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## The UL97 Gene Product of Human Cytomegalovirus Is an Early-Late Protein with a Nuclear Localization but Is Not a Nucleoside Kinase

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**The temporal expression of the UL97 gene product during human cytomegalovirus (HCMV) infection of human foreskin fibroblasts (HFF) and subcellular localization of this protein were analyzed by using a polyclonal antiserum raised against a truncated UL97 protein of 47 kDa. The UL97 protein was detectable 16 h after infection by Western blot (immunoblot) analysis. Since only reduced UL97 expression occurred in the presence of two inhibitors of DNA replication, phosphonoacetic acid and ganciclovir, we conclude that UL97 is an early-late gene, requiring DNA replication for maximum expression. By indirect immunofluorescence, the protein could be visualized in the nuclei of virus-infected HFF 22 h after infection. Nuclear localization of the UL97 protein was also detected in thymidine kinase-deficient 143B cells infected with a recombinant vaccinia virus containing the entire UL97 open reading frame (ORF), as well as in HFF transiently expressing the entire UL97 ORF under the control of the HCMV major immediate-early promoter. However, transiently expressed 5'-terminal deletion mutants of the UL97 ORF in addition showed a cytoplasmic localization of the UL97 protein, confirming the presence of a nuclear localization site in the N-terminal region of the protein. Our high-pressure liquid chromatography analyses confirmed the ganciclovir phosphorylation by the UL97 protein, but no specific phosphorylation of natural nucleosides was observed, indicating that the UL97 protein is not a nucleoside kinase. During plaque purification of recombinant UL97-deficient HCMV, this virus was growth defective; hence, we presume that UL97 may be essential for the viral life cycle.**

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is a major pathogen in immunosuppressed or immunodeficient individuals. The nucleoside analog ganciclovir (GCV) is one of two drugs licensed currently for the treatment of HCMV infections. One problem encountered after long-term treatment of immunocompromised patients with GCV is the emergence of drug-resistant virus variants (8). The action of GCV depends largely on virus-controlled phosphorylation in infected cells, and it has been shown that the UL97 open reading frame (ORF) of HCMV encodes a protein that has homologies to protein kinases but is able to phosphorylate GCV to the monophosphate (3, 17, 29). Several mutations in the coding region of UL97 that induce resistance of HCMV to GCV have been described (2, 18, 29). We reported previously that the UL97 ORF alone is sufficient to confer GCV susceptibility on the naturally GCV-resistant vaccinia virus (21).

However, almost nothing is known about the natural function and the intracellular localization of the UL97 gene product. For varicella-zoster virus, another member of the herpesvirus family, it was reported that a protein of 35 kDa exhibited thymidine kinase (TK) activity in infected cells. Immunofluorescence staining with antiserum against the viral TK showed that the protein was mainly located in the nuclei of infected cells (26). In contrast to this result, herpes simplex virus (HSV) TK, which phosphorylates aciclovir (ACV), penciclovir, and GCV, was detected mainly in the cytoplasm (10).

The aim of this study was to further characterize the UL97 gene product. To our knowledge, this is the first study showing the temporal expression of the UL97 protein and the intracel-

lular localization in HCMV-infected cells, providing evidence for the presence of a nuclear localization site (NLS) at the 5' end of the protein. Our results also show that viral DNA replication is necessary for maximum expression of the UL97 protein. Nucleoside kinase activity is not the normal biological function of the protein, but we suspected that the UL97 gene function might be essential for the HCMV replication cycle.

### MATERIALS AND METHODS

**Cells and viruses.** Experiments with the laboratory HCMV strain AD169 and the recombinant HCMV UL97-Orc were done with confluent human foreskin fibroblasts (HFF) between passages 11 and 20. If not stated otherwise, the cell monolayers were grown in minimal essential medium (Gibco, Eggenstein, Germany) supplemented with 2 mM glutamine, 0.1 mg of streptomycin per ml, 0.1 mg of penicillin per ml, 1% nonessential amino acids (Seromed, Berlin, Germany), and 10% fetal calf serum (Gibco). 143B TK-deficient cells were also grown as described above and infected with recombinant vaccinia viruses containing a wild-type (T1) and a mutated (A5) UL97 ORF, respectively (21). In other experiments, 143B cells were infected with HSV type 1 strains K1 (ACV and GCV sensitive) and K1r (ACV and GCV resistant [11a]).

**Generation of recombinant HCMV.** Recombinant HCMV was produced as described by Lurain et al. (19). Briefly, HFF monolayers were infected with the wild-type strain AD169 and after 3 h transfected with 30 µg of plasmid p223 containing the mutant UL97 (pOrc). Recombinant virus was selected in the presence of 50 µM GCV. For monitoring, primers UL97-435 (5'-TTA CCA CCG CTT GCG CTG-3') and UL97-1599 (5'-TTA TTG CAT GTC GGA GCT G-3'), amplifying a fragment containing the deletion of p223 (pOrc), were used. For amplification, 35 cycles were performed at 94°C for 30 s, 58°C for 45 s, and 72°C for 50 s, with an initial step of denaturation at 94°C for 2 min.

