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UL74 of Human Cytomegalovirus Contributes to Virus Release by Promoting Secondary Envelopment of Virions▽†

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The glycoprotein (g) complex gH/gL represents an essential part of the herpesvirus fusion machinery mediating entry of cell-free virions and cell-associated viral spread. In some herpesviruses additional proteins are associated with gH/gL contributing to the cell tropism of the respective virus. Human cytomegalovirus (HCMV) gH/gL forms complexes with either gO (UL74) or proteins of the UL128-131A gene locus. While a contribution of UL128-131A to endothelial cell tropism is known, the role of gO is less clear. We studied the role of gH/gL-associated proteins in HCMV replication in human foreskin fibroblasts (HFF) and human umbilical vein endothelial cells (HUVEC). Deletions of UL74 alone or in combination with mutations of the UL128-131A gene region were introduced into bacterial artificial chromosome vectors derived from the endotheliotropic strain TB40/E. Deletion of UL74 caused a profound defect regarding virus release from infected HFF and HUVEC. Large numbers of capsids accumulated in the cytoplasm of infected HFF but failed to acquire an envelope. Clear cell type differences were observed in the cell-associated spread of the UL74-defective virus. In HFF, focal growth was severely impaired, whereas it was normal in HUVEC. Deletion of UL131A abolished focal growth in endothelial cells. UL74/UL128-131A dual mutants showed severely impaired reconstitution efficiency. Our data suggest that gO plays a critical role in secondary envelopment and release of cell-free virions independent of the cell type but affects cell-associated growth specifically in HFF, whereas UL128-131A contributes to cell-associated spread in HFF and HUVEC.

Human cytomegalovirus (HCMV) is a β-herpesvirus causing significant morbidity in immunocompromised individuals and in congenitally infected newborns. Like other β-herpesviruses, HCMV is restricted to its natural host, but in infected humans it can infect virtually every organ due to its broad cell tropism, which includes endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, macrophages and monocytes (21). With respect to modes of viral transmission, it appears that HCMV can spread either by a cell-to-cell route, which is characteristic for recent clinical isolates, or by release of enveloped progeny virions, which is the predominant route after cell culture adaptation (26). With other herpesviruses, glycoproteins have been identified as major determinants of these transmission modes (11, 17).

At least three virally encoded glycoprotein complexes are incorporated into the envelope of HCMV during secondary envelopment in the cytoplasm (9, 28), and these complexes are assumed to be engaged in adsorption to and subsequent fusion with the target cell membrane (6). HCMV glycoproteins are not only engaged in penetration of cell-free virions but are also assumed to contribute to cell-associated spread (15). Concordant with this supposition, we could show transfer of cytoplasmic material from infected to uninfected cells through cell-cell fusions (7), but the molecular basis of this transmission mode remained unclear. In epithelial cell lines, gH/gL alone was sufficient to induce cell-cell fusion (15). Thus, it appears that gH and gL together provide the basic fusion machinery of HCMV (15), very similar to other herpesviruses in which additional compounds may then determine a cell type specificity of the fusogenic gH/gL complex (4, 20). In the virion envelope of HCMV, gH and gL are complexed either with gO (pUL74) (14) or with proteins of the UL128-131A gene locus (1, 32). UL128-131A-encoded proteins are essential for growth in endothelial and epithelial cells (1, 12, 32). In contrast, the specific contribution of gO is less clear. In particular, a cell-type-dependent function might be assumed also for this protein. UL74, has been identified as a hypervariable locus in the HCMV genome, and transient expression assays have initially suggested a contribution of gO to gH/gL-mediated fusion (19). This function of gO is, however, a matter of debate, as in immortalized cell lines gO did not increase the fusogenic activity of gH and gL (15). Transposon mutagenesis on the background of HCMV AD169 and deletion on the background of HCMV Towne have classified UL74 a nonessential but replication-enhancing open reading frame (ORF) (8, 33), with disruption of gO resulting in a small-plaque phenotype (13). The functional defect causing this phenotype is unknown. In the context of a point mutation in envelope glycoprotein gN, a

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block in secondary envelopment and virus release was recently reported as the underlying reason for restricted growth (16). It is tempting to speculate that a similar mechanism applies to the reported effect of gO deletions.

