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UL69 of Human Cytomegalovirus, an Open Reading Frame with Homology to ICP27 of Herpes Simplex Virus, Encodes a Transactivator of Gene Expression

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The UL69 open reading frame of human cytomegalovirus (HCMV) is homologous to the immediate-early protein ICP27 of herpes simplex virus, an essential viral regulatory protein involved in the transition from early to late gene expression. Genes with homology to ICP27 have been detected in all subclasses of herpesviruses so far. While the respective proteins in alpha- and gammaherpesviruses have been defined as *trans*-regulatory molecules, nothing is known about these genes in betaherpesviruses. This study was therefore undertaken in order to investigate expression from the UL69 gene locus of HCMV. Northern (RNA) blot experiments revealed a complex pattern of transcripts that changed during the time course of the HCMV replicative cycle: two transcripts of 2.7 and 3.5 kb that were regulated differentially could be detected as early as 7 h after infection. However, these transcripts could not be detected in the presence of cycloheximide. Additional, larger transcripts were present exclusively at late times after infection. To analyze protein expression from the UL69 gene region, the UL69 open reading frame was expressed as a histidine-tagged protein in *Escherichia coli*. A specific antiserum was generated and used to detect the UL69 protein in HCMV-infected cells which revealed its localization within the intranuclear inclusions that are characteristic for HCMV infection. In cotransfection experiments, an HCMV true late promoter could not be activated by UL69, whereas an early promoter and several heterologous promoters were stimulated about 10-fold. Complementation studies showed that the UL69 protein cannot substitute for ICP27 in the context of the HSV infection, suggesting functional differences between these two proteins. In summary, these experiments define a novel regulatory protein encoded by HCMV that is expressed as an early-late gene and appears to exert a broad stimulatory effect on gene expression.

Human cytomegalovirus (HCMV), a member of the beta subgroup of herpesviruses, is of considerable medical importance in immunosuppressed patients and newborns. Gene expression of HCMV occurs in a cascade with at least three main phases, commonly referred to as immediate early (IE), early, and late (15, 36, 73, 74). At IE times, gene expression is restricted to a few loci of the genome (1, 29, 64, 67, 68, 77). The most abundantly expressed IE region, termed the major IE gene region of HCMV, encodes several proteins with *trans*-regulatory functions: the best characterized proteins are the 72-kDa IE-1 protein and the 86-kDa IE-2 protein that are both involved in the activation of promoters of the next temporal phase, the early phase (25, 33, 46, 65, 66). The third class of genes, the late genes, can be further classified: whereas gene expression from true late genes (also called γ-2 genes) is strictly dependent on viral DNA replication, leaky late promoters (γ-1 genes) are active prior to DNA replication and show an increase in activity at late times. While IE-1/2 gene products can efficiently activate early and some leaky late promoters of HCMV, they fail to stimulate gene expression from true late promoters which, however, are readily turned on by superinfection of transfected cells with HCMV (16, 47). This suggests that additional regulatory proteins encoded by HCMV may play a role.

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In herpes simplex virus (HSV), the immediate-early protein ICP27 has been identified as an essential viral protein that is involved in the switch from early to late gene expression. This is based on studies with ICP27 temperature-sensitive and deletion mutants which have shown that this protein is required for efficient expression of the later classes of genes (35, 41, 48, 53). In addition, cotransfection experiments have demonstrated an activation or repression of reporter genes in the presence of ICP27 (19, 41, 48, 55, 70). Although there is some experimental evidence for a transcriptional effect of ICP27 (35, 41, 57), recent results suggest that the action of ICP27 is also via a posttranscriptional mechanism: reporter gene activation is correlated with the presence of weak polyadenylation signals, whereas repression of reporter genes is correlated with the presence of introns (9, 40, 54, 57).

Genes with homology to ICP27 have been detected in all classes of herpesviruses so far. These include the IE genes BMLF1 and IE-52k of the gammaherpesviruses Epstein-Barr virus (6, 11, 12, 32) and herpesvirus saimiri (2, 42), ORF4 and UL3 of the alphaherpesviruses varicella-zoster virus (27, 39) and equine herpesvirus 1 (71), and the UL69 gene of HCMV (Fig. 1) (10). The amino acid identities among the encoded proteins range from 17 to 36%. The relationship among these genes is further emphasized by their positional conservation as parts of a conserved gene block that codes for several proteins involved in DNA replication, such as a component of the helicase-primase complex or dUTPase (2, 10). While the respective homologs of ICP27 in alpha- and gammaherpesviruses have been defined as regulators of gene expression,

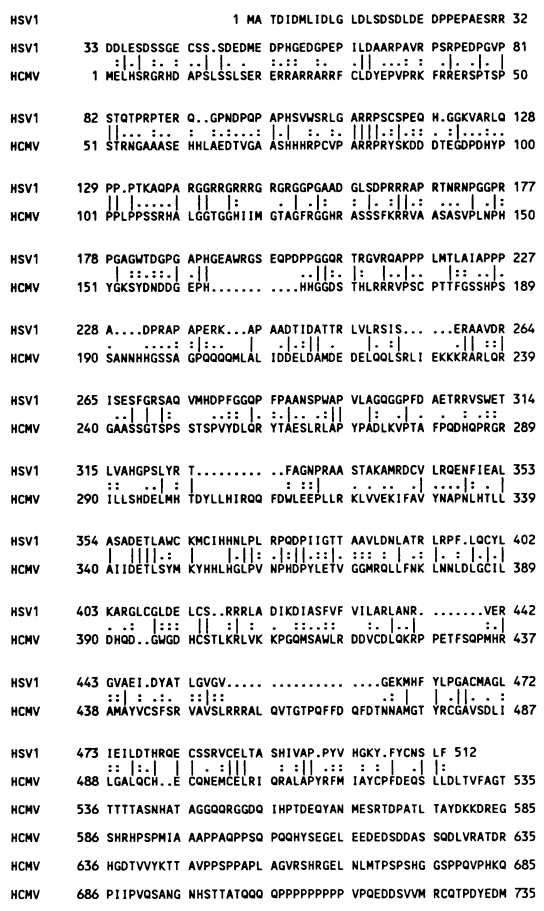

FIG. 1. Alignments of the amino acid sequences of ICP27 of HSV-1 (upper lines) and UL69 of HCMV (lower lines). The alignment shown was obtained by using the program Bestfit of the University of Wisconsin Genetics Computer Group software package. Identical residues in the two aligned sequences are interconnected by vertical lines, and similar residues are connected by dots; gaps introduced to give the alignments are indicated by dots.

nothing is known about the expression of these genes and the function of the encoded proteins in betaherpesviruses (11, 12, 14, 32, 43, 44, 58, 81). It is interesting that HCMV UL69 protein differs from its counterparts in other herpesviruses in having a unique carboxy-terminal amino acid sequence (Fig. 1).