**Plasmids.** A region containing the entire 2.2-kb UL97 ORF (positions 140367 to 142607 according to the AD169 complete genome [GenBank accession number X17403]) was amplified by PCR and inserted into the *EcoRI-HindIII* sites of pEMBL18 as described previously (21), resulting in construct pUL97. For generation of N-terminal deletions, plasmid pUL97 was cut with *HindIII* at the very 5' end of the UL97 insert and subjected to exonuclease III digestion as described by Henikoff (13). For generation of internal UL97 deletion mutants, the plasmid was cut at an artificial *SalI* recognition site, which had been introduced previously (21), 981 bp downstream of the putative UL97 translation start. For transient expression experiments, the truncated inserts were subcloned into plasmid pBK-

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CMV $\Delta$ NS (Stratagene, Heidelberg, Germany) (see Fig. 4A) under the control of the HCMV major immediate-early (MIE) promoter, resulting in constructs p214 (containing wild-type UL97), p213 (11 bp of the ORF deleted, including the first ATG), and p221 (314 bp of the ORF deleted, including the first three of the four ATGs) and internal deletions p223 (pOrc), p235 (in-frame mutants carrying deletions of 831 and 477 bp, respectively), and p236 (out-of-frame mutant, 368-bp internal deletion). Construct p212 is the same as p214 but lacking the promoter.

**Monoclonal antibodies and polyclonal antiserum against UL97 protein.** Plasmid pUL97 containing the entire UL97 ORF was cut with *Sph*I and *Pst*I, resulting in a 1.3-kb fragment (coding for a truncated protein of 47 kDa [see Fig. 4A]). The fragment was fused to the histidine tag of the bacterial expression vector pQE31 (Qiagen, Düsseldorf, Germany), resulting in plasmid p47kD. Expression and purification of the 47-kDa protein were performed as recommended by the manufacturer. Subcutaneous rabbit immunization was carried out according to standard protocols (12), using aluminum hydroxide as the adjuvant. Antiserum was harvested 10 days after the second booster injection. Mouse monoclonal antibodies against the immediate-early (IE) HCMV 76-kDa protein (E-13; Biogenesis), early (E) HCMV protein p52 (CCH2), and the early-late protein pp65 (AAC10) (Dako Glostrup, A/S, Denmark) were used for immunoprecipitation. Horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG; Amersham, Braunschweig, Germany) and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG F(ab')<sub>2</sub> (Dianova, Hamburg, Germany) were used as secondary antibodies in Western blot (immunoblot) analyses and for indirect immunofluorescence, respectively.

**Western blot analysis.** Total cell lysates were extracted as described by Sambrook et al. (24) from mock-infected or HCMV-infected cell cultures at different times postinfection (p.i.), separated on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel (16), and semidry transferred to nitrocellulose membranes. The membranes were probed with UL97 antiserum (dilution of 1:300) and then incubated with secondary horseradish peroxidase-conjugated IgG (dilution of 1:5,000). The protein-antibody complex was detected by using an enhanced chemiluminescence Western blotting detection system (Amersham).

**Metabolic labeling.** For metabolic labeling experiments, HFF were infected with AD169 at a multiplicity of infection (MOI) of 5 in the presence of different metabolic inhibitors allowing expression of HCMV IE and/or E proteins, or without metabolic inhibitors for sequential expression of all viral proteins (28, 30). Fibroblasts were maintained in medium with metabolic inhibitors throughout the washing procedures and during pulse-labeling. For expression of only HCMV IE proteins, monolayers were preincubated for 30 min with medium containing 100  $\mu$ g of cycloheximide (CHx) per ml and infected with AD169 in the presence of CHx for 2.5 h. Actinomycin D (AcD; 20  $\mu$ g/ml) was then added, and after 30 min, the monolayers were washed to remove the CHx, and medium containing AcD was added and the mixture was incubated for a further 2 h. To allow expression of HCMV IE and E proteins, HFF were infected in the presence of phosphonoacetic acid (PAA; 250  $\mu$ g/ml; Sigma, Deisenhofen, Germany) or GCV (125  $\mu$ g/ml; Cymeven, Syntex, Germany) for 48 h. For expression of HCMV late proteins, HFF monolayers were incubated for 48 h without metabolic inhibitors. Radioactive labeling was performed basically as described by Coligan et al. (5). In summary, after incubation, HCMV-infected HFF were washed two times with warm phosphate-buffered saline (PBS; 37°C) and once with warm labeling medium (minimal essential medium without L-methionine and L-cysteine, supplemented with 5% dialyzed fetal calf serum). Cells were then incubated in the same medium for 30 min at 37°C and pulsed with 250  $\mu$ Ci of [<sup>35</sup>S]methionine-cysteine (Pro-mix; Amersham) for 90 min at 37°C. After triple washing with cold PBS (4°C), cellular and viral proteins were extracted from cells by incubation in lysis buffer (1 ml/10<sup>6</sup> cells) (50 mM Tris-HCl [pH 8.3], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, ABTS at 0.1 mg/ml, 1  $\mu$ g of leupeptin per ml, 1  $\mu$ g of aprotinin per ml [protease inhibitors were purchased from Boehringer, Mannheim, Germany]) for 1 h at 4°C. The lysates were centrifuged at 14,000  $\times$  g for 10 min in a tabletop centrifuge, and supernatants were either stored at -20°C or used directly for immunoprecipitation experiments.