Based on the fact that gO is part of the glycoprotein complex III and that its deletion/disruption results in a small-plaque phenotype, we hypothesized that gO is necessary for maturation and release of progeny virions, thus explaining the small-plaque phenotype previously reported for UL74 deletion mutants. In addition we wanted to test whether gO also contributes to cell-associated spread of HCMV. To test these hypotheses, we generated a UL74 deletion mutant of HCMV strain TB40E by using bacterial artificial chromosome (BAC) technology, which was then analyzed by immunofluorescence and electron microscopy in order to determine the role of gO in production of cell-free progeny virions and cell-associated spread in fibroblast and endothelial cell cultures. Our results indicate that gO greatly contributes to secondary envelopment and release of cell-free virions and that it facilitates cell-associated spread specifically in fibroblasts. UL128-131A, previously linked to endothelial cell tropism of HCMV (1, 12, 32), rescues cell-associated growth in fibroblasts in the absence of UL74.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFF) were isolated from foreskins of infants by trypsin treatment and were used for experiments at passages 10 to 25. HFF were cultured in minimal essential medium (Invitrogen, Karlsruhe, Germany) containing 5% fetal calf serum, 2.4 mmol/liter glutamine, and 100 μg/ml gentamicin (MEM5). Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins by chymotrypsin treatment and were used for experiments at passages 4 to 7. HUVEC were cultured in RPMI 1640 (Invitrogen) medium containing 100 μg/ml gentamicin, 5 IU/ml heparin, 50 μg/ml endothelial cell growth supplement (Becton Dickinson, Heidelberg, Germany), and 10% human serum (seronegative for HCMV). All cell preparations tested negative for mycoplasms by 4′,6′-diamidino-2-phenylindole (DAPI) staining.

The highly endotheliotropic HCMV-TB40/E-derived BAC TB40-BAC4 and its poorly endotheliotropic counterpart TB40-BAC1 (24) as well as the AD169-derived BAC pHB5 (3) were used for the generation of mutant strains. Stocks of cell-free viruses HCMV-TB40-BAC4 and HCMV-TB40-BAC4-ΔUL74nt1-37-rev were prepared as follows: HFF were infected at a multiplicity of infection (MOI) of 0.1, and supernatants of infected cultures were harvested at day 6 postinfection and stored at −80°C after removal of cell debris by centrifugation for 10 min at 2,800 × *g*. The infectious titer in HCMV preparations was determined by 50% tissue culture infective dose ($TCID_{50}$) assays in HFF on 96-well plates. For supernatant-mediated infection of cell cultures, the medium was replaced by infectious supernatant at the appropriate MOI, incubated for 2 h at 37°C, and finally replaced with fresh medium (MEM5).

BAC mutagenesis. The nucleotide sequences of all primers used for the generation of BAC mutants are given in Data S1 in the supplemental material.

For the knockout of UL74 in the genetic backbone of TB40-BAC4, a linear PCR fragment containing a kanamycin resistance gene flanked by FLP recombination target sites and HCMV homology sequences was generated from plasmid pCP15 (5) using appropriate primers. The resulting recombination fragment was inserted into TB40-BAC4 by homologous recombination in *Escherichia coli* as described previously (31), thereby deleting the start codon and the following 34 bp of the UL74 ORF. The kanamycin resistance gene was subsequently excised by FLP-mediated site-directed recombination, yielding mutant TB40-BAC4-ΔUL74nt1-37.

To reinsert the deleted 37 bp of UL74 back into TB40-BAC4-ΔUL74nt1-37, the I-SceI-*aphAI* cassette from plasmid pEPKan-S was PCR amplified with appropriate primers, adding 77 bp of HCMV sequence extensions that contained the respective 37-bp UL74 sequence. In a first Red recombination, this PCR product was inserted into TB40-BAC4-ΔUL74nt1-37, resulting in a selectable BAC with an I-SceI restriction site and a kanamycin cassette flanked by a duplication of the UL74 target sequence. After successful kanamycin selection, all non-HCMV sequences were removed from this BAC by an intrabacterial I-SceI digest and a subsequent Red recombination, resulting in a scarless "repair" of the UL74 gene in the background of TB40-BAC4-ΔUL74nt1-37. This markerless mutagenesis technique, which has been previously described in detail by Tischer et al. (30), yielded the revertant TB40-BAC4-ΔUL74nt1-37-rev.

For the generation of UL131A deletion mutants of TB40-BAC4 and TB40-BAC4-ΔUL74nt1-37, the I-SceI-*aphAI* cassette from plasmid pEPKan-S was PCR-amplified with appropriate primers. Using this amplification product, nucleotides (nt) 49 to 447 of UL131A were then replaced by the kanamycin cassette of plasmid pEPKan-S employing Red recombination. The respective mutants were designated TB40-BAC4-ΔUL131kan and TB40-BAC4-ΔUL74nt1-37-ΔUL131kan.

In a similar way, the whole UL128-131A gene region was deleted from the backbone of TB40-BAC4 and TB40-BAC4-ΔUL74nt1-37, resulting in mutants TB40-BAC4-ΔUL128-131kan and TB40-BAC4-ΔUL74nt1-37-ΔUL128-131kan.

An alternative UL74-UL131A double mutant of TB40-BAC4 was generated by markerless modification of codon number 7 of UL131A into a stop codon and subsequent replacement of the N-terminal 533 nt of UL74 with a kanamycin resistance cassette. This mutant was designated TB40/BAC4-UL131stop-ΔgO-kan.

A UL74 deletion mutant of AD169-derived BAC pHB5 was generated by markerless mutagenesis as described above. The resulting mutant BAC pHB5-ΔUL74nt1-42 carries a deletion equivalent to that of TB40-BAC4-ΔUL74nt1-37. Due to the interstrain polymorphism in the N terminus of UL74, the first 42 bp had to be removed in order to delete a second ATG in the AD169 background.