This study was therefore aimed to investigate both RNA expression and protein expression from the UL69 gene locus of HCMV. We show that expression from UL69 occurs at both the RNA level and the protein level with early-late kinetics. The encoded protein is localized within the nucleus of both transfected and infected cells, indicating a potentially regulatory function. In cotransfection experiments, we were not able to observe transactivation of the true late UL86 promoter of HCMV. However, the early UL112 promoter and several heterologous promoters were stimulated about 10-fold. In addition, we found that UL69 could not complement ICP27 mutants of HSV. These results suggest that there are functional differences between the UL69 protein and ICP27. In summary, these experiments define a novel regulatory protein encoded by HCMV that is not expressed as an immediate-early protein.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). The following oligonucleotides (5′ to 3′ sequences) were used for cloning and PCRs: PR1, TATTAGGATCCCCATGGAGCTGCACTCAC; PR2, TGTTGGAATTCGGACAGCAATCATCACGC; PR3, ACC AGGAATTCTCAATAAGCGCCAGCATC; PR4, CGGTAT CATCGTCTTTGC; PR5, AAAGTGAGGCTCAGACGG; PR7, AACTGGATCTGCGTAACC; E1, ATAGAAGCTTC GCACAGAGGTAACAACGTG; and E2, TTCAGAGCTC CCGTGGAGCGAGTG.

PCR and plasmid constructions. PCR was used to generate specific subfragments of the UL69-UL70 gene region of HCMV. Amplification was performed by using Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) and 50 pmol of each primer. Buffer conditions were as recommended by the manufacturer. Cosmid clone pCM1029 served as the template in these reactions (21). Primers PR1 and PR2 were used to amplify the entire UL69 open reading frame (ORF). The addition of either primer pair PR1-PR3 or PR4-PR7 resulted in subfragments of the UL69-UL70 gene region. PCR cycling was accomplished with a DNA thermal cycler (Biotech, Greifenberg, Germany) under conditions described previously (61). After purification of the amplification products of PR1-PR2 and PR1-PR3 as described previously (61) and cleavage with *Bam*HI and *Eco*RI, whose recognition sequences were contained within the primers, the respective fragments were cloned into the Bluescribe vector (Stratagene, Heidelberg, Germany). The resulting constructs were designated pHM159 and pHM157, respectively. Plasmid pHM162 was generated by insertion of the *Bam*HI-*Eco*RI fragment of plasmid pHM159 into the vector pBluescript KS II (Stratagene). For procaryotic expression of UL69 as a histidine-tagged protein, a *Bam*HI-*Hin*dIII fragment of plasmid pHM162 was ligated into the *Bam*HI-*Hin*dIII-cleaved procaryotic expression vector pQE11 (Diagen, Hilden, Germany). The eucaryotic UL69 expression vector pHM160 was created by insertion of the same *Bam*HI-*Hin*dIII fragment into the vector pCB6, which contained the HCMV enhancer/promoter (5, 72) to drive gene expression and was kindly provided by M. F. Stinski (Iowa City, Iowa). Luciferase reporter plasmids with HCMV promoter sequences were constructed by ligation of the respective fragments into the *Hin*dIII-*Sac*I-cleaved luciferase expression vector p19luc (kindly provided by J. A. Nelson, Portland, Oreg.). Plasmid pHM142 contained a fragment corresponding in sequence to nucleotides (nt) −352 to +37 of the early UL112 promoter of HCMV upstream of the luciferase ORF (62). This fragment had been generated by PCR with the primer pair E1-E2. Plasmid pHM145 had the true late UL86 promoter of HCMV inserted as a *Hin*dIII-*Sac*I fragment (corresponding to nt −393 to +39 relative to the major cap site) that was isolated from plasmid pBXCAT (52). Luciferase constructs containing heterologous promoters such as pRSVluc (Rous sarcoma virus long terminal repeat [LTR] upstream of luciferase [17]), pHIVluc (human immunodeficiency virus LTR upstream of luciferase), pTKluc (thymidine kinase promoter of HSV type 1 [HSV-1] upstream of luciferase), pβactin-luc (β-actin promoter upstream of luciferase), and pPGKluc (phosphoglycerol kinase promoter upstream of luciferase) were obtained from J. A. Nelson, C. Aepinus (Erlangen, Germany), and H. Fickenscher (Erlangen, Germany), respectively. Plasmid p19luc was used to construct pHIVluc, pTKluc, and pPGKluc. All reporter plasmids contained the small t-antigen intron and early poly(A) region from simian virus 40 at the 3′ end. Plasmid pRR47 was used in cotransfec-

tion experiments to express IE-1 and IE-2 proteins of HCMV (61). To be able to generate a luciferase-specific antisense RNA for RNase protection analysis, plasmid pRSVluc was cleaved with *Nde*I and *Dra*I. After filling in the recessed ends with Klenow polymerase, the specific fragment containing the luciferase gene was inserted into the *Xba*I site of the pBluescript vector which had also been filled in with Klenow enzyme. This resulted in plasmid pHM173R. All plasmid constructs were confirmed by DNA sequence analysis with a commercially available T7 sequencing kit (Pharmacia, Freiburg, Germany).

Virus and cell culture. Primary human foreskin fibroblasts (HFFs), HeLa cells, and Vero cells were cultured as described previously (20, 48, 61). U373 MG (human glioblastoma) and COS7 cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in minimal essential medium (Gibco/BRL, Eggenstein, Germany) supplemented with 5% fetal calf serum. Infection of HFF cells with HCMV (AD169) and treatment of cells with cylcoheximide or phosphonoformic acid were performed exactly as described previously (47, 61).

RNase protection and Northern (RNA) blot analysis. Isolation of total cellular RNA and RNase protection analyses were performed as described previously (60). RNA was analyzed for UL69-specific transcripts by using a T7 RNA polymerase-generated antisense RNA probe derived from plasmid pHM157 that was linearized with *Mlu*I. This riboprobe was 231 nt in size and corresponded in sequence to 221 nt located within the 5′ region of the UL69 ORF. For detection of luciferase-specific transcripts, plasmid pHM173R was linearized with *Eco*RV and transcribed with T7 polymerase. This resulted in a riboprobe of approximately 570 nt that was homologous to approximately 360 nt of the 3′ end of luciferase RNA.

For Northern blot analysis, total cellular RNA was separated by using 1.2% formaldehyde agarose gels as described previously (61). The RNA was transferred to Hybond-N membranes (Amersham, Braunschweig, Germany), fixed to the filters by heat (2 h, 80°C), and prehybridized for at least 4 h at 43°C in prehybridization solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 5× Denhardt's solution, and 1 mg of yeast RNA per ml. As radioactive probes, double-stranded DNA fragments corresponding either to the entire UL69 ORF or to a *Pvu*II fragment of UL70 (UL70B probe) were labeled by nick translation as described previously (61). Single-strand-specific probes were generated by runoff synthesis according to Sturzl and Roth (69), using the primer PR2 and plasmid pH162 which was linearized with *Bam*HI. Alternatively, primer PR5 and the amplification product of the primer pair PR4-PR7 were used to obtain the UL70A probe. Hybridizations were performed for 24 h at 43°C in hybridization solution containing 50% formamide, 5× SSC, 20 mM sodium phosphate (pH 6.5), 1× Denhardt's solution, 500 μg of yeast RNA per ml, and the radioactive probe at 500,000 cpm/ml. The filters were washed several times after hybridization with buffer containing 20 mM sodium phosphate, 0.1% sodium dodecyl sulfate (SDS), and decreasing concentrations of SSC. For reprobing of the filters, the radioactive probe was removed by incubation for 3 h at 65°C in a buffer containing 5 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0), and 0.1× Denhardt's solution with several changes of the buffer.

