defective in the expression of late genes. IE2 86Δ427-435 and IE2 86Δ505-511 express increased levels of selected late transcripts at early times but do not express early gene products.

MATERIALS AND METHODS

Cells. Human foreskin fibroblasts (HFFs) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 200 U of penicillin, 200 μg of streptomycin, 1.5 μg of amphotericin B, and 50 μg of gentamicin per ml and were grown as described previously (69).

Molecular cloning. Construction of the shuttle plasmids for use in the BAC mutagenesis procedure began with the plasmid pHCMV *Eco*RI J (69), which contains the HCMV strain AD169 MIE region. The 10-kbp J fragment was liberated from this plasmid by *Eco*RI digestion, gel purified, and blunt ended with Klenow enzyme. *Kpn*I linkers were ligated onto both ends of the fragment, and it was subcloned into the unique *Kpn*I site present in pST76K_SR (a gift of Martin Messerle, Ludwig-Maximilians-Universität München), resulting in the shuttle plasmid wtJ-pST76K_SR. The pST76K_SR parental plasmid contains a kanamycin resistance marker, the *sacB* gene under the control of the *lac* promoter, and a gene encoding a temperature-sensitive RecA mutant that provides recombinase activity at 30°C but not above 37°C. This plasmid also contains an origin of replication and a *rep ts* gene, which permit replication at 30°C but not above 37°C.

TABLE 1. Quantitative real-time RT-PCR primer and TaqMan probe sequences

Transcript	Sequence		
	Forward primer	Reverse primer	TaqMan probe
IE1	CAAGTGACCGAGGATTGCAA	CACCATGTCCACTCGAACCTT	TCCTGGCAGAACTCGTCAAACAGA
IE2	TGACCGAGGATTGCAACGA	CGGCATGATTGACAGCCTG	TGGCAGAACTCGGTGACATCCTCGCC
UL112-113	TGACGGACGTGGCCG	CAATCATTGAGCATTTTGGTCAA	CCGACGGAATCCGCGGCGCCTCAG
UL89	GGCGCTTTTTGCCAGTTG	ACCAGCAGCAAGTGGAAGTTTT	TACAACACCAACAGCATCCGAGGAC
R160461	CAGTCCGCCGCCCAA	AGATTTCTGTTCGTAAACTTATCCGTT	TGCGCGTCCCAGGTACCACCCGTCGA
UL77	CGTTGCCCGGGAACG	GGTGTGAAAGCGGATAAAGGG	ACCTAGCTACTTTGGAATCACGCAGAACGA
G6PD	TCTACCGCATCGACCACTACC	GCGATGTTGTCCCGGTTC	ATGGTGCTGAGATTTGCCAACAGGA

For construction of a template for site-directed mutagenesis, an 875-bp *Fse*I-*Stu*I fragment isolated from pHCMV *Eco*RI J was cloned into pFastBacI (Invitrogen, Carlsbad, Calif.). Mutagenic oligonucleotide primers were used in conjunction with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) to create four plasmids, each containing one of the following deletions or a 3-aa substitution in the UL122 open reading frame: Δ356-359, Δ427-235, Δ505-511, or R356/357/359A. The numbering used here indicates the amino acids that were removed from the IE2 86 protein, and the plasmids are pFB-86Δ356-359, pFB-86Δ427-435, pFB-86Δ505-511, and pFB-86R356/7/9A. These constructs were cycle sequenced by use of the Thermo Sequenase radiolabeled terminator cycle sequencing kit (United States Biochemical, Cleveland, Ohio) to verify the presence of the correct mutations. The sequences of the mutagenic primers (Integrated DNA Technologies, Coralville, Iowa) are as follows: for pFB-86Δ356-359, sense, 5′ GCACACCCAACGTGCAGACTGTCAA GATTGACGAGGTGAG 3′, and antisense, 5′ CTCACCTCGTCAATCTTGA CAGTCTGCACGTTGGGTGTGC 3′; for pFB-86Δ427-435, sense, 5′ GTGAA GAGTGAGGTGGATGCGGTGCTGGCCCTCTCCACTCCCTTCCTC 3′, and antisense, 5′ GAGGAAGGGAGTGGAGAGGGCCAGCACCGCATCCA CCTCACTCTTCAC 3′; for pFB-86Δ505-511, sense, 5′ GCCACCCCCGTGG ACCTGTTGGGCCTGATGCAAAAGTTTCCCAAACAG 3′, and antisense, 5′ CTGTTTGGGAAACTTTTGCATCAGGCCCAACAGGTCCACGGGGG TGGC 3′; and for pFB-86R356/7/9A, sense, 5′ CCCAACGTGCAGACTGCG GCGGGTGCCGTCAAGATTGACGAG 3′, and antisense, 5′ CTCGTCAATC TTGACGGCACCCGCCGCAGTCTGCACGTTGGG 3′.

pFB-86Δ356-359, pFB-86Δ427-435, pFB-86Δ505-511, and pFB-86R356/7/9A were digested with *Fse*I and *Stu*I, and the resulting 863-, 848-, 854-, and 875-bp fragments were gel purified. The fragments were then ligated with the 15.5-kbp fragment resulting from an *Fse*I and *Stu*I digest of wtJ-pST76K_SR to produce the four shuttle plasmids containing the 12-, 27-, and 21-bp deletions or the three substitutions in the IE2 86 coding region. These shuttle plasmids were sequenced as described above to confirm that the mutations were present and that the reading frame had been preserved.

BAC mutagenesis. Mutagenesis of the wild-type HCMV strain AD169 BAC(pHB5), obtained from M. Messerle (10), was performed by using the shuttle plasmids described above. Briefly, *Escherichia coli* strain DH10B containing pHB5 was transformed by electroporation with one of the shuttle plasmids. Transformants were selected on Luria-Bertani (LB)–chloramphenicol (30 μg/ml) or –kanamycin (30 μg/ml) plates at 30°C and then were replated onto LB-chloramphenicol and -kanamycin plates and grown at 43°C to select for bacteria in which a BAC-shuttle plasmid cointegrate had formed. The resulting clones were transferred to LB-chloramphenicol plates and grown at 30°C to allow recombination and resolution of the cointegrates. Two rounds of sucrose selection and the confirmation of kanamycin sensitivity were used to isolate mutants, and the resulting BACs (p86Δ356-359, p86Δ427-435, p86Δ505-511, and p86R356/7/9A) were sequenced as described above to confirm that each was a pure mutant containing the correct sequences.

Rescued BACs were generated from the deletion mutants by the same method. *E. coli* strain DH10B was transformed with one of the three deletion mutant BACs (p86Δ356-359, p86Δ427-435, or p86Δ505-511) and was made electrocompetent. The resulting bacteria harboring a mutant HCMV BAC were each transformed with wtJ-pST76K_SR. Clones containing a rescued BAC were isolated by the selection procedure described above, and the presence of wild-type IE2 86 sequences in the resulting BACs, p86Δ356-359rescue, p86Δ427-435rescue, and p86Δ505-511rescue, was verified by sequencing. Wild-type, mutant, and rescue BAC DNAs were amplified and purified as described previously (57). Each BAC was subjected to digestion with *Eco*RI and gel electrophoresis to ascertain that no major alterations to the DNA were sustained during the cloning procedure.