**Immunoprecipitation.** Immunoprecipitation of HCMV proteins was performed as described by Anderson and Blobel (1). Briefly, the radioactively labeled lysates (containing 10  $\mu$ Ci) were precleared by incubation with 5  $\mu$ l of nonimmune rabbit serum for 30 min. All incubations were done with constant shaking at 4°C. After 30 min, 30  $\mu$ l of protein A-Sepharose (50% slurry suspension) was added, and the mixture was incubated for 30 min. Immune complexes bound to protein A-Sepharose were removed by centrifugation. To each supernatant, 3  $\mu$ l of UL97 antiserum (undiluted) or 3  $\mu$ l (0.5  $\mu$ g) of monoclonal antibodies against viral antigens was added. After 2 h, 50  $\mu$ l of protein A-Sepharose slurry was added, and the samples were incubated for 1 h. Immune complexes adsorbed to the protein A-Sepharose were collected by centrifugation and washed twice in 50 mM Tris-HCl (pH 7.5)-150 mM NaCl-0.1% Nonidet P-40, then twice in high-salt buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Nonidet P-40), and finally once in 10 mM Tris-HCl (pH 7.5). Adsorbed antigens were eluted by boiling for 5 min at 95°C in 50  $\mu$ l of 50 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, 200 mM dithiothreitol and a trace of bromophenol blue dye. The immunoprecipitated proteins were subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE). The gels were visualized by fluorography after being fixed in 40% methanol-10% acetic acid for 30 min,

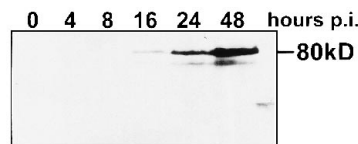


FIG. 1. Time course of UL97 protein expression in HCMV-infected fibroblasts. Cells were harvested at the indicated times. Total cell lysates from infected tissue cultures were analyzed by SDS-PAGE and Western blotting with the UL97 antiserum as described in Materials and Methods.

impregnated with Amplify (Amersham) for 30 min, and dried in a gel dryer at 80°C for 2 h.

**Indirect immunofluorescence and transient expression experiments.** Visualization of UL97 expression was done by indirect immunofluorescence with the UL97 antiserum. Before infection or transfection for transient expression, cells were grown for 1 day on glass coverslips in 24-well plates. Cultures were infected with HCMV at MOIs of 0.1 to 0.5 and with recombinant vaccinia virus at an MOI of 0.1 and fixed with methanol-acetone (1:1) for 10 min at -20°C at 0, 6, 22, 48, 72, 96, and 120 h p.i. for HCMV and at 0, 2, 5, and 22 h p.i. for vaccinia virus. For transient expression experiments, cells were transfected with 30  $\mu$ g of plasmid as described by Sambrook et al. (24) by calcium phosphate precipitation followed by glycerol shock. Immunofluorescence was carried out at room temperature 24 h posttransfection. The UL97 antiserum (diluted 1:100 with PBS-0.1% bovine serum albumin) was added, and the coverslips were incubated for 60 min at 37°C. The coverslips were then washed three times with PBS and incubated with the secondary fluorescein isothiocyanate-conjugated IgG F(ab')<sub>2</sub> (dilution of 1:30) for 60 min at 37°C. The coverslips were covered with glycerol-PBS (9:1). Microscopy was performed with a Zeiss microscope equipped for fluorescence and photography.

**HPLC analysis of nucleoside anabolism in virus-infected cells.** 143B cells were infected with the UL97-expressing vaccinia virus recombinants or HSV at an MOI of 1. At 1 h p.i., 5  $\mu$ Ci of [<sup>3</sup>H]ganciclovir or [<sup>3</sup>H]-labeled natural nucleosides (dA, dG, dC, and dT) was added at a specific activity of 100 mCi/mmol. After 20 h of incubation, cells were harvested and washed three times with PBS. The cells were resuspended in PBS and extracted with 0.5 M perchloric acid. The extracts were adjusted to pH 6.5 with 2.5 M KOH in 1.5 M KH<sub>2</sub>PO<sub>4</sub> and centrifuged for 5 min at 14,000  $\times$  g, and the supernatants were used for high-pressure liquid chromatography (HPLC). HPLC was performed on a Prep Pac reversed-phase column (250 by 4.6 mm; Pharmacia, Uppsala, Sweden) with 20 mM KH<sub>2</sub>PO<sub>4</sub> (pH 6.0)-7.5% methanol (GCV and dC) or 15% methanol (dA, dG, and dT) as the mobile phase, using isocratic elution at a flow rate of 1 ml/min. Fractions containing the nucleoside phosphates (mono-, di-, and triphosphates) were collected after 2 min, and the unphosphorylated compounds after their respective retention times were measured by external standard runs. The radioactivity of each fraction was determined by liquid scintillation counting.