Protein expression and purification, immunization, immunoblotting, and indirect immunofluorescence analysis. Procaryotic expression and purification of UL69 as a histidine-tagged protein were performed as described previously (31). The isolated protein was dialyzed against 1% SDS and stored in aliquots at −80°C. In order to generate specific antisera, rabbits were injected intramuscularly with 200 μg of protein five to seven times at 28- and/or 14-day intervals and bled after approximately 4 months. Immunoblotting was performed essentially as described previously (31). Briefly, proteins were separated by SDS-8% polyacrylamide gel electrophoresis (SDS-8% PAGE) and electrophoretically transferred from acrylamide gels to nitrocellulose filters. In order to diminish nonspecific reactions, rabbit antisera were preincubated with total *Escherichia coli* lysate that had been transferred to nitrocellulose filters. Blocking of filters and immunostaining were done as described previously except that goat anti-rabbit antibodies coupled with alkaline phosphatase (Dianova, Hamburg, Germany) were used for staining (31). For indirect immunofluorescence, cells were fixed with methanol, and a 1:200 dilution of rabbit antisera was layered over the cells for 30 min at 37°C. Nonspecific binding as observed with infected HFF cells was blocked by preincubation with 12.5% horse serum for 1 h at 37°C. Fluorescein-conjugated anti-rabbit immunoglobulin (Dako GmbH, Hamburg, Germany) (1:40 dilution) was added for 30 min. After each incubation step, cells were washed extensively with phosphate-buffered saline.

Transfection of cells and luciferase assays. COS7, HFF, HeLa, and U373 cells were plated the day before transfection either in 60-mm-diameter plastic dishes at 4×10^5 cells per dish or in 100-mm-diameter plastic dishes at 1.2×10^6 cells per dish. DNA transfection was performed by the calcium phosphate precipitation method as described previously (4). Routinely, 5 μg of the luciferase target genes and 2 μg of the cotransfected plasmids were employed. For the establishment of stably transfected U373 cell lines, G418 (Gibco/BRL) was added 48 h after transfection at a concentration of 500 μg/ml. Four weeks later, individual colonies were isolated and analyzed for UL69 protein expression by indirect immunofluorescence.

For luciferase assays, cells were washed twice with phosphate-buffered saline, harvested in 1 ml of extraction buffer containing 100 mM potassium phosphate (pH 7.8) and 1 mM dithiothreitol, and collected by low-speed centrifugation. Extracts were prepared in 100 μl of extraction buffer by three cycles of freezing and thawing and were cleared by centrifugation at 10,000 × *g* at 4°C for 5 min. An equal amount of protein extract was mixed with 100 μl of reaction buffer containing 100 mM potassium phosphate (pH 7.8), 15 mM $MgSO_4$, and 5 mM ATP. Luciferase activity was determined by injection of 100 μl of reaction buffer containing 1 mM luciferin (Boehringer GmbH, Mannheim, Germany) with a luminometer (Berthold, Wildbad, Germany) as described previously (17).

Complementation of HSV mutants with cloned genes. Complementation of HSV ICP27 mutants by proteins expressed transiently from cloned genes was carried out as described previously (49). Briefly, Vero cells (25-cm^2 flasks) were transfected with 6 μg of each expression plasmid by calcium phosphate coprecipitation. At 24 h posttransfection, cells were infected with HSV-1 ICP27 mutants d27-1 or n504R at a multiplicity of infection of 2 PFU per cell. After adsorption at 37°C for 1 h, cells were washed at 2 h postinfection (hpi) with acid-glycine solution (pH 3) in order to inactivate unadsorbed virus. Progeny virus was then released 24 h after infection by three cycles of freeze-thaw, and its titer was determined on V27 cells, which have the ICP27 gene stably integrated.

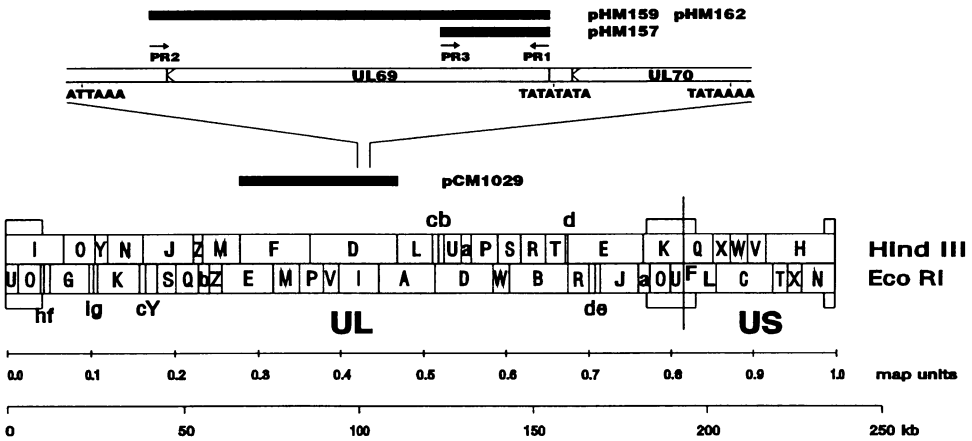

FIG. 2. Prototype arrangement of the HCMV AD169 genome and positions of DNA fragments generated by PCR in this study. The prototype arrangement of the HCMV AD169 genome is shown in the lower half; the position of the cosmid clone pCM1029 used for PCR is indicated as a black bar. In the upper half, a schematic diagram of the UL69 gene region and the positions of possible transcription control signals are shown. The PCR amplification products are represented as black bars, and the derived vectors are named at the right. The arrows indicate the positions and directions of the primers used for amplification.

RESULTS

Analysis of transcripts from the UL69 gene region. The ICP27 transcript of HSV-1, which encodes an activator of late gene expression, is expressed at IE times of infection. In order to investigate the time course of expression of its homolog in HCMV, RNA from HCMV-infected fibroblast cells was analyzed. To obtain probes for RNase protection and Northern blot experiments, two fragments from the UL69 gene region were amplified by PCR and subcloned into the Bluescribe vector (Stratagene). The insert of plasmid pHM159, which corresponded in sequence to the entire UL69 ORF, was generated by using the primer pair PR1-PR2; pHM157 contained a fragment derived from the 5′ end of the UL69 ORF (Fig. 2). In an initial experiment, RNA was analyzed by quantitative RNase protection with a probe derived from plasmid pHM157 (Fig. 3B). As shown in Fig. 3A, lanes 4 to 8, signals corresponding to transcripts from the UL69 gene region could be detected as early as 7 h after infection and showed an increase in intensity during the late phase of the viral replicative cycle. No transcripts were seen with mock RNA or RNA harvested in the presence of cycloheximide (Fig. 3A, lanes 9 and 10). When phosphonoformic acid was used to block viral DNA replication, the transcript levels were about the same as those observed at early times. Thus, we observed an early-late kinetics of expression from the UL69 gene region; however, no transcripts could be detected under IE conditions.

Northern blot experiments were then performed to determine the size(s) of transcripts from UL69. Total cellular RNA, harvested at the indicated times after infection, was separated on 1.2% formaldehyde agarose gels, transferred to nylon filters, and hybridized with the insert of plasmid pHM159 that was labeled by nick translation (Fig. 4A). As observed in RNase protection experiments, no transcripts were present when RNA from cells harvested at 3 h after infection, RNA from mock-infected cells, or RNA from cells infected in the presence of cycloheximide was used (Fig. 4A, lanes 1, 6, and 7). At 7 h after infection, two signals corresponding to transcripts of 2.7 and 3.5 kb could be detected (Fig. 4A, lane 2), which appeared about equal in abundance at this time point. At 24 h after infection, a decrease in steady-state levels of the 3.5-kb transcript was obvious, whereas the amount of the 2.7-kb transcript remained about constant (Fig. 4A, lane 3; note that less RNA was loaded in this lane, as can be deduced from the control hybridization with GAPDH [glyceraldehyde-3-phosphate dehydrogenase] as the probe [lower panel of Fig. 4B]).