Electroporation time course with BAC viruses. HFFs (3.2×10^6) were transfected by electroporation with 2 μg of BAC DNA and 1 μg of pcDNApp71tag (a gift of Bodo Plachter, University of Mainz) for RNA isolation or with 6.25 μg of BAC DNA and 3.75 μg of pcDNApp71tag for immunofluorescence and genome quantification in a BTX ECM-600 electroporator (Genetronics, Inc.). Briefly, confluence-synchronized HFFs (56) were trypsinized, pelleted, washed in MEM plus 100 mM HEPES with no antibiotics or serum, and resuspended in the same medium at a concentration of 4×10^6 cells/ml. Eight-hundred-microliter samples of resuspended cells were added to 4-mm-gap cuvettes containing BAC DNA and pcDNApp71tag and were mixed. Cells and DNA were electroporated at 300 V, 2500 μF, and 72 Ω, and the resulting pulse lengths were typically between 26 and 28 ms. Immediately after electroporation, cells were recovered in MEM supplemented as described above plus 100 mM HEPES and were seeded into 75-cm^2 flasks or onto sterile coverslips in 12-well dishes for immunofluorescence assays (IFAs). Mock-treated cells received the pp71 expression vector alone. One, 3, 6, and 9 days postelectroporation, cells were harvested from flasks for RNA analysis or fixed on coverslips for IFAs.

Quantitative real-time RT-PCR. Total RNA was isolated from cell pellets by using a NucleoSpin RNA II kit (Clontech, Palo Alto, Calif.) according to the manufacturer's instructions. The concentration of each sample was determined by UV spectrophotometry. Quantitative real-time reverse transcription (RT)-PCR was performed in an Applied Biosystems ABI Prism 7700 sequence detection system, using the TaqMan One-Step RT-PCR master mix reagents kit (Applied Biosystems), oligonucleotide primers, and TaqMan dual-labeled (5′-FAM and 3′-black hole quencher) probes (Integrated DNA Technologies, Coralville, IA) designed with Primer Express software (Applied Biosystems) (Table 1). Each probe spanned a splice junction in the transcript of interest. RT-PCRs contained 50 ng of total RNA each and were performed in duplicate. Additionally, the RNA isolated at 1 day postelectroporation from cells that received pHB5 BAC DNA was used to generate a standard curve for each gene examined. The standard curve was used to calculate the relative amount of specific RNA present in a sample, from which the fold induction of transcription of the gene was calculated by comparison to wile-type values at 1 day postelectroporation. As an additional control for the inclusion of equal amounts of RNA in each reaction, samples were analyzed with primers and a TaqMan probe specific for the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD).

Normalization of transfected viral genomes. At 24 h postelectroporation, cells seeded on coverslips were washed in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde in PBS, and then stained for MIE protein expression as described below. Cells in flasks were harvested and nuclear DNA was isolated as previously described (63). Fifty nanograms of nuclear DNA was analyzed by quantitative real-time PCR with primers and a TaqMan probe specific for the unspliced UL77 gene. The ratio of viral DNA recovered to the number of IE-expressing cells by immunofluorescence varied less than twofold among the transfected BACs tested.

Immunofluorescence. At 24 h or 9 days postelectroporation, cells seeded on coverslips were washed in PBS and fixed in 2% paraformaldehyde in PBS. Cells were permeabilized in 0.2% Triton X-100 and stained as previously described (57). After 30 min of blocking in 10% normal goat serum in PBS, cells were incubated with a primary antibody in 5% normal goat serum at the following dilutions: CH16.0 monoclonal antibody (MAb) specific for the region shared by IE1 72 and IE2 86, 1:1,000; IE1 72-specific MAb p63-27, 1:500; IE2 86-specific MAb IE 2.9.5, no dilution; IE2 86 rabbit polyclonal antibody 1218, 1:250; UL112-113 MAb m23, 1:5; UL44 MAb, 1:1,000; and pp28 MAb 41-18, no dilution. Monoclonal antibodies p63-27, 2.9.5, and 41-18 were gifts from William Britt (University of Alabama, Birmingham), m23 was a gift from Masaki Shirakata (Tokyo Medical and Dental University), and the polyclonal antibody directed against IE2 86 was a gift from Jay Nelson (Oregon Health Sciences University). The CH16.0 and anti-UL44 antibodies were purchased from the Goodwin Institute (Plantation, Fla.). After three washes in PBS, coverslips were incubated with Hoechst and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse

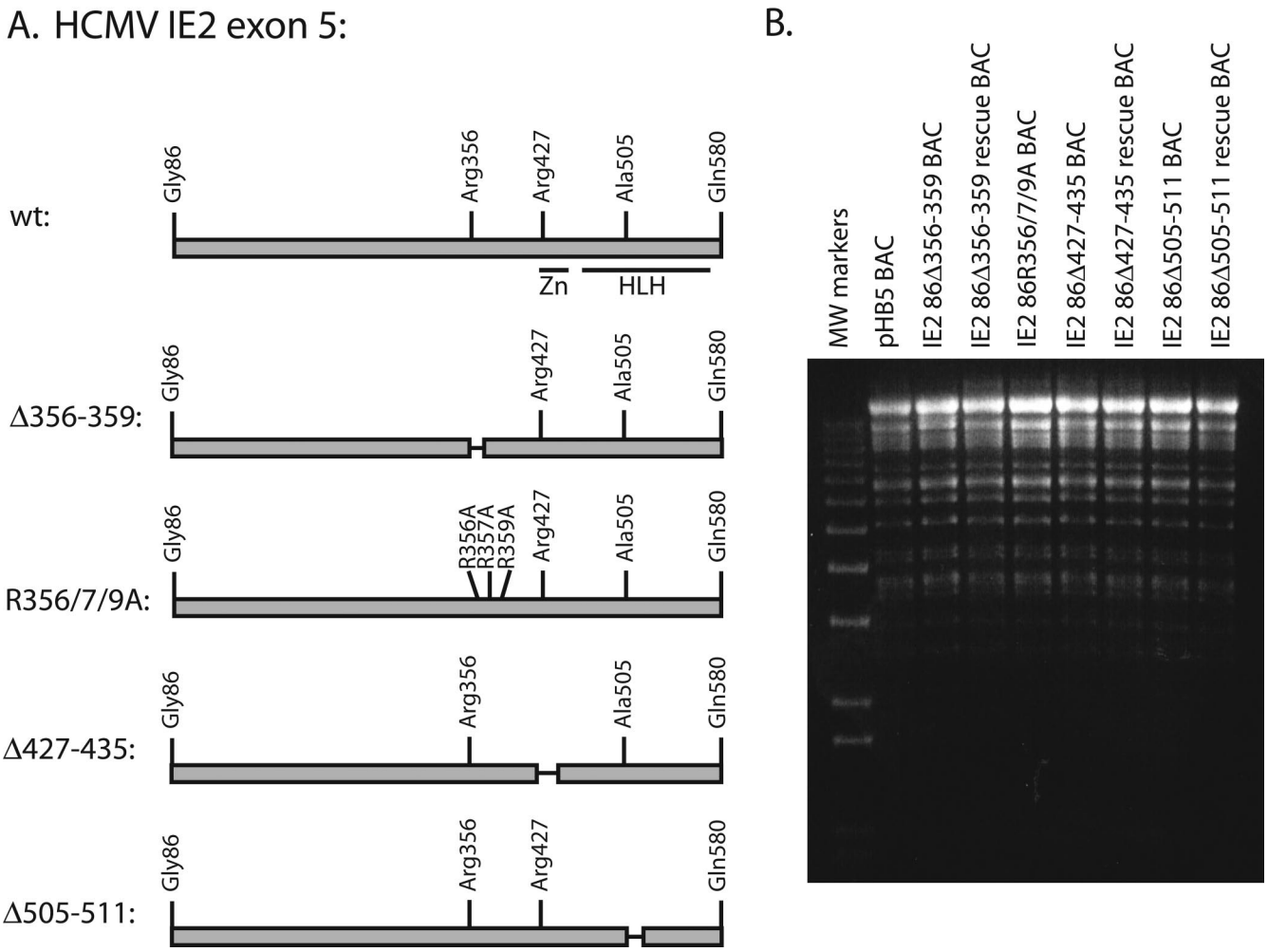

FIG. 1. Construction of the HCMV IE2 86 deletion mutant BACs. (A) Schematic of exon 5 of the HCMV MIE region. For construction of the four mutants, IE2 86Δ356-359, IE2 86R356/7/9A, IE2 86Δ427-435, and IE2 86Δ505-511, regions of the exon were deleted or amino acids were substituted as shown. Zn, zinc finger domain; HLH, helix-loop-helix motif. (B) *Eco*RI digests of the HCMV BAC clones. Three micrograms of wild-type (pHB5), mutant, or rescued mutant BAC DNA was cut with *Eco*RI, and the resulting digests were separated on a 0.6% agarose gel in 1× Tris-borate-EDTA. MW, molecular weight markers.