## RESULTS

**Temporal expression of the UL97 gene product after HCMV infection of HFF.** To study the expression pattern of the UL97 gene product, we expressed a truncated UL97 protein in a heterologous prokaryotic system. This plasmid, p47kDa, directs the expression of a protein (amino acids 106 to 529) with a predicted molecular mass of 47 kDa. We failed to express full-length UL97 protein or proteins that contain the N-terminal part of UL97. The antiserum produced against the purified 47-kDa protein reacted in Western blot experiments with a protein from HCMV-infected HFF of approximately 80 kDa, which is the predicted value for UL97 as shown by Littler et al. (17). The temporal expression of the protein was examined during one cycle of infection in HFF. For this purpose, cell cultures were infected with AD169 and harvested at different times p.i. The UL97 protein was detectable 16 h p.i. by Western blotting, and the intensity of the signal increased during the following hours (Fig. 1). In cell lysates from HFF cultures infected with different clinical HCMV isolates, the UL97 protein was equally detectable (data not shown). This 80-kDa protein was not detected in mock-infected cells. By using a preimmune serum, no signal was observable in mock-infected or in HCMV-infected fibroblasts (data not shown).

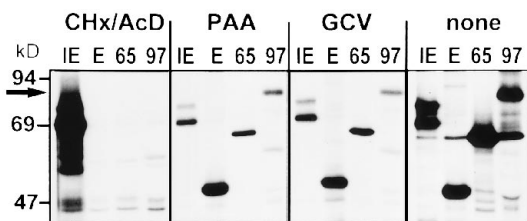


FIG. 2. Immunoprecipitation of different HCMV proteins after metabolic labeling at different stages of the viral life cycle in HFF. Cells were infected at an MOI of 5 and treated with different metabolic inhibitors: CHx, AcD, PAA, and GCV. For detection of IE proteins, cells were incubated with CHx and AcD; for detection of E proteins, infected cells were incubated with PAA or GCV for 48 h; and for detection of late proteins, infected cells were grown without metabolic inhibitors. Labeling with [ $^{35}$ S]methionine-cysteine was done 90 min before cells were harvested. Immunoprecipitations were performed with the monoclonal antibodies against IE protein (p76), E protein (p52), and early-late (65) protein (pp65) and with UL97 antiserum (97). SDS-PAGE was carried out for separation of proteins. Molecular masses are indicated at the left. The position of the UL97 band is marked by an arrow.

**UL97 is an early-late protein, since it is maximally expressed after onset of DNA replication.** To determine in which stage of the HCMV replicative cycle the UL97 protein is expressed, immunoprecipitation of different viral proteins from cell cultures treated with different metabolic inhibitors was carried out. Sequential blockade with CHx and AcD was used to selectively express IE gene products in HCMV-infected HFF (immediate-early conditions). Metabolic blockade with PAA (250  $\mu$ g/ml) or GCV (125  $\mu$ g/ml or 500  $\mu$ M) was used to allow expression of HCMV E proteins, since these substances selectively inhibit the viral DNA polymerase (early conditions). For expression of late proteins, infected cultures were incubated for 48 h without metabolic inhibitors. The viral proteins were immunoprecipitated with different monoclonal antibodies or with the UL97 antiserum (Fig. 2). As expected, no signal of UL97 protein was detectable under immediate-early conditions. Expression of UL97 occurred also in the presence of DNA polymerase inhibitors; however, without metabolic inhibitors, significantly higher expression levels could be observed. In view of its mechanism of action, it is interesting that also GCV, the substrate of the UL97 protein, reduces the expression of the UL97 protein. The expression pattern of UL97 was very similar to that of the HCMV early-late protein pp65 (6). Therefore, these results suggest that the UL97 gene is an early-late gene which expresses lower levels of protein under early conditions, with maximum expression occurring after the onset of DNA replication.

**The HCMV UL97 protein is located in the nuclei of infected fibroblasts.** Thus far, nothing has been known about the intracellular localization of the UL97 protein. To determine the subcellular localization, uninfected and HCMV-infected HFF were analyzed by indirect immunofluorescence. After infection, cells were fixed at different time points. No signal was visible in mock-infected cells (0 and 22 h p.i.) or in cells 6 h after HCMV infection (Fig. 3B). Staining of the nuclei was detected 22 h p.i. (Fig. 3C), with increasing intensity up to 48 h p.i. (Fig. 3D), and no clear signal was observed in the cytoplasm. Infected cells at 72 h p.i. showed further increased staining of the nuclei, but with increasing time cytoplasmic signals became visible, particularly in the perinuclear compartment (data not shown). To detect nonspecific staining, i.e., staining due to binding to viral Fc receptors, control experiments were performed. HCMV-infected cultures were examined with rabbit preimmune serum; only faint staining of the perinuclear cytoplasm was detectable 48 to 72 h p.i., and no

signal was visible in the nuclei (Fig. 3A). No signal was observed in HCMV-infected cells after incubation with the secondary monoclonal anti-rabbit antibody only. Furthermore, no signal was observed in the nuclei of HSV-infected cells 24 and 48 h p.i. after staining with the UL97 antiserum. However, the faint perinuclear staining was visible at later time points. In conclusion, this perinuclear staining at later time points may be attributed to the expression of viral Fc receptors (Fig. 3A).