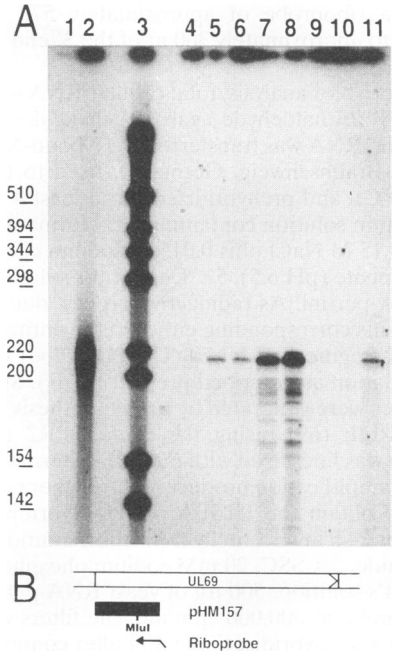

FIG. 3. RNase protection analysis with RNA isolated during the HCMV replicative cycle in order to detect UL69-specific transcripts. (A) Lanes: 1, UL69-specific antisense transcript, digested with RNase; 2, UL69-specific antisense transcript; 3, molecular weight marker; 4 to 8, 10 μg of RNA harvested at 3, 7, 24, 48, and 72 hpi, respectively; 9, 10 μg of RNA harvested from uninfected cells; 10, 10 μg of RNA harvested at 6 hpi in the presence of cycloheximide (150 μg/ml); 11, 10 μg of RNA harvested at 72 hpi in the presence of phosphonoformic acid (200 μg/ml). Sizes of molecular weight markers (in nucleotides) are indicated on the left. (B) Schematic diagram of the riboprobe generated by transcription of *Mlu*I-digested pHM157.

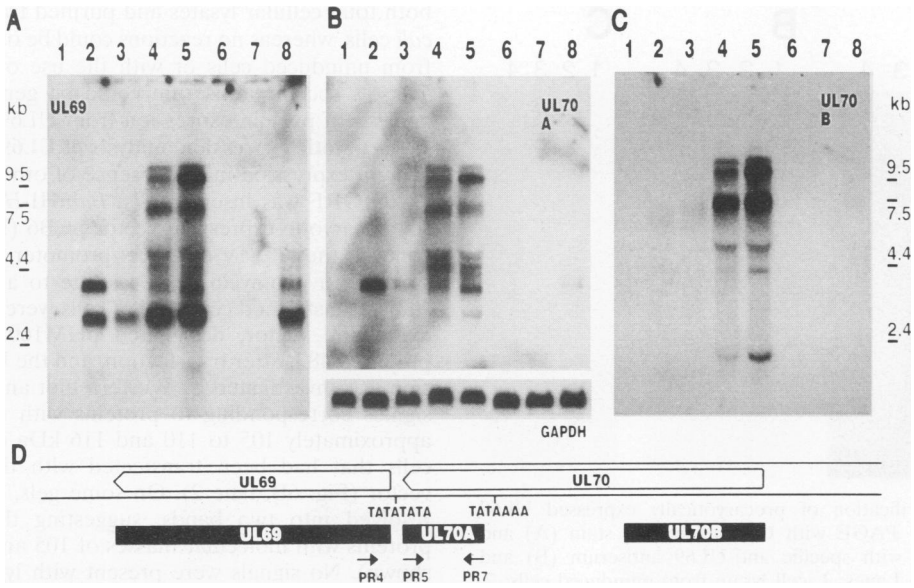

FIG. 4. Northern blot analysis with RNA isolated during the HCMV replicative cycle. Probes specific for the UL69 ORF (A), the 3′ half of the UL70 ORF (B), and the 5′ half of the UL70 ORF (C) were used. The lower panel in panel B shows a control hybridization with a probe specific for GAPDH mRNA. Lanes: 1 to 5, 30 μg of RNA harvested at 3, 7, 24, 48, and 72 hpi, respectively; 6, 30 μg of RNA harvested from uninfected cells; 7, 30 μg of RNA harvested at 6 hpi in the presence of cycloheximide (150 μg/ml); 8, 30 μg of RNA harvested at 72 hpi in the presence of phosphonoformic acid (200 μg/ml). Sizes of molecular weight markers are indicated on both sides. (D) The probes used for hybridization are indicated as black bars in the schematic diagram of the UL69-UL70 genomic region. The primers used for generation of the single-stranded probe UL70A are shown as arrows. The single-stranded probe was generated by runoff synthesis using the primer PR5 and the amplification product of the primer pair PR4-PR7 as the template.

Later on during the infectious cycle, additional RNAs of up to 11 kb appeared (Fig. 4A, lanes 4 and 5). These transcripts could not be detected when RNA was harvested in the presence of phosphonoformic acid, suggesting their regulation as true late transcripts (Fig. 4A, lane 8). Identical results were obtained when a single-stranded probe was used for hybridization, indicating that the detected RNAs originate from the strand of the DNA predicted to encode UL69 (data not shown).

Two further hybridizations were then performed to localize the detected RNAs. When a probe upstream of the UL69 ORF corresponding to 3′ sequences of the UL70 ORF was used (UL70A) (Fig. 4D), the 2.7-kb transcript could no longer be detected (Fig. 4B). In hybridizations with the UL70B probe (Fig. 4D), only the true late transcripts were present (Fig. 4C). This result suggests that the 2.7-kb early transcript has its start site downstream of the UL70 ORF, whereas the 3.7-kb RNA may originate from within UL70. Consensus TATA sequences could be detected both within UL70 and between UL69 and UL70 (Fig. 4D).

Thus, several transcripts contribute to the early-late kinetics of expression from UL69 that was observed in the RNase protection experiments. Whereas some of these RNAs seem to be regulated as true late transcripts that originate from a promoter upstream of UL70, the two RNAs of 2.7 and 3.5 kb appear at early times after infection. These transcripts could not be detected under IE conditions. Later on during the infectious cycle, these two early RNAs were expressed differentially: whereas the abundance of the 2.7-kb transcript increased at late times of infection, the 3.5-kb RNA was downregulated. In summary, these results demonstrate a complex expression of transcripts from UL69 that appear with early-late kinetics during the replicative cycle of HCMV.