immunoglobulin G (IgG) (Southern Biotech, Birmingham, Ala.) (for CH16.0, UL112-113, UL44, and pp28 stains), FITC-conjugated goat anti-mouse IgG2a and tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse IgG3 (for IE1 and IE2 stains of IE2 86Δ356-359 and IE2 86Δ505-511 BAC-electroporated cells), or TRITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (for IE1 and IE2 stains of IE2 86Δ427-435 BAC-electroporated cells), diluted 1:75 to 1:200 (Jackson ImmunoResearch Laboratories, West Grove, Pa). Coverslips were washed and mounted onto slides with SlowFade antiphotobleaching reagent (Molecular Probes, Eugene, Oreg.). Images were captured with a Nikon Eclipse E800 microscope and a Photometrics CoolSnap fx charge-coupled device camera (for IE2 86Δ427-435 electroporations) or with a Leica DMRB microscope and a Hammamatsu C5810 Color 3CCD camera (for IE2 86Δ356-359 and IE2 86Δ505-511 electroporations) by use of Metamorph software (Universal Imaging Corp., Downington, PA) and were processed with Adobe Photoshop.

RESULTS

Recombinant HCMV mutant and rescue BAC production. To construct the four mutant BACs, we used site-directed mutagenesis to make in-frame deletions of aa 356 to 359, 427 to 435, and 505 to 511 from the coding region of IE2 86 or mutations of the arginines at positions 356, 357, and 359 to alanines (Fig. 1A). We chose to focus on deletions rather than amino acid substitutions to reduce the possibility of mutants reverting to the wild-type sequence, as we find that in our hands, substitution mutants with changes in the IE2 86 sequence rapidly undergo reversion at one or more of the altered sites. While the parent BAC used in this study was derived from HCMV strain AD169, the numbering we used reflects the more commonly used Towne amino acid sequence, which differs from AD169 primarily by the deletion of one residue in the set of serines at aa 258 to 264 (7). The intermediate constructs were sequenced to confirm the presence of the correct deletion and preservation of the reading frame, and a portion of the IE2 gene containing the deletion was then cloned into the shuttle vector wtJ-pST76K_SR in place of the corresponding wild-type sequence. *E. coli* strain DH10B containing the wild-type HCMV BAC pHB5 was transformed with a shuttle vector, and

mutant BACs were isolated as described in Materials and Methods. The BACs were isolated and checked for the presence of the correct sequences by DNA sequencing. A comparison of the *Eco*RI restriction digest patterns of the newly constructed mutants to that of pHB5 was also used to confirm that no large-scale rearrangements of the BAC DNA had occurred during the cloning procedure (Fig. 1B). To confirm that the phenotypes observed in this study were not due to mutations that had occurred elsewhere in the HCMV genome, we rescued the deletion mutant BACs, returning each to the wild type as described in Materials and Methods.

Each of these deletions removes or disrupts a region predicted to be needed by IE2 86 to transactivate downstream promoters. The aa 356 to 359 deletion removes a region that has been shown to be important for the activation of the viral early promoter for the UL54 gene (65), and the arginine-to-alanine substitution mutant similarly disrupts this region without introducing a deletion that might result in a large-scale alteration of the IE2 86 structure. Removal of aa 427 to 435 disrupts a predicted zinc finger, which when altered in other studies resulted in a protein that can activate transcription of the MIE region but cannot bind DNA or autorepress transcription (34, 42). Finally, the aa 505 to 511 deletion mutant is missing part of a leucine-rich region that is predicted to form a helix-loop-helix structure (21).

To determine the effects of these deletions on the viability of the virus, we transfected the wild-type BAC, mutant BACs, and BACs generated by rescue of the deletion mutants into HFFs along with a pp71 expression construct and observed the cultures for the development of a cytopathic effect. Beginning at 6 days postelectroporation, plaques were visible in cultures that received the wild type or each of the rescued BACs. In contrast, plaques were not observed in cultures transfected with any of the mutants for several repetitions of the experiment. Since this result indicates that the residues that were deleted from IE2 86 are required for a productive infection, we proceeded to determine at what stage during infection was the life cycle of each of these mutants blocked.

IE gene expression is increased in IE2 86 mutant-electroporated cells. To investigate the kinetics of IE gene expression from the IE2 86 deletion mutants, we electroporated the wild-type, mutant, and rescued BACs into G_0 confluence-synchronized primary human fibroblasts along with a pp71 expression vector to promote viral replication. A mock sample received the pp71 construct alone. Total RNAs were isolated from cells harvested at 1, 3, 6, and 9 days postelectroporation, and IE1 and IE2 transcript levels were measured by quantitative real-time RT-PCR. Each RT-PCR was performed in duplicate and standardized to threshold cycle values obtained for reactions containing a known amount of RNA. In addition, RNA samples were analyzed by real-time RT-PCR with G6PD-specific primers and probe to ensure that RNA concentrations in all samples were approximately equal (Fig. 2C). Rescued viruses behaved essentially as the wild type did for each target analyzed by RT-PCR or stained by immunofluorescence.

All three mutants displayed, to various degrees, upregulation of IE1 72 and IE2 86 transcripts at early times postelectroporation. The IE2 86Δ356-359 and IE2 86R356/7/9A mutants showed a pattern of IE1 72 gene expression that was most similar to that of the wild type at early times postelectroporation (Fig. 2A). Both mutants expressed three to four times more IE1 72 1 day after electroporation than the wild-type and IE2 86Δ356-359 rescue viruses did, and the two mutants continued to express slightly more IE1 72 transcript than the wild type 3 days after electroporation. After 6 days, visible plaques indicated that the wild-type and IE2 86Δ356-359 rescue viruses had spread to infect neighboring cells in the second round of infection. IE1 72 transcript levels increased to approximately 12 times their day 1 values as a result of this spread. In contrast, IE1 72 expression from the IE2 86Δ356-359 and IE2 86R356/7/9A mutants decreased after day 3 postelectroporation, consistent with the lack of spread of these two recombinants.

The pattern of IE1 72 gene expression by the IE2 86Δ427-435 and IE2 86Δ505-511 mutants was similar, but was upregulated more compared to the wild type than was that of the aa 356 to 359 region mutant 1 day after electroporation of the cells. Specifically, the mutants expressed approximately 10-fold more IE1 72 at this time (Fig. 2A). IE1 72 expression in the cells that received these mutant BACs then fell to levels approximating those in cells with the wild-type and rescue viruses by day 3 and continued to decrease for the duration of the time course. In contrast, IE1 72 transcript levels in the cells that were electroporated with wild-type and rescue viruses were induced 75- to 300-fold over their day 1 levels by the end of the time course.

IE2 expression by the four mutants and the corresponding rescue viruses was characterized similarly after electroporation into HFFs (Fig. 2B and data not shown). Like that of IE1 72, IE2 86 expression 1 day after electroporation was slightly elevated in the cells that received IE2 86Δ356-359 (8-fold upregulation) or IE2 86R356/7/9A (2.5-fold upregulation) relative to those that received the wild-type and rescue viruses. The IE2 86Δ427-435 and IE2 86Δ505-511 mutants similarly produced 14 to 24 times more IE2 RNA at this time than did the wild-type virus. IE2 expression fell to wild-type levels by day 3 postelectroporation for all four viruses and then decreased or remained level throughout the time course.