**In cells infected with recombinant vaccinia virus T1 or A5, the UL97 protein also shows nuclear localization.** To determine whether the nuclear localization of the UL97 protein is dependent on other HCMV proteins, we performed indirect immunofluorescence analysis by using the previously described recombinant vaccinia viruses T1 and A5 (21). Wild-type vaccinia virus is resistant against GCV, but T1 carrying the entire wild-type UL97 ORF of HCMV is sensitive to the antiviral drug. In the recombinant vaccinia virus A5, 12 bp of the UL97 ORF are deleted (29). Consequently, the recombinant vaccinia virus A5 shows impaired GCV phosphorylation and is resistant to GCV, although the UL97 protein is expressed. TK-deficient 143B cells were infected with recombinant vaccinia virus T1 or A5 and fixed 0, 2, 5, and 22 h p.i. No signal was observed in mock-infected cells (Fig. 3E) or in infected cells up to 2 h p.i. Staining was detectable first in nuclei of single cells 5 h p.i. (Fig. 3F) in both T1- and A5-infected cell cultures. At 22 h p.i., nearly all nuclei were positive for the UL97 protein (Fig. 3G). These findings indicate that no other HCMV proteins are required for nuclear localization of the UL97 protein.

**Only the first ATG of the UL97 ORF is used as a translation start site in vivo, and the UL97 protein contains an NLS.** To further test whether the UL97 gene product is able to enter the nucleus without the presence of other viral proteins (vaccinia virus or HCMV) and whether an NLS could be identified, transient expression experiments with different UL97 constructs (Fig. 4A) under the control of the HCMV MIE promoter were performed. Furthermore, we wanted to clarify whether different proteins are expressed from the UL97 gene as described for the TK of HSV (11, 20).

With plasmid p214 carrying the wild-type UL97, the protein was detectable in the nuclei of HFF 24 h after transfection (Fig. 3K), very similar to what was found in HCMV-infected cells. In contrast, after transfection with the 5'-truncated constructs of the UL97 ORF p213 and p221, the protein was also retained in the cytoplasm, since it was equally visible in the cytoplasm and in the nuclei (Fig. 3L and M). These results suggest the existence of an NLS in the N-terminal region of the UL97 protein. Sequence analysis of this region revealed a putative NLS which showed significant homology to appropriate sites in human retroviruses and influenza A virus as well as to one of the bipartite NLSs of the HCMV protein pp65 (Table 1).

Western blot experiments were performed with lysates of cells transiently transfected with the constructs described above. The construct that contains the full-length UL97 ORF expressed the 80-kDa protein (Fig. 4B), whereas both constructs p213 (lacking the first ATG) and p221 (lacking first three ATGs) expressed a protein of the same molecular mass of approximately 70 kDa although p213 contains two more in-frame ATG codons in the 5' region than p221. In addition, we could not detect proteins of a lower molecular mass in HCMV-infected HFF; therefore, we conclude that in contrast to the TK of HSV, only one kind of protein is naturally expressed from the UL97 gene and that only the fourth AUG can be used for the start of translation if the first one is missing. These results also explain why constructs p213 and p221 expressed proteins with identical retention in the cytoplasm.