Procaryotic expression of UL69 and generation of a specific antiserum. In order to obtain protein for the generation of a specific antiserum, the UL69 ORF was expressed in *E. coli*. Initially, the entire UL69 ORF was inserted into the procaryotic expression vectors pGEX3X and pROS, which should result in β-galactosidase (pROS) and glutathione *S*-transferase (pGEX3X) fusion proteins. The vector pROS has previously been used for high-level expression of several fusion proteins (18, 30). After induction of protein expression with IPTG [isopropyl-β-D-thiogalactopyranoside] and analysis of the expression pattern by SDS-PAGE and Coomassie blue staining, no additional proteins of the expected molecular mass could be observed (data not shown). We then decided to express UL69 as a histidine-tagged protein, as this system allows for very efficient purification under denaturing conditions of even small amounts of expressed protein via metal chelate affinity chromatography. For this purpose, the UL69 ORF was inserted as a *Bam*HI-*Hin*dIII fragment into the procaryotic expression vector pQE11, which resulted in an in-frame fusion to 13 vector-encoded amino acids at the N terminus, including the histidine tag. Expression from this construct was analyzed by induction of protein synthesis in the presence of IPTG, followed by SDS-PAGE and Coomassie blue staining of the gel. As can be seen in Fig. 5A, lanes 1 and 2, there was no recognizable fusion protein synthesis in the presence of IPTG. We then tried to purify the histidine-tagged protein from induced *E. coli* cells via metal chelate affinity chromatography as described in Materials and Methods. Uninduced *E. coli* cells were harvested and treated in parallel as a negative control. The elution fractions from the metal chelate affinity columns were analyzed for the presence of protein by SDS-PAGE and Coomassie blue staining (Fig. 5A, lanes 3 and 4). This revealed a protein with a molecular mass of approximately 105 kDa corresponding to the expected molecular mass of a UL69 fusion polypeptide in the elution fraction from induced *E. coli*

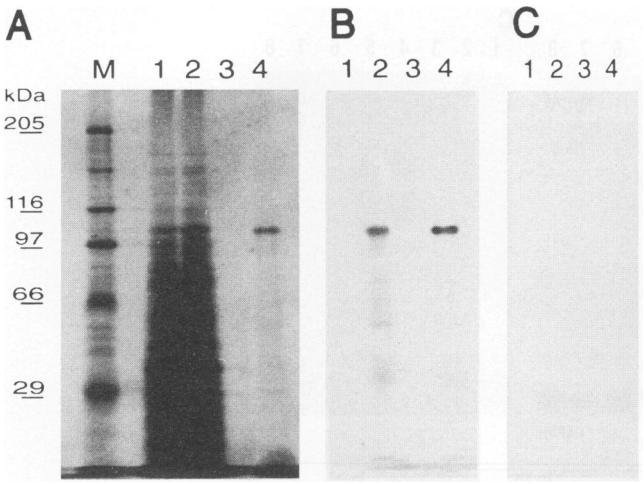

FIG. 5. Affinity purification of procaryotically expressed UL69 protein. Eight percent PAGE with Coomassie blue stain (A) and Western blot analysis with specific anti-UL69 antiserum (B) and preimmune serum (C). Lanes: 1, cell lysate from uninduced cells; 2, cell lysate from IPTG-induced cells; 3, affinity-purified proteins from uninduced cells; 4, affinity-purified proteins from IPTG-induced cells. Lane M shows the molecular mass marker, with the sizes indicated on the left.

cells, whereas no protein was present in fractions from uninduced *E. coli* cells. The purified protein was then used to immunize rabbits. The resulting rabbit antiserum against the UL69 fusion protein was tested for its specificity by Western blot (immunoblot) analysis. As shown in Fig. 5B, the specific antiserum exclusively detected the UL69 fusion protein within both total cellular lysates and purified fractions of induced *E. coli* cells, whereas no reactions could be observed with proteins from uninduced cells or with the use of preimmune serum. Thus, a specific antiserum could be generated for a further analysis of protein expression from UL69.

Eucaryotic expression analysis of UL69. To be able to study protein expression in the absence of other viral functions, the UL69 ORF was inserted as a *Bam*HI-*Hin*dIII fragment into the eucaryotic expression vector pCB6 (Fig. 6A). This vector contains the HCMV enhancer/promoter to drive gene expression and a neomycin gene cassette to allow the selection of stably transformed cells. COS7 cells were transfected with this expression vector, designated pHM160. Cell extracts were prepared 48 h after transfection, and the UL69 protein expression was investigated by Western blot analysis (Fig. 6B). Two signals corresponding to proteins with molecular masses of approximately 105 to 110 and 116 kDa could be detected in cells that had been transfected with the UL69 expression vector (Fig. 6B, lane 2). On some gels, the lower signal was resolved into two bands, suggesting the existence of two proteins with molecular masses of 105 and 110 kDa (data not shown). No signals were present with lysates from cells that had been transfected with the pCB6 expression vector itself (Fig. 6B, lane 3). In addition, the preimmune serum did not react with any of the lysates, demonstrating again the specificity of the antiserum used (Fig. 6B, lanes 4 to 6). The 105-kDa form of the eucaryotically expressed protein (Fig. 6B, lane 2, lower band) comigrated with the procaryotic UL69 polypeptide (Fig. 6B, lane 1), suggesting that UL69 exists in several modified forms within the eucaryotic cell.

To determine the subcellular localization of UL69, indirect immunofluorescence analyses of both transiently and stably transfected cells were performed. COS7 cells that were transfected with either pCB6 or the UL69 expression vector

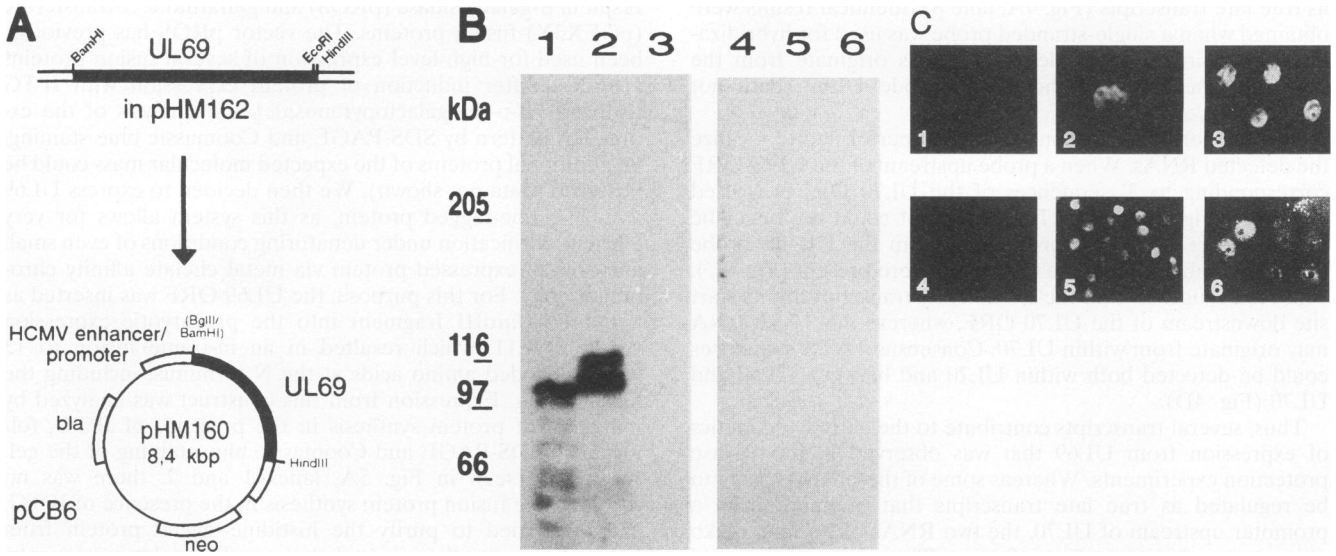

FIG. 6. Eucaryotic expression analysis of the UL69 protein in COS7 and U373 cells. (A) Cloning of UL69 into the eucaryotic expression vector pCB6. The derived expression plasmid pHM160 is shown in the lower half. Genes for selection in procaryotic (bla, β-lactamase) and eucaryotic (neo, neomycin resistance) cells are indicated. (B) Western blot analysis with specific anti-UL69 antiserum (lanes 1 to 3) and preimmune serum (lanes 4 to 6). Lanes: 1 and 4, procaryotically expressed UL69 protein; 2 and 5, extracts from COS7 cells that were transfected with the UL69 expression vector pHM160; 3 and 6, extracts from COS7 cells that were transfected with the vector pCB6. (C) Immunofluorescence analysis of transiently transfected COS7 cells (panels 1 to 3) or stably transfected U373 cells (panels 4 to 6). Specific antiserum was used for the staining of COS7 cells transfected with vector pCB6 (panel 1) or the UL69 expression plasmid pHM160 (panels 2 and 3). Stably transfected U373 cells were probed with preimmune serum (panel 4) or specific antiserum (panels 5 and 6). (Magnification in panels 4 and 5 is five times less than in the other panels).