To determine the transfection efficiencies and relative expression levels of IE1 and IE2 in individual cells, we further examined the cultures by IFAs with antibodies specific for IE1 and IE2. At 1 day postelectroporation, single IE1- and IE2-positive cells were present in cultures that were electroporated with each of the IE2 deletion mutants (Fig. 3 and data not shown). The number of positive cells was approximately equal to the number of IE1- and IE2-positive cells electroporated with the wild-type BAC. By the end of the time course, IE1- and IE2-positive plaques were evident in wild-type-transfected cultures. In cells that were electroporated with the IE2 mutants, only single IE1- and IE2-positive cells were observed, indicating that no spread of the virus from the first transfected cell to neighboring cells had occurred (Fig. 4).

As an additional control to ensure that the changes in gene expression observed in mutant BAC-electroporated cells were not due to variations in transfection efficiency, we performed test transfections of each of the wild-type or deletion mutant BACs and harvested cells for isolation of nuclear DNA and for IFA at 24 h postelectroporation, before replication of the viral genome had begun. Equal amounts of DNA isolated from the nuclei of wild-type- or deletion mutant-electroporated cells were used as templates in quantitative real-time PCRs (data not shown) with primers and probes specific for the portion of

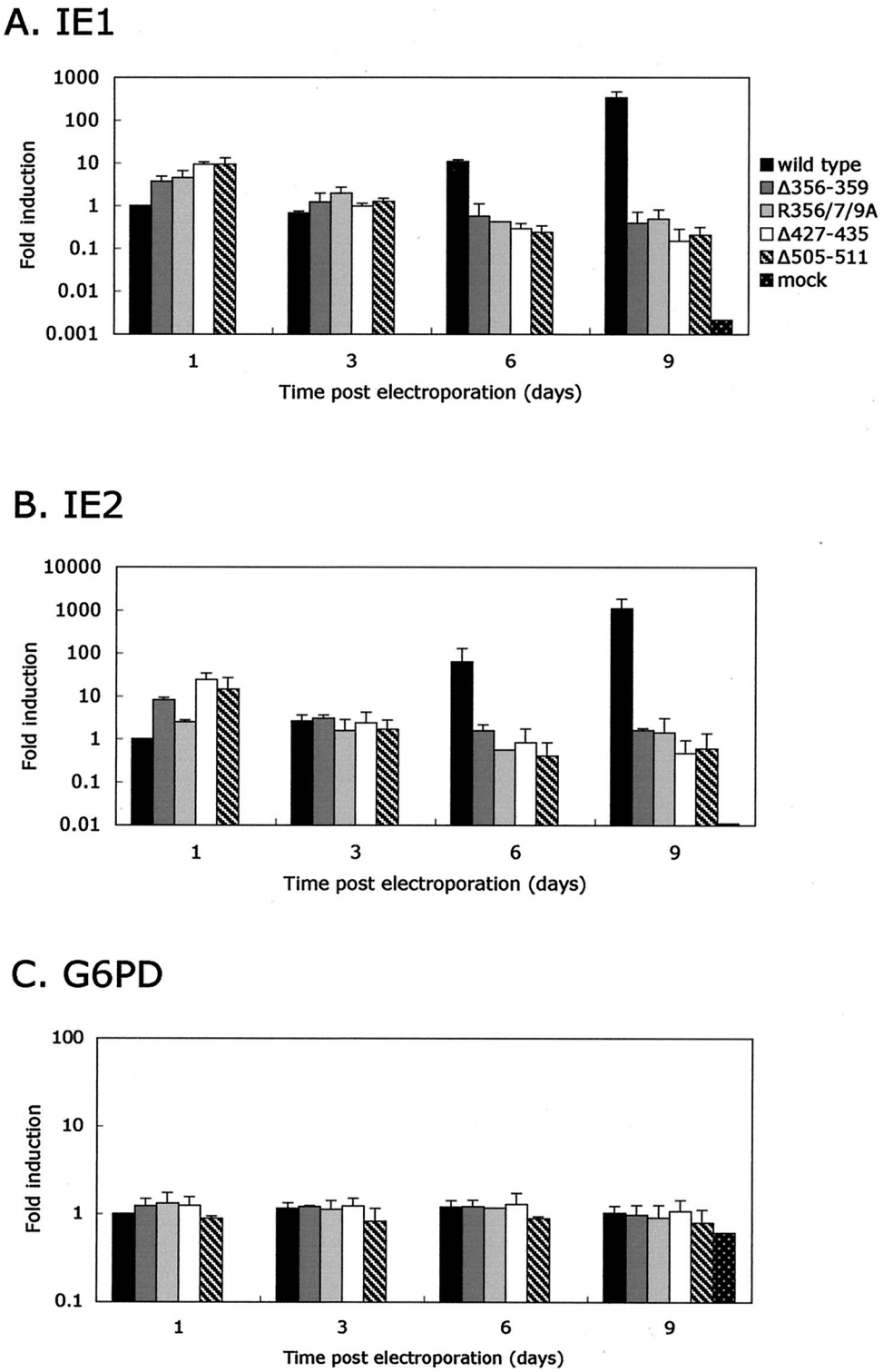

FIG. 2. Increased IE1 and IE2 transcription at IE times in cells electroporated with the IE2 86 mutant BACs. G_0-synchronized HFFs were electroporated with pHB5, IE2 86Δ356-359, IE2 86R356/7/9A, IE2 86Δ427-435, or IE2 86Δ505-511 BAC and pcDNApp71tag and were harvested at the indicated time points postelectroporation. Total cellular RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the presence of IE1 72 (A) or IE2 86 (B) transcripts. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate 1 standard deviation from the means of the combined experiments. To ensure that an equal amount of RNA was included in each reaction, we analyzed samples with G6PD-specific primers and probe (C).