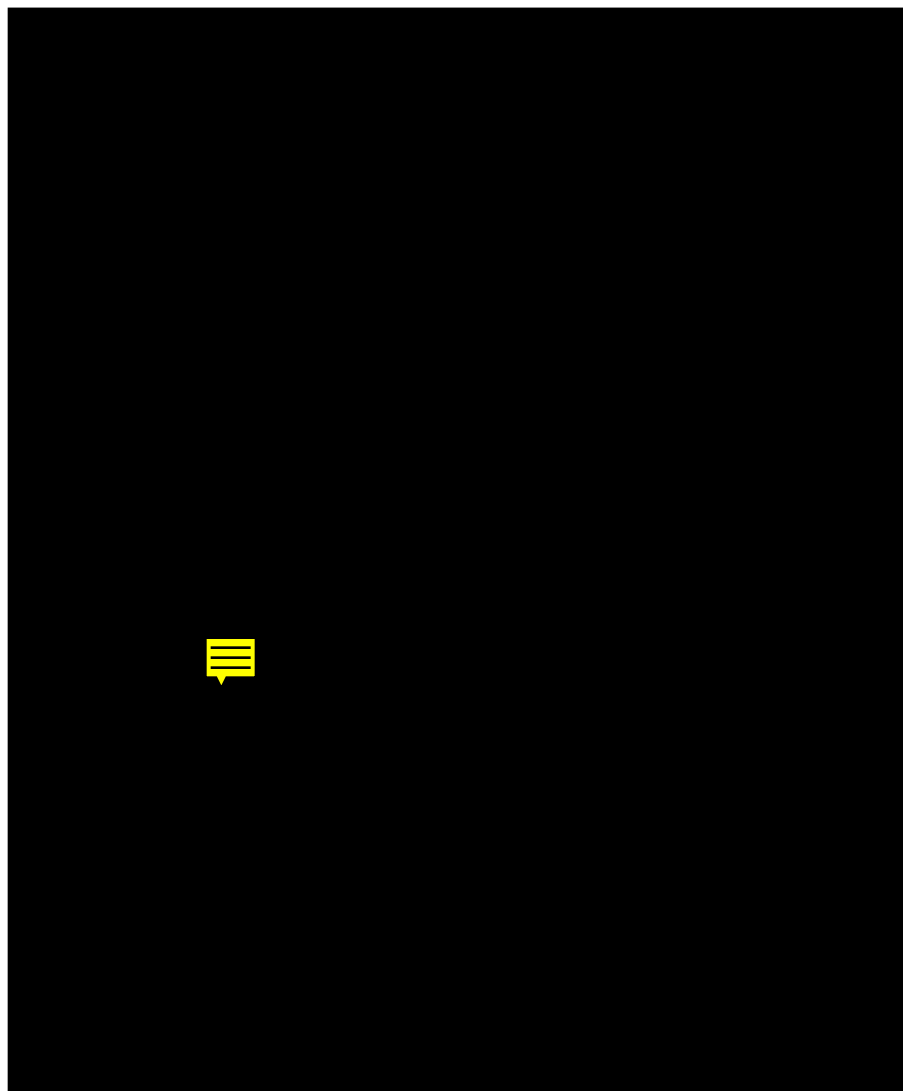


FIG. 3. Indirect immunofluorescence with UL97 antiserum. (A to D) HCMV-infected fibroblasts at different times p.i. (A) At 72 h p.i. with preimmune serum; (B) at 6 h p.i.; (C) at 22 h p.i.; (D) at 48 h p.i. with UL97 antiserum. (E to G) 143B (TK-deficient) cells infected with recombinant vaccinia virus T1 carrying the entire UL97 ORF. (E) Mock infected at 22 h p.i.; (F and G) virus infected at 5 (F) and 22 (G) h p.i. (H to M) Fibroblasts transiently transfected with different UL97 constructs. (H) pOrc (981-bp deletion); (K) p214 (UL97 wild type); (L) p213 (deletion of 11 bp at the 5' end of the ORF containing the first ATG); (M) p221 (deletion of 314 bp of the 5' end, including the first three ATG codons of the ORF). Magnification,  $\times 630$ .

**The UL97 protein does not specifically phosphorylate natural nucleosides.** The UL97 protein has been found to phosphorylate the nucleoside analog GCV. To determine the natural substrate of the protein, we tested the UL97-dependent phosphorylation of the natural nucleosides in our vaccinia virus system, since phosphorylation experiments could not be performed quantitatively in HCMV-infected HFF because of a low specific phosphorylation activity. We found that GCV was phosphorylated by the UL97 protein, but surprisingly, no UL97-specific phosphorylation of natural nucleosides was observed (Table 2). As a control, we tested the phosphorylation of natural nucleosides and GCV in 143B cells infected with HSV, comparing the sensitive strain (K1) and the TK-deficient, resistant strain (K1r). As expected, thymidine and cytosine as well as GCV were phosphorylated by the HSV TK, whereas adenosine and guanosine were not (Table 2). These results suggest that the UL97 protein is not a nucleoside kinase

and therefore not an analog of HSV TK with respect to natural function.

**Recombinant HCMV with extended deletion of the UL97 ORF is growth defective.** The best way to study the natural function of a protein is often by generating and characterizing deficient mutants. However, for UL97, only point mutations or small deletions which confer impaired susceptibility of HCMV to GCV have been described (2, 18, 29). Such mutants may produce a UL97 protein with intact natural function although GCV phosphorylation is hampered. To generate a mutant that is deficient for natural UL97 function, we transformed wild-type AD169 by transfection with the UL97-derived p223 (pOrc). This construct contains a large internal deletion of the UL97 ORF (Fig. 4A). After transformation, the recombinant viruses were passaged several times in the presence of 50  $\mu$ M (12.5  $\mu$ g/ml) GCV. This concentration of GCV is 10- to 20-fold higher than the 50% inhibitory dose of the laboratory strain