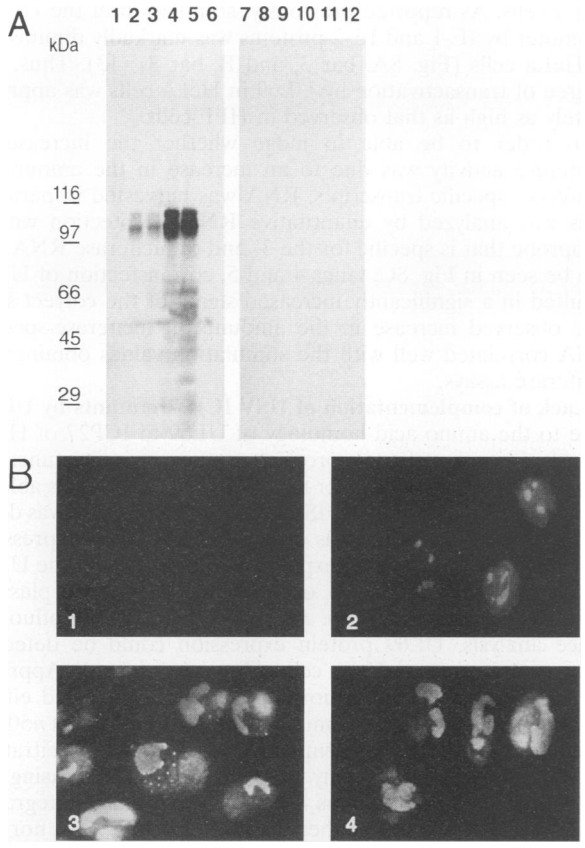

FIG. 7. Expression analysis of the UL69 protein during the HCMV replicative cycle. (A) Western blot analysis of protein extracts harvested at different times after infection of human fibroblast cells with HCMV AD169. Lanes 1 to 6 were probed with specific anti-UL69 antiserum, whereas preimmune serum was used for lanes 7 to 12. Lanes: 1 and 7, 3 hpi; 2 and 8, 7 hpi; 3 and 9, 24 hpi; 4 and 10, 48 hpi; 5 and 11, 72 hpi; 6 and 12, uninfected fibroblast cells. Sizes of molecular mass markers are shown on the left. (B) Immunofluorescence analysis of HCMV-infected fibroblast cells. Panels: 1, probed with preimmune serum 48 hpi; 2, 3, and 4, probed with specific anti-UL69 antiserum 24, 48, and 72 hpi, respectively.

pHM160 were incubated with the specific UL69 antiserum and then stained with fluorescein-conjugated anti-rabbit antibodies. This revealed the strong fluorescence of the nuclei of cells transfected with pHM160, whereas the pCB6-transfected cells showed no reaction (Fig. 6C, panels 1 to 3). The nuclear staining was diffuse with the exception of nucleolar structures, which seemed to be excluded from staining. The same pattern of nuclear expression was also observed with U373 cells that had the plasmid pHM160 stably integrated into the genome (Fig. 6C, panels 4 to 6). Thus, UL69 is expressed as a nuclear protein that exists in several modified forms within the eucaryotic cell.

Expression of the UL69 protein during the HCMV replicative cycle. UL69 protein expression during the HCMV replicative cycle was investigated by both Western blot and indirect immunofluorescence analysis. HFFs were infected with HCMV, and cell extracts were harvested or the cells were fixed for subsequent staining at different times after infection. As shown in Fig. 7A, the UL69-specific antiserum detected at least two polypeptides with molecular masses of approximately 105 to 110 and 116 kDa that appeared with early-late kinetics within the infected cell. No signals were present with cell extracts from cells infected for 3 h with HCMV or from mock-infected cells or with preimmune serum used for staining (Fig. 7A, lanes 1 and 6 and 7 to 12, respectively). In immunofluorescence analysis, the protein was also located within the nucleus of infected cells. It, however, was not distributed evenly within the nucleus but concentrated in foci that appeared at about 24 h after infection and enlarged during the late phase of the replicative cycle (Fig. 7B).

Thus, the kinetics of protein expression during the HCMV replicative cycle parallels the expression of RNA from UL69. The subcellular localization, however, differed from the pattern that was observed after the isolated expression of UL69 in transfected cells.

Transactivation of gene expression by UL69. In order to test whether UL69 of HCMV has any regulatory activities, transient expression assays were performed with HFFs. We were especially interested to see whether UL69 is able to activate late gene expression. For this reason, the promoter of the UL86 gene (encoding the major capsid protein), which had previously been identified as a true late promoter, was cloned upstream of the luciferase reporter gene (52). In addition, the UL112 promoter which represents a well-characterized early promoter of HCMV was used (62). Cotransfection of the UL69 expression vector pHM160 with the luciferase reporter plasmid pHM145 containing the late UL86 promoter of HCMV did not result in any significant stimulation of luciferase activities compared with cotransfection with the expression vector pCB6 without the UL69 ORF (Fig. 8A, bars 4 and 5). As also shown for other true late promoters, the UL86 promoter could not be activated by coexpression of IE-1 and IE-2 polypeptides (Fig. 8A, bar 6). This promoter, however, was strongly stimulated by superinfection with HCMV (Fig. 8A, bar 7). Even a combination of IE-1/2 together with UL69 did not exert any significant effect (data not shown). When the luciferase plasmid pHM142 containing the early UL112 promoter was used for cotransfection, UL69 was able to increase luciferase activities about 10-fold, indicating a *trans*-acting function of UL69 (Fig. 8A, bars 1 and 2). As an additional negative control, the vector p19luc that served as the cloning vector for the HCMV true late and early promoters was tested. Neither UL69 nor the IE-1 and IE-2 polypeptides were able to stimulate gene expression from this construct (Fig. 8A, bars 8 to 10).

To further investigate the specificity of the observed transactivation by UL69, several luciferase reporter constructs containing heterologous promoters were tested. These included the thymidine kinase promoter of HSV-1 (TKluc), the human immunodeficiency virus type 1 LTR (HIVluc), the Rous sarcoma virus LTR (RSVluc), and the cellular promoters of β-actin and phosphoglycerol kinase (βactin-luc and PGK-luc, respectively). All these constructs had the small t-antigen intron and early poly(A) region from simian virus 40 as the 3′ processing signal. Surprisingly, gene expression from each of these promoters increased in the presence of UL69 (Fig. 8A, bars 11 to 20). There were, however, quantitative differences in the levels of induction. Whereas the cellular promoters contained within the luciferase plasmids pβactin-luc and pPGKluc were stimulated only marginally, the viral promoter sequences were activated about 10-fold as was also observed with the HCMV early promoter.