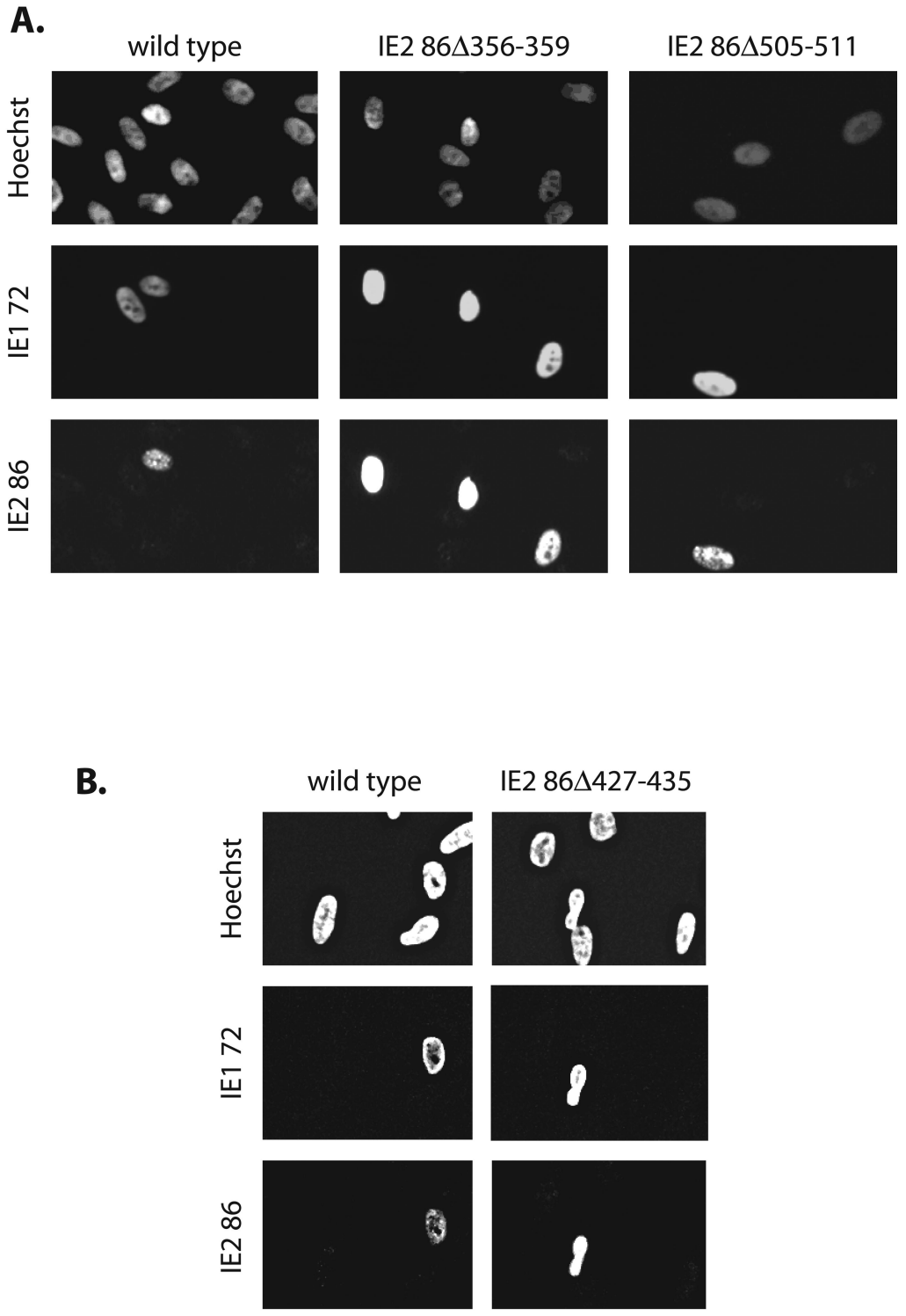

FIG. 3. IE1 72 and IE2 86 expression in wild-type- and IE2 86 mutant BAC-electroporated cells at 1 day postelectroporation. Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359 (A), IE2 86Δ505-511 (A), or IE2 86Δ427-435 (B), and 24 h later they were fixed with 2% paraformaldehyde in PBS. Cells were stained with Hoechst dye to visualize nuclei, anti-IE1 72 monoclonal antibody p63-27, and either anti-IE2 86 monoclonal antibody IE 2.9.5 (A) or anti-IE2 86 rabbit polyclonal antibody (B), followed by appropriate FITC- or TRITC-conjugated secondary antibodies (see Materials and Methods). Magnification, ×400.

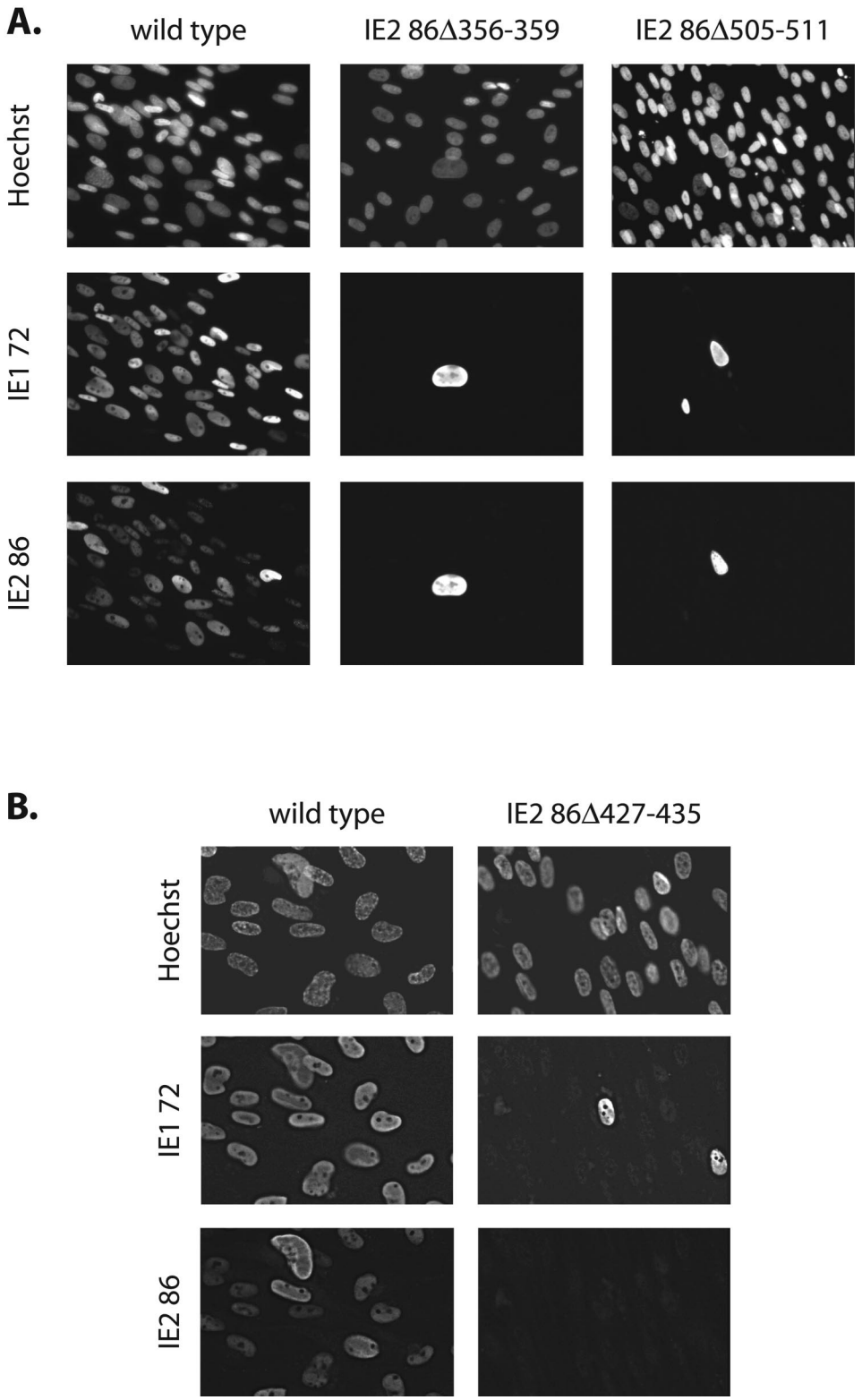

FIG. 4. IE1 72 and IE2 86 expression in wild-type- and IE2 86 mutant BAC-electroporated cells at 9 days postelectroporation. Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359(A), IE2 86Δ505-511 (A), or IE2 86Δ427-435 (B), and 9 days later they were fixed with 2% paraformaldehyde in PBS. Coverslips were processed as described above (in the legend for Fig. 3 and Materials and Methods). Magnification, ×400.

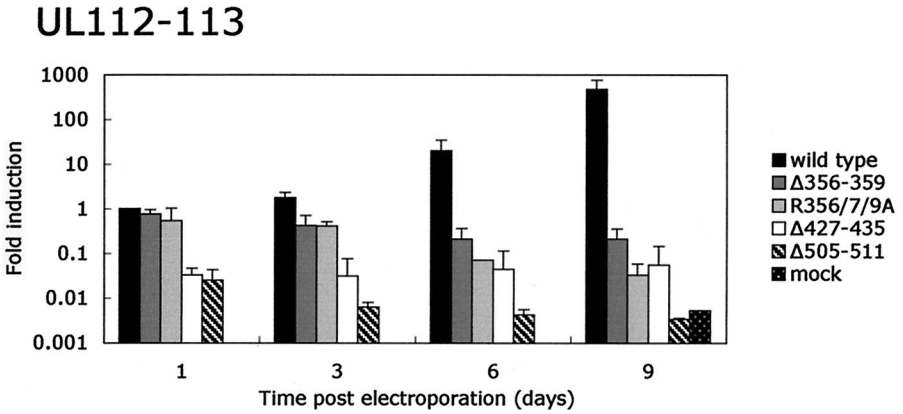

FIG. 5. Reduced UL112-113 transcription in IE2 86 mutant BAC-electroporated cells. Total cellular RNAs were isolated from electroporated cells as described in Materials and Methods and the legend for Fig. 2 and were analyzed by quantitative real-time RT-PCR with UL112-113-specific primers and a TaqMan dual-labeled probe. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate 1 standard deviation from the means of the combined experiments.

the HCMV genome encoding the unspliced UL77 gene. Cells fixed for immunofluorescence were stained with an antibody detecting the region common to the IE1 72 and IE2 86 proteins, and the number of cells expressing IE proteins by IFA was compared to the relative amount of the transfected viral genome determined by quantitative PCR. The ratio of DNA transfected to the number of IE-positive cells varied less than twofold among the four viruses tested, indicating that transfection efficiency does not significantly impact the observed changes in viral gene expression.