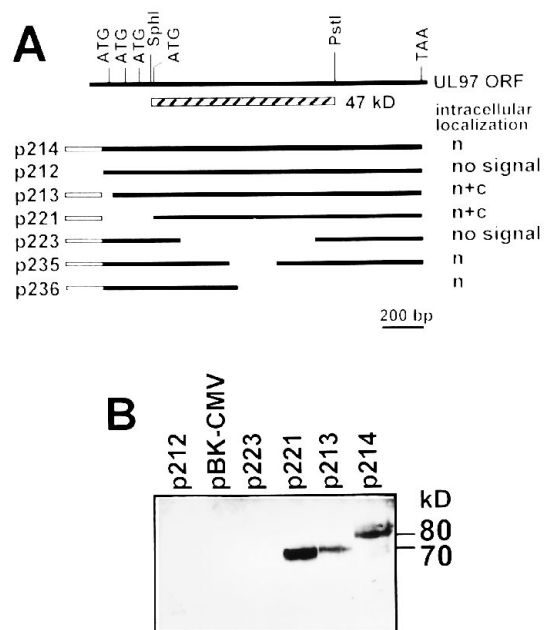


FIG. 4. (A) Deletion mutants of the UL97 ORF for transient expression experiments, driven by the HCMV MIE promoter. At the top, a schematic drawing of the UL97 ORF is shown. The putative translation start (first ATG) and three further in-frame ATGs are indicated. The region for generating the UL97 antiserum (47 kDa) is represented by the hatched bar. The MIE promoter is shown as a white box. Subcellular localization was determined by indirect immunofluorescence with the UL97 antiserum. n, nucleus; n+c, nucleus and cytoplasm. (B) Immunoblotting analysis after transient expression of different UL97 deletion mutants in HFF. Cells were harvested 24 h after transfection. Total cell extracts were separated by SDS-PAGE, and proteins were visualized by chemiluminescence in Western blotting analysis with the UL97 antiserum.

AD169 (4, 18, 27). However, even after several rounds of passage, wild-type virus and UL97-deficient virus were found in the same culture as determined by PCR. To separate the mutants from the wild type, plaque purifications were performed. But as soon as the wild-type virus was no longer detectable by PCR, the mutant virus also disappeared. These results suggest that the UL97-deficient recombinant viruses are growth defective, and therefore this gene may be essential for the HCMV life cycle.

## DISCUSSION

It has been shown that the protein encoded by the UL97 ORF of HCMV is able to phosphorylate GCV, but almost

TABLE 1. Homology in the N-terminal region of the UL97 protein to NLSs

Virus <sup>a</sup>	Protein	Position of signal <sup>b</sup> (aa)	Deduced signal sequence	Reference
HCMV	UL97	31–36	R A R R R Q	This study
HTLV-1	Rex	2–7	K T R R R P	26a
HIV-1	Rev	39–44	R N R R R R	4a
Influenza A virus	NS1	35–38	R L R R	9a
HCMV	pp65 (UL83)	537–542	P K R R R R H <sup>c</sup>	26

<sup>a</sup> HTLV-1, human T-cell leukemia virus type 1; HIV-1, human immunodeficiency virus type 1.

<sup>b</sup> Region of the protein which was shown to contain an NLS. aa, amino acids.

<sup>c</sup> It has been shown that this sequence is part of a bipartite NLS.

TABLE 2. Phosphorylation of GCV and natural nucleosides in 143B (TK-deficient) cells infected with recombinant vaccinia viruses or different HSV strains

Compound or nucleoside	Vaccinia virus UL97 recombinants		HSV			
	pmol of nucleoside phosphates/10 <sup>5</sup> infected cells		Ratio, T1/UL97-Orc	pmol of nucleoside phosphates/10 <sup>5</sup> infected cells		
	T1	UL97-Orc		K1	K1r	
GCV	75	7	11	900	5	180
dT	28	27	1	450	9	50
dC	25	25	1	55	16	3
dA	790	800	1	788	790	1
dG	385	389	1	405	398	1

nothing is known about the biological function of this protein during the HCMV life cycle. By sequence comparisons, homologies with protein kinases have been detected, but no natural substrate has been identified. Previously we reported that the UL97 protein alone is able to confer GCV susceptibility to the naturally GCV-resistant vaccinia virus (21). We wanted to further characterize this protein by using a polyclonal antiserum raised against a bacterial UL97 fusion protein. The aims of the presented study were to (i) determine the intracellular localization and the temporal expression pattern of UL97 protein, (ii) determine a possible role of viral DNA replication for the expression of the protein, since the initiation of the viral DNA replication is the well-defined time point separating early from late events during the HCMV life cycle, and (iii) clarify whether natural nucleosides are substrates which are specifically phosphorylated by the UL97 protein.

By indirect immunofluorescence analysis, in HCMV-infected cells, an often spotty fluorescence was observed in the nuclei, suggesting local accumulation of UL97 protein. However, the nature and the composition of these spots are not known.