To see whether there are cell type-specific differences in the degree of transactivation, as for example, reported for IE-1 and IE-2 proteins of HCMV, HeLa cells were used for cotransfection experiments. As shown in Fig. 8B, UL69 stimulated gene expression from the UL112 promoter ninefold in

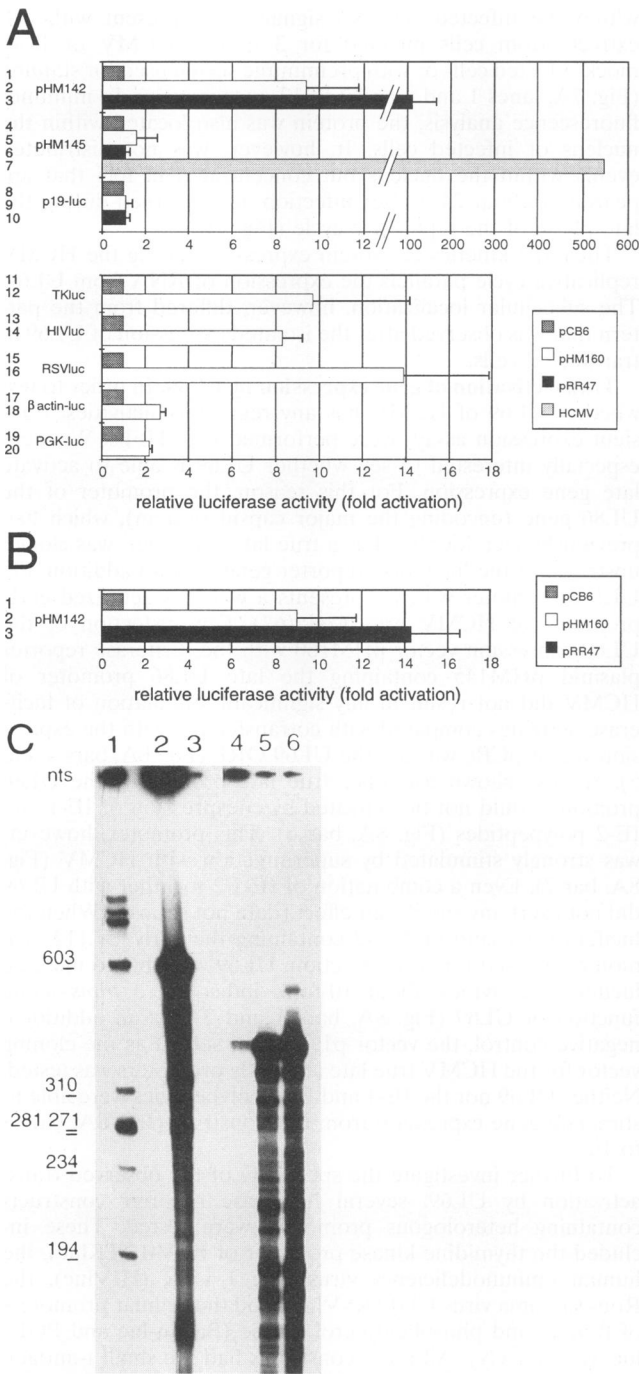

FIG. 8. Luciferase and RNase protection analyses after cotransfection of various luciferase reporter constructs with the UL69 expression vector. (A) Luciferase assays of cotransfected and superinfected human fibroblast cells. Five micrograms of each of the reporter gene constructs indicated at the left was either cotransfected with 2 μg of the plasmids shown in the box or superinfected with HCMV. Standard deviations of at least three experiments are shown as bars. pHM142, UL112 promoter upstream of luciferase; pHM145, UL86 promoter upstream of luciferase; p19luc, no promoter; TKluc, thymidine kinase promoter of HSV-1 upstream of luciferase; HIVluc, human immunodeficiency virus type 1 LTR upstream of luciferase; RSVluc, Rous sarcoma virus LTR upstream of luciferase; β actin-luc, β-actin promoter upstream of luciferase; PGK-luc, phosphoglycerol kinase promoter upstream of luciferase; pCB6, vector used for expression of UL69; pHM160, UL69 expression vector; pRR47, IE-1/2 expression vector; HCMV, superinfection with HCMV. (B) Luciferase assays of cotransfected HeLa cells. Conditions used for HeLa cell transfections were as described for panel A. (C) RNase protection analysis using RNA from HeLa cells after cotransfection of the luciferase expression plasmid pHM142 (containing the early UL112 promoter of HCMV) with various expression vectors. Lanes: 1, molecular weight marker (sizes are indicated on the left); 2, luciferase-specific antisense transcript; 3, luciferase-specific antisense transcript, digested with RNase; 4 to 6, plasmid pHM142 cotransfected with vector pCB6 (lane 4), UL69 expression vector pHM160 (lane 5), or IE-1/2 expressing vector pRR47 (lane 6).

HeLa cells. As reported previously, stimulation of the UL112 promoter by IE-1 and IE-2 proteins was markedly diminished in HeLa cells (Fig. 8A, bar 3, and B, bar 3) (13). Thus, the degree of transactivation by UL69 in HeLa cells was approximately as high as that observed in HFF cells.

In order to be able to judge whether the increase in luciferase activity was due to an increase in the amount of luciferase-specific transcripts, RNA was harvested in parallel. This was analyzed by quantitative RNase protection with a riboprobe that is specific for the 3′ end of luciferase RNA. As can be seen in Fig. 8C, lanes 4 and 5, cotransfection of UL69 resulted in a significantly increased signal of the correct size. The observed increase in the amounts of luciferase-specific RNA correlated well with the stimulation values obtained in luciferase assays.

Lack of complementation of HSV ICP27 mutants by UL69. Due to the amino acid homology of UL69 to ICP27 of HSV, we asked whether the two proteins perform a similar function. We therefore tested whether the UL69 gene product is able to complement the growth of HSV ICP27 mutants. This was done by transfection of Vero cells with either the UL69 expression vector pHM160, the pCB6 expression vector without the UL69 ORF as a negative control, or the ICP27 expression plasmid pM27 as a positive control. As judged from immunofluorescence analysis, UL69 protein expression could be detected after transfection of Vero cells (data not shown). Approximately 24 h after transfection, the cells were infected either with the HSV-1 ICP27 null mutant *d*27-1 or the mutant *n*504R, which encodes a carboxy-terminally truncated ICP27. Titration of the resulting virus progeny was then performed by using the cell line V27, which contains the ICP27 gene stably integrated into the chromosome. Neither the null mutant, *d*27-1, nor the carboxy-terminal mutant, *n*504R, could be efficiently complemented by the UL69 protein, whereas there was significant complementation in the presence of the intact ICP27 as expressed from plasmid pM27 (Table 1) This indicates that there are functional differences between the UL69 protein of HCMV and the ICP27 of HSV.

DISCUSSION

In this study, we analyzed gene expression from the UL69 ORF of the betaherpesvirus HCMV. This reading frame encodes the homolog of the IE protein ICP27 of HSV-1 (10). The relationship between these two proteins is based on an amino acid identity of about 24% and on their location at identical positions within a gene block that is conserved between the various subclasses of herpesviruses (2, 10).

Gene expression from UL69 was analyzed at both the RNA level and the protein level. RNA expression was rather complex: several transcripts that appear with an overall early-late kinetics during the HCMV replicative cycle could be detected.

TABLE 1. Complementation of ICP27 mutants of HSV-1 by expression plasmids containing the coding sequence of UL69 or ICP27[a]

Plasmid	Virus	Titer
pCB6	*d*27-1	4×10^1
pCB6	*d*27-1	$<2 \times 10^1$
pHM160	*d*27-1	2×10^1
pHM160	*d*27-1	4×10^1
pM27	*d*27-1	5×10^5
pM27	*d*27-1	4×10^5
pCB6	*n*504R	4×10^3
pCB6	*n*504R	8×10^3
pHM160	*n*504R	1×10^4
pHM160	*n*504R	8×10^3
pM27	*n*504R	4×10^5
pM27	*n*504R	6×10^5

[a] Duplicate samples were used for each plasmid-virus combination.