Early gene expression levels are low in IE2 86Δ356-359- and IE2 86R356/7/9A-transfected cells and are severely reduced in IE2 86Δ427-435- and IE2 86Δ505-511-transfected cells. Transcription of the UL112-113 region, which is expressed with early kinetics, was also measured by using real-time RT-PCR (Fig. 5). The IE2 86Δ356-359 and IE2 86R356/7/9A mutants expressed near-wild-type levels of UL112-113 transcripts 1 day after electroporation. After day 3, wild-type and rescue virus transcript levels increased dramatically, while in cells electroporated with either mutant, the amount of UL112-113 transcript decreased after day 1 and remained low throughout the time course. In contrast, in cells electroporated with IE2 86Δ427-435 and IE2 86Δ505-511, the amount of UL112-113 RNA present after 1 day was >10-fold less than that in cells electroporated with the wild-type or rescued BAC DNA. By day 3 or 6, UL112-113 induction remained approximately constant or had decreased further in IE2 86Δ427-435- and IE2 86Δ505-511-electroporated cells. In contrast, the wild-type and rescue viruses had spread rapidly and progressed through a second round of replication, as demonstrated by the 2- to 3-log induction of UL112-113 expression in each of these cultures.

To examine early gene expression in the individual cells following electroporation with the mutant BACs, we used immunostaining directed against the UL112-113 and UL44 proteins. Consistent with the UL112-113 RT-PCR data, we observed UL112-113 expression by IFA in a few IE2 86Δ356-359-transfected cells 9 days after electroporation, but not in cells that received IE2 86Δ427-435 or IE2 86Δ505-511 BAC DNA (Fig. 6A and data not shown). The pattern of expression of UL112-113 in cells that were electroporated with the IE2 86Δ356-359 mutant was comparable to that in cells that were electroporated with the wild-type BAC, but remained restricted to single isolated cells due to the inability of the mutant virus to spread to neighboring cells. Similarly, UL44-positive plaques were evident in cells 9 days after electroporation with wild-type BAC, but UL44 expression was restricted to single isolated cells following electroporation with IE2 86Δ356-359 and was not observed after electroporation with the other deletion mutants (Fig. 6B and data not shown). IE2 86Δ356-359 DNA replication in BAC-transfected cells, however, was not detectable by slot blot analysis (data not shown).

Delayed early and late gene expression is increased at early times and decreased at late times in IE2 86Δ427-435- and IE2 86Δ505-511-electroporated cells. The expression of UL89, the DNA terminase that is involved in viral genome maturation, begins at early times and increases through late times postinfection. The R160461 gene is expressed exclusively with late kinetics, and both the UL89 and R160461 transcripts are spliced (54). We measured the induction of UL89 and R160461 transcripts in cells that were electroporated with each of the four IE2 mutant BACs at various times postelectroporation. As seen previously for the characterization of IE and early gene expression, the IE2 86Δ356-359 and IE2 86R356/7/9A mutants were most similar to the wild type, with slightly elevated levels of expression of both genes 1 day after electroporation (Fig. 7). These mutants showed variable increases in the amounts of both UL89 and R160461 RNAs between days 1 and 3 and then maintained this level throughout the time course. In contrast, by the end of the time course in cells electroporated with the wild-type BAC, the levels of UL89 and R160461 transcripts were nearly 1,000- or 10,000-fold higher than at day 1. This strong induction results from normal late gene expression in addition to the spread of the wild-type and rescue viruses through the cultures.

Surprisingly, at 1 day postelectroporation of the IE2 86Δ427-435 or IE2 86Δ505-511 mutant, UL89 and R160461 expression levels were 11- to 21-fold higher than those observed in cells electroporated with the wild-type and rescued viruses. This suggests that these mutants either activate or fail to repress expression of these genes at very early times in the viral life

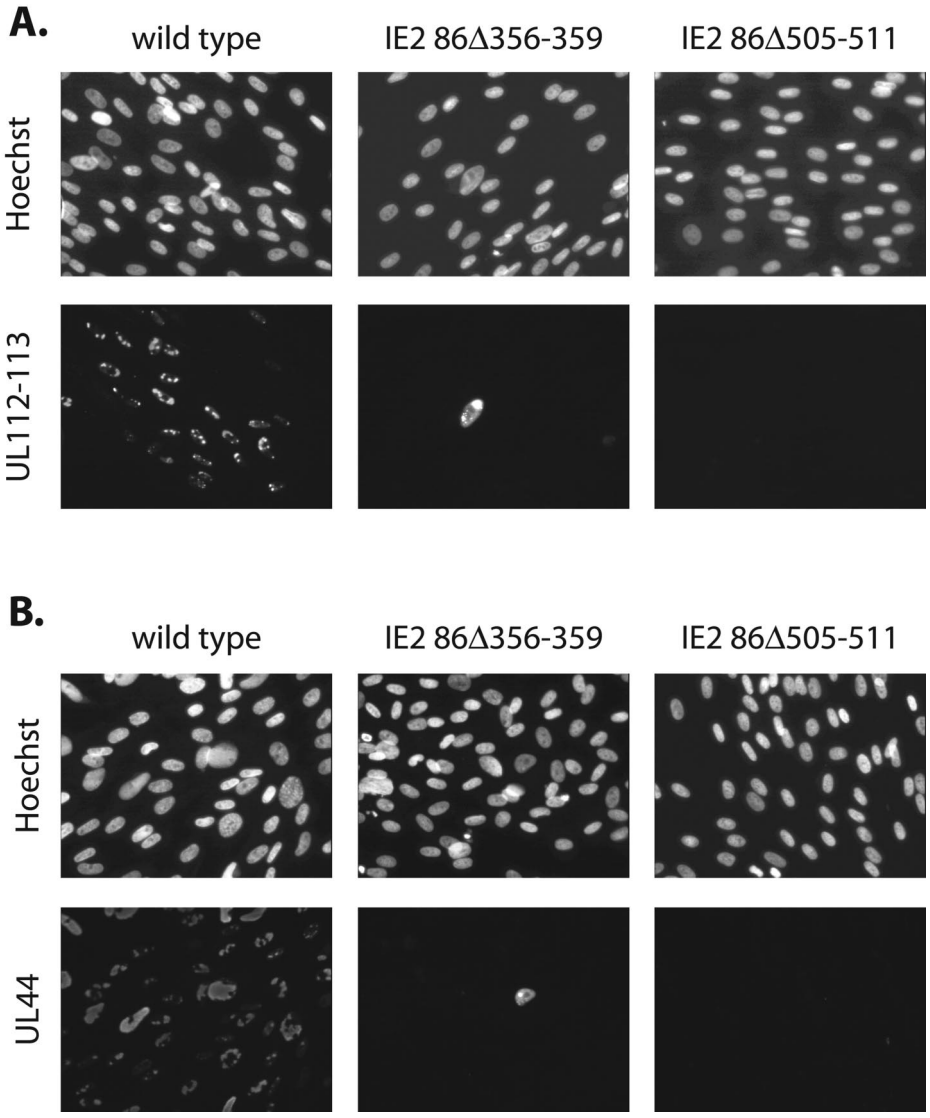

FIG. 6. UL112-113 and UL44 expression in IE2 86Δ356-359 BAC-electroporated cells. Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359, or IE2 86Δ505-511, and 9 days later they were fixed with 2% paraformaldehyde in PBS. Coverslips were stained with Hoechst dye to visualize nuclei and with anti-UL112-113 monoclonal antibody m23 (A) or anti-UL44 monoclonal antibody (B) followed by FITC-conjugated anti-mouse secondary antibody. The blank fields for UL112-113 and UL44 staining of IE2 86Δ505-511-electroporated cells are included for comparison and are representative of the lack of UL112-113- and UL44-positive cells that were electroporated with either the IE2 86Δ427-435 or IE2 86Δ505-511 BAC. Magnification, ×400.

cycle (Fig. 7). The presence of a consensus CRS element in the UL89 promoter suggests that the defect may be a lack of repression, and this will be discussed below. In contrast to case with the aa 356 to 359 region recombinant viruses, as the time course progressed, these deletion mutants did not maintain elevated levels of UL89 and R160461 transcripts. Rather, the concentrations of the UL89 and R160461 RNAs decreased gradually throughout the experiment, in each case approaching the wild-type day 1 levels of expression by the last time point examined. In the case of all four mutant viruses, both transcripts were still clearly present above the limit of detection when compared to the uninfected control at 9 days postelectroporation.