Our results reveal that the protein encoded by the UL97 ORF has the intrinsic capacity for the nucleus even in the absence of other HCMV proteins. This was clearly shown in cells which transiently expressed the protein as well as by experiments using the vaccinia virus-UL97 recombinants. In the latter experiments, nuclear localization was seen in the absence of any other HCMV proteins in a different cell line. The nuclear uptake of karyophilic proteins has been demonstrated to be a highly selective, signal-mediated process (7, 22). Proteins with a molecular mass higher than 60 kDa must be actively translocated into the nucleus, and such proteins have been shown to possess a specific NLS (9, 14). We found significant homology in the N-terminal region of the UL97 protein with NLSs of other viral proteins, especially with the *trans*-acting proteins of the retroviruses human T-cell leukemia virus type 1 and human immunodeficiency virus type 1 and the tegument protein pp65 of HCMV. A second basic region of the UL97 protein (RGGRKRPLRP, amino acids 190 to 199) carrying homology to sequences that have a positive influence on nuclear localization was identified. It has been shown that the polyomavirus large T antigen contains two regions (bipartite) which cooperate allowing transport into the nucleus (23). Mutation of either region impaired but did not eliminate the ability of the protein to enter the nucleus; mutations of both result in only cytoplasmic localization. A similar mechanism could be suggested for the UL97 protein, since by using 5'-

truncated UL97 ORFs, the expressed protein is clearly retained in the cytoplasm, but entry into the nucleus is not completely abolished. Schmolke et al. (25) have identified two structurally and functionally distinct bipartite NLSs within protein pp65 (A-B and C-D), which can direct nuclear uptake of large amounts of the protein immediately after infection. Deletion mutants of pp65 lacking motif C-D were impaired in nuclear import, whereas all proteins containing this sequence were detected exclusively within the nucleus. Furthermore, motif A-B contributed to the rapid nuclear accumulation of pp65, possibly influencing the kinetics of the import; however, removal of this motif did not change the nuclear accumulation.

Wing and Huang (31) performed transcription analysis of the region containing ORFs UL93 to UL99 of HCMV. The authors found a group of overlapping transcripts and suspected a transcript of 4.7 kb to be the mRNA of UL97. In Northern (RNA) blots, this transcript was detectable as early as 5 h p.i., but it was absent in the presence of CHx at 5 h p.i. Furthermore, the level of the assumed UL97 transcript appeared to be unaltered by GCV treatment at 72 h p.i. It has to be mentioned, however, that four members of the overlapping transcripts identified by Wing and Huang were transcribed at reduced levels in the presence of GCV, and all of them contain the coding region of the UL97 protein. The results of Wing and Huang, with respect to expression during viral infection, suggested an early kinetic rather than an immediate-early kinetic of the transcript. Our results based on temporal expression of the protein are in agreement with these data, since we first detected the protein 16 h p.i. Furthermore, in experiments using metabolic inhibitors, in contrast to the data obtained on the transcriptional level, we found that expression of the UL97 protein was indeed significantly affected by GCV treatment as well as by treatment with PAA, a well-known inhibitor of the viral DNA replication. This finding indicates an influence of viral DNA replication on UL97 expression. Comparing UL97 expression with that of the HCMV pp65 protein, which has been described as an early-late protein (25), we have to classify the UL97 protein also as an early-late protein. Another interesting point is the fact that GCV, which itself is phosphorylated by the UL97 protein to some extent, suppresses the expression of UL97. The important question of whether this might influence the therapeutic potential of GCV cannot be answered at the moment.

It has been shown for the TK of HSV that translation is initiated at three separate AUG codons, resulting in different polypeptides (11, 20). All of these proteins are associated with TK activity. It was suggested that the possible functions of the different polypeptides could extend the range of the potential complex of the TK, which is suspected to form dimers (11). Our results show that the first ATG of the HCMV UL97 ORF is essential especially for the restricted nuclear localization of the protein, and obviously no other proteins are expressed if this codon is present. On the other hand, the second and third AUG codons are not used for initiation of translation, but the fourth AUG may be used in the absence of the first one, resulting in a smaller protein in vitro.

High background phosphorylation of natural nucleosides in HFF and a low specific kinase activity of the UL97 protein prevented determination of the natural substrate of UL97 in the homologous system. However, the results gained from phosphorylation studies of TK-deficient cells infected with recombinant vaccinia virus strongly suggest that the natural function of the UL97 protein is not directly linked to the phosphorylation of natural nucleosides and may only fortuitously lead to the phosphorylation of GCV. A similar observation, namely, that GCV in varicella-zoster virus-infected cells is

phosphorylated not by the varicella-zoster virus TK but by a viral protein kinase, has been published recently (15).

For determination of the region responsible for phosphorylation of GCV, we wanted to use our constructs in transient expression experiments. But although we tried extensively to measure phosphorylation of GCV in cells transiently expressing the different constructs, we did not succeed in this attempt since the phosphorylating capacity of these cells is not high enough. In the future, we will try to overcome this problem by using the different UL97 constructs described above to produce recombinant vaccinia viruses.

Questions remaining to be answered are whether a correct localization of the UL97 protein is essential for the biological function of the protein and what this function is. The facts that our extensive attempts to select a recombinant UL97-deficient HCMV failed and that such recombinants could be found only as long as wild-type HCMV was present in the same culture may indicate that the UL97 ORF could be, in contrast to the HSV TK gene, essential for virus replication. Generation of a cell line stably expressing the UL97 protein as a rescue system for UL97-deficient HCMV could allow us to clarify the natural function of the protein in the viral life cycle, which is presently under investigation.

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