These transcripts could not be detected under IE conditions. All of these transcripts have the potential to code for UL69 and could therefore contribute to the level of UL69 protein within an infected cell. Regulation of these transcripts was differential. Two transcripts of 2.7 and 3.5 kb could be detected as early as 7 h after infection. Several true late RNAs were also observed with the UL69-specific probe. Remarkably, while the 2.7-kb RNA showed an increase in abundance at late times after infection, expression of the 3.5-kb transcript was rather transient, with maximum RNA levels detected at 7 h after infection and a rapid decline at 24 h. These kinetics of expression differ from previously described early transcription units of HCMV, for which transcript levels were either constant or increased between 8 and 24 h, suggesting a unique regulation of the 3.5-kb UL69 RNA (8, 26, 37, 59, 63).

A search for polyadenylation sequences 5′ and 3′ of the UL69 ORF revealed a sequence of ATTAAA approximately 500 nt downstream of the UL69 ORF as the only sequence conforming with a described polyadenylation signal (56). Thus, the detected transcripts may have a coterminal 3′ end as has previously been described for other transcription units of herpesviruses (6, 75). In agreement with that finding, hybridizations with two probes 5′ of the UL69 ORF could not detect the 2.7- and 3.5-kb RNAs, suggesting possible transcriptional start sites of these RNAs either between UL69 and UL70 (for the 2.7-kb RNA) or within UL70 (for the 3.5-kb RNA). However, although typical consensus sequences were not detected, the location of splice acceptor sites at these positions cannot be excluded entirely.

The UL69 ORF is able to encode a protein of 744 amino acids. Procaryotic expression of UL69 as a histidine-tagged protein revealed a polypeptide of 105 kDa, which is in good agreement with the calculated molecular mass of 96 kDa. In immunoblot analysis of extracts from transfected COS7 and infected fibroblast cells, at least three polypeptides of 105, 110, and 116 kDa could be observed. This suggests that the UL69 protein is posttranslationally modified, most probably, as observed for ICP27 (78), by phosphorylation. A search for phosphorylation consensus sequences within the UL69 ORF detected a cluster of signals for protein kinase C and casein kinase II at the amino terminus of the protein which is also present in the respective homologs of other herpesviruses. Similar to its counterparts in HSV and Epstein-Barr virus, the protein was also localized within the nucleus of cells (12, 24, 50, 80). Interestingly, however, there was a difference between infected cells and the isolated expression of UL69 in transfected cells. In transfected cells, a rather even distribution of immunofluorescence signals was observed, whereas in infected cells, UL69 appeared to accumulate in intranuclear inclusions. Intranuclear inclusions have been described as being characteristic of cytomegalovirus-infected cells and are thought to represent viral DNA replication compartments interfaced with nucleocapsid assembly regions (3, 7, 22, 34, 38, 51, 76). Few proteins of HCMV have been localized to intranuclear inclusions so far. An identical distribution in intranuclear inclusions of infected cells has been demonstrated for the major DNA-binding protein of HCMV (UL57), which is homologous to ICP8 of HSV (3, 10) and is essential for DNA replication. This could indicate an association of UL69 either with the replication machinery or with chromatin; whether such an association would be direct or mediated by other viral proteins remains to be determined.

This study was initiated in order to identify an HCMV-encoded protein that is involved in the regulation of late gene expression as has been described for the homologous protein in HSV, ICP27 (19, 35, 41, 48, 53, 55, 70). In transient expression assays, the UL69 polypeptide was not able to activate a true late promoter of HCMV but could stimulate an HCMV early promoter as well as several heterologous promoters. Thus, UL69 codes for a protein with a *trans*-acting function. However, it appears to be distinct from the IE protein ICP27 in several aspects. These include the expression of UL69 as an early-late gene and its inability to complement ICP27 mutants of HSV. In addition, the mechanism used by UL69 to stimulate gene expression seems to be different from that of ICP27. Whereas ICP27 depends on posttranscriptional signals and represses gene expression when an intron is contained either 5′ or 3′ to the target gene-coding sequences (54), UL69 had no negative effect, although all the reporter constructs used contained the small t-antigen intron of simian virus 40. Instead, expression of most reporter constructs was stimulated; this, however, was dependent on the promoter sequence.

The mode of action of UL69 could be on the transcriptional level by targeting a common signal such as the TATA box. Such a mechanism has been proposed to explain the broad spectrum of promoters that are activated by the IE-2 protein of HCMV and was further emphasized after the detection of a direct interaction between the TATA-binding protein TBP and the IE-2 polypeptide (23). This, however, seems to be insufficient to explain the action of UL69 since the nonresponsive true late promoter contains a TATA sequence. Alternatively, a posttranscriptional mechanism such as RNA stabilization could account for the broad stimulation that was observed. In the case of RNA stabilization, for example, expression from all promoters that have some constitutive activity would increase. The failure to see transactivation from the true late promoter would then be due to its lack of constitutive activity. For ICP27 of HSV, a posttranscriptional mode of action has been demonstrated: genes that contain a weak polyadenylation signal are activated, whereas genes with introns are repressed (54). This, however, does not seem to be the case for UL69, as most of the used target genes that were activated had the small t-antigen intron and early poly(A) region from SV40 at its 3′ end. In addition, deletion of the small t-antigen intron sequence from the 3′ processing signal did not alter the induction levels significantly (79). Although the ICP27 homolog BMLF1 of Epstein-Barr virus has also been described as a protein with broad transactivator properties that is able to activate an early-class Epstein-Barr virus target promoter (12, 32), the mechanism of UL69 appears to differ from that of BMLF1

with respect to one result. For BMLF1, a disproportional effect on chloramphenicol acetyltransferase mRNA levels compared with chloramphenicol acetyltransferase enzyme levels has been described (6, 28). This could not be observed for UL69. After cotransfection of UL69, the increase in luciferase RNA correlated well with the results of luciferase assays that were performed in parallel, excluding a major effect on the translational level. UL69, however, may be more similar to the ORF4 protein of varicella-zoster virus. Two studies have reported that this protein failed to stimulate gene expression from a late promoter but could activate an early promoter of varicella-zoster virus (14, 45). Moreover, several heterologous promoters were induced by ORF4, thus resembling the broad activation pattern of UL69 (14, 27).

UL69 is the first early gene product of HCMV that has a *trans*-acting function on other early promoters to be described. The biological relevance of such an autoregulatory circuit within the early kinetic class of genes may be to boost gene expression after the initial trigger exerted by IE gene products. However, an influence on late gene expression cannot be excluded. Assuming that there is a posttranscriptional mechanism such as RNA stabilization, UL69 may further increase late gene expression as soon as the respective promoters are activated. Such a function could explain the strong expression of UL69 at late times of the replicative cycle. In order to answer these questions, it will be necessary to generate UL69 null mutants of HCMV. This will also reveal whether UL69 is an essential gene as has been demonstrated for ICP27 of HSV (53).

In summary, we have defined a novel regulatory protein encoded by HCMV that seems to exert a broad activation pattern. Further studies will help to elucidate the molecular mechanism used by UL69. This will be of interest in order to understand the biological function of this group of homologous proteins.

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