We also assessed the expression of pp28, which is encoded by UL99 and expressed with late gene kinetics. Because there are multiple colinear transcripts from this region, we used immunofluorescence to examine its expression (1). While pp28 was strongly expressed in wild-type electroporated cells at 9 days postelectroporation, none of the mutant viruses can support pp28 expression, even in single cells (Fig. 8 and data not shown). This is consistent with the finding that gene expression by all of these mutants is blocked before replication of the viral DNA.

DISCUSSION

The HCMV IE2 86 protein has been extensively characterized in efforts to better understand its role as an essential regulatory factor in the viral life cycle. Much of this work has been performed in vitro, using mutational analysis to define regions of the protein that are required for the transcription of other viral genes. These studies showed that both protein-

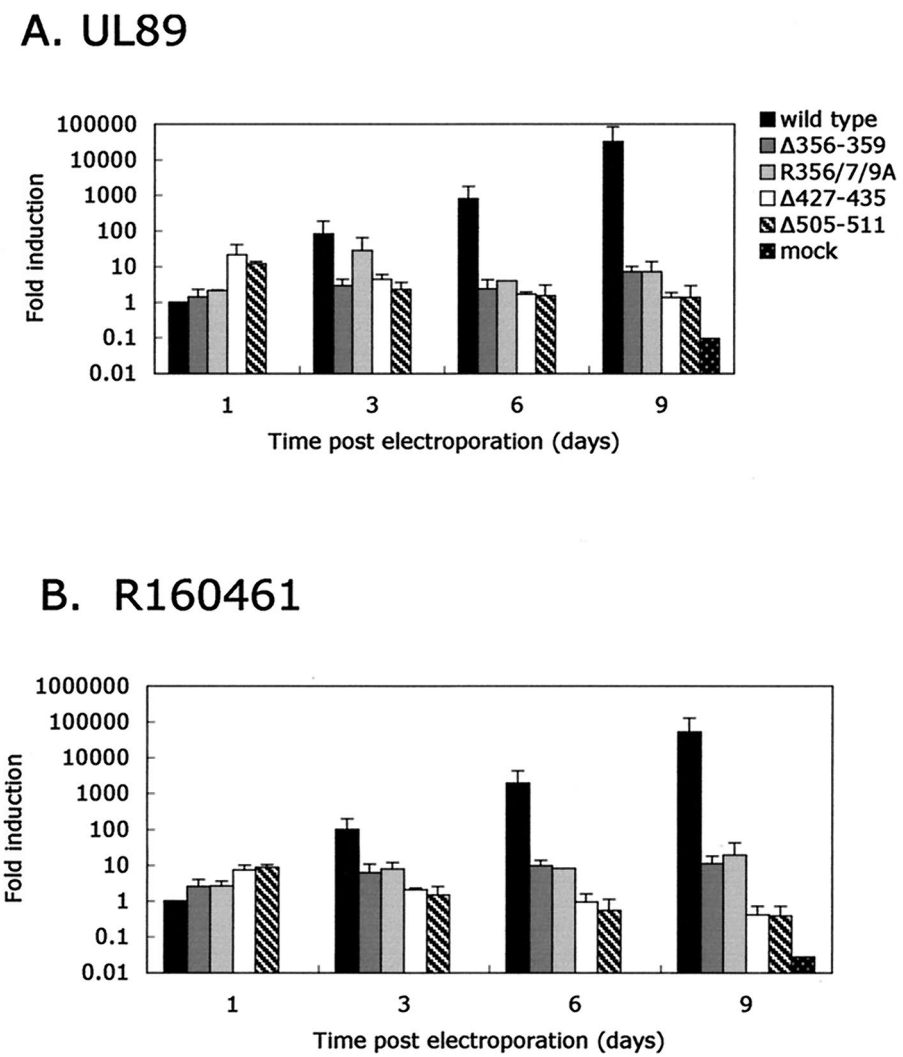

FIG. 7. UL89 and R160461 transcription is increased at IE times in mutant BAC-electroporated cells. Total cellular RNAs were isolated from electroporated cells as described in Materials and Methods and the legend for Fig. 2 and were analyzed by quantitative real-time RT-PCR with UL89 (A)- or R160461 (B)-specific primers and TaqMan dual-labeled probes. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate 1 standard deviation from the means of the combined experiments.

protein and protein-DNA interactions are involved in IE2 86's transactivation capabilities, but the assays were performed with either bacterially expressed, purified IE2 86 protein or IE2 86 expression vectors in transient transfection experiments. In either case, the mutant protein was not examined in the context of the infected cell. We have taken advantage of the cloning of the HCMV genome as a BAC to construct recombinant viruses with mutations in the IE2 86 gene residing in the viral genome. The mutations remove aa 356 to 359, 427 to 435, and 505 to 511 or mutate arginines at residues 356, 357, and 359 to alanines and were chosen based on previous studies from our laboratory and others suggesting that these regions are particularly important for the ability of IE2 86 to transactivate other viral promoters. The region near aa 356 to 359 has been shown to affect both the transactivation and autoregulatory functions of IE2 86. In transient transfection assays, the insertion of four residues between aa 359 and 360 both reduces the ability of IE2 86 to transactivate the UL54 (DNA polymerase) promoter and limits its capacity to repress its own promoter (65). Similarly, an IE2 86 mutant in which the three residues from aa 358 to 360 are changed to an alanine, serine, and alanine has been examined by use of transient assays, in vitro work, and yeast one- and two-hybrid systems. This mutant appears able to dimerize but has little to no DNA binding capacity (as assayed by an electrophoretic mobility shift assay and yeast one-hybrid experiments, respectively) and does not repress transcription from the MIEP in transient transfection assays (3, 71).

The deletion of aa 427 to 435 removes residues involved in the formation of a predicted zinc finger, while the removal of aa 505 to 511 disrupts a putative helix-loop-helix. The zinc finger has been altered previously by the mutation of histidines 446 and 452 to leucine, resulting in a protein which can neither repress transcription of the MIEP in vitro nor bind DNA in an electrophoretic mobility shift assay (42). The helix-loop-helix region was recently analyzed by the mutation of cysteine 509 to glycine (C510G in AD169) in an HCMV BAC, resulting in a

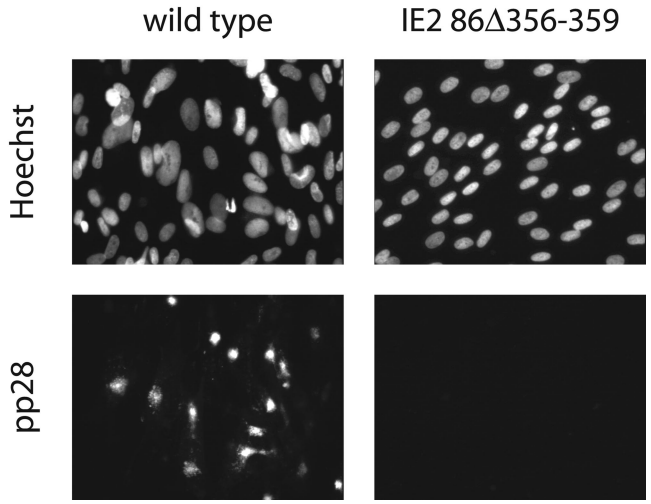

FIG. 8. pp28 expression in mutant BAC-electroporated cells. Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5 or IE2 86Δ356-359, and 9 days later they were fixed with 2% paraformaldehyde in PBS. Coverslips were stained with Hoechst dye to visualize nuclei and with anti-pp28 monoclonal antibody 41-18 followed by FITC-conjugated anti-mouse secondary antibody. The blank field indicating the lack of pp28 staining in the IE2 86Δ356-359-electroporated cells is included for comparison and is representative of pp28 staining in cells electroporated with the IE2 86Δ427-435 or IE2 86Δ505-511 BAC. Magnification, ×400.

temperature-sensitive virus (28). At the nonpermissive temperature, cells infected with this virus contain elevated levels of IE transcripts but no detectable early (UL44 and UL54) RNAs. Correspondingly, the C509G mutant does not replicate its DNA. In all three cases, it is important to note that three of the mutants we characterized here contain internal deletions in the IE2 gene rather than the insertions or point mutations described in other reports. We chose to introduce deletions based on the observation that in our system, single and multiple point mutations in the IE2 coding region undergo spontaneous reversion to the wild type in the infected cell, but we have also included the construction and analysis of a substitution mutant, IE2 86R356/7/9A, and have shown that it behaves essentially the same as the corresponding deletion mutant. In this case, it appears that the phenotype observed is not due to a simple change in protein structure generated by deletion of these amino acids.

In the present report, we show that the three regions of IE2 86 described above are crucial to its function as a key regulator of HCMV infection. Mutation of any of these regions results in a recombinant virus that is not viable and is blocked in its replication early in the viral life cycle. To determine the locations of these blocks, we used quantitative real-time RT-PCR and IFA to examine the expression of selected IE, early, and late genes at the RNA and protein levels. To ensure that only viral RNA and not input or replicated viral DNA was detected during RT-PCR analysis, we examined the transcription of selected spliced viral genes only and used real-time PCR probes spanning the splice junctions for each target.

All four mutants upregulated IE1 72 and IE2 86 expression compared to the wild type at early times. During the first round of infection, IE2 86Δ356-359 and IE2 86R356/7/9A expressed transcripts from the IE1 and IE2 regions at slightly elevated levels relative to those observed for the wild type (Fig. 2). Immunostaining for IE1 72 and IE2 86 also indicated that the protein levels in the mutants and the wild type were comparable (Fig. 3 and data not shown). In contrast, RT-PCR analysis of UL112-113 transcripts indicated near-wild-type transcription of this locus in IE2 86Δ356-359- or IE2 86R356/7/9A-electroporated cells (Fig. 5). By IFA, both UL112-113 and UL44 were detectable in single cells that received the deletion mutant (Fig. 6). We could not, however, detect DNA replication by slot blotting (data not shown) with cells transfected with the IE2 86Δ356-359 BAC. The transcription of delayed early (UL89) and late (R160461) loci by the IE2 86Δ356-359 or IE2 86R356/7/9A mutant was induced only slightly and remained low throughout the time course, and a representative late protein (pp28) was not detectable by immunostaining in IE2 86Δ356-359-electroporated cells (Fig. 7 and 8). Taken together, these data suggest that the IE2 86Δ356-359 mutant can support IE and some early gene expression but that expression is blocked before DNA replication begins. The IE2 86R356/7/9A mutant behaved very similarly, indicating that the changes in gene expression induced by the deletion of these crucial amino acids are not simply the result of a structural alteration resulting from the deletion. These data are consistent with previous work in which this region of IE2 86, when disrupted and tested in transient assays, resulted in the decreased activation of early promoters and the 10-fold upregulation of MIE transcription (65).

IE gene expression in the IE2 86Δ427-435- and IE2 86Δ505-511-electroporated cells was also different from that observed in cells containing the wild-type virus. At the earliest time examined postelectroporation, IE1 and IE2 transcripts were >10-fold higher in cells that received the mutant BACs than in those transfected with the wild-type or rescued construct (Fig. 2). While this induction was not observed for UL112-113 transcription, a similar pattern was seen when UL89 and R160461 transcripts were examined by RT-PCR. Early in the time course, UL89 and R160461 transcription was significantly increased compared to that of the wild type. This effect was most apparent at the transcriptional level at 1 day postelectroporation, less apparent after 3 days, and not detectable later in the time course as a result of the spread of the wild-type and rescue viruses through the culture. While this phenotype was also detectable in cells transfected with the IE2 86Δ356-359 and IE2 86R356/7/9A BACs, these more N-terminal disruptions resulted in an intermediate phenotype and a less striking upregulation of selected delayed early and late genes.

Since the IE2 86Δ427-435 and IE2 86Δ505-511 mutations disrupt the zinc finger and helix-loop-helix motifs in the DNA binding domain of IE2 86, we speculate that the deleted regions, and possibly the amino acids near residue 356, are required for IE2 86 to bind the CRS. The deletion of these amino acids results in the loss of the capacity of IE2 86 to repress its own promoter and other CRS-containing promoters. This hypothesis is supported by the presence of a consensus CRS element directly upstream of the UL89 transcription start site. Many CRS elements are also located near the proposed site of initiation of R160461 transcription (54). These sequences are both upstream and downstream of the proposed TATAA box, although they do not appear to be in a location that would be expected to affect transcription. However, since the start site of the transcript is based on the sequence of a cDNA clone

generated from infected cell RNA, it is possible that R160461 transcription initiates at a nearby, but different, site and may therefore be regulated by the same mechanism. The analysis of UL83 transcription at 1 day postelectroporation provided further support for this idea. UL83 encodes the matrix phosphoprotein pp65 and is expressed with delayed early kinetics, but it lacks a CRS element in its promoter region. We observed that at early times, UL83 transcripts were present at approximately equal levels in cells electroporated with the wild type, any of the three IE2 deletion mutants, or the rescued BACs (data not shown). Although we have not examined all possible late gene targets of regulation by IE2 86, these data suggest that the presence of a CRS upstream of the promoter of a delayed early or late gene may determine whether the locus will be overexpressed at early times in the mutant-electroporated cell. There are other possibilities, including alternative regulation of spliced late genes by the mutant virus, and experiments to resolve this question are in progress.

In contrast to the data described above, UL112-113 transcription was severely reduced in cells electroporated with the IE2 86Δ427-435 and IE2 86Δ505-511 mutants. The cells electroporated with these mutants contained <10% the amount of UL112-113 RNA in the wild-type-electroporated cells 1 day after transfection, and the expression of this transcript dropped to the limit of detection by days 3 and 6 for the IE2 86Δ427-435 and IE2 86Δ505-511 mutants, respectively (Fig. 5). This result is also reflected at the protein level, since neither one of the early genes examined (UL112-113 and UL44) was detectable by immunostaining (Fig. 6 and data not shown). Therefore, the introduction of either of these mutations results in a recombinant virus that is blocked even earlier in its replication than the IE2 86Δ356-359 virus.

These results indicate that the three regions of IE2 86 examined are essential for viral replication and the progression of a productive infection. Results to date suggest that the disruption of some or all of these regions can decrease the ability of IE2 86 to bind to DNA and can result in a protein that is able to activate early promoters minimally or not at all. The IE2 86Δ356-359 mutation has a less severe phenotype than the other deletion mutants examined, as the recombinant virus containing this deletion drives some early gene transcription and supports the expression of both UL112-113 and UL44 proteins.

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