For generation of UL74-UL128 dual mutants of TB40-BAC4, an adenine insertion was introduced at position 332 of UL128, previously shown to destroy the function of this gene (24), and subsequently the first 37 nt of UL74 were removed, both by markerless mutagenesis. This mutant was designated TB40-BAC4-ΔUL74nt1-37-UL128insA332.

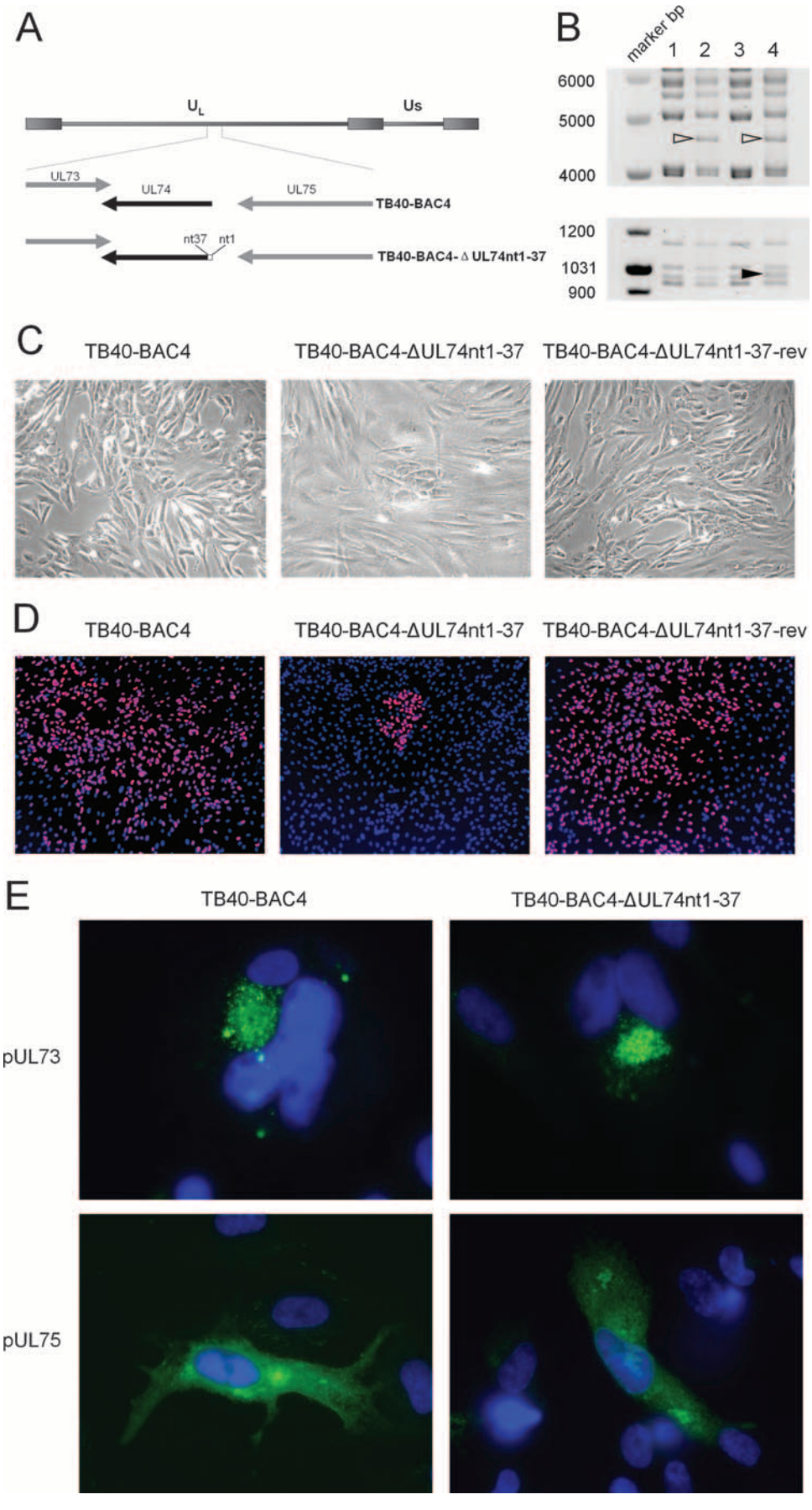
A similar UL74-UL128 dual mutant was generated on the genetic backbone of TB40-BAC1, which has a single nucleotide insertion compared to TB40-BAC4, leading to a frameshift and a nonfunctional UL128 protein. A linear PCR fragment containing the kanamycin resistance gene and HCMV-homologous sequences was amplified from plasmid pAYCY177 using appropriate primers. By homologous recombination the PCR fragment was inserted into TB40-BAC1, thereby replacing the first 533 nt from UL74 with the kanamycin resistance gene. This mutant was designated TB40-BAC1-ΔUL74kan.

From each mutant several independent clones were generated and checked for the correctness of recombinations by restriction pattern analysis, PCR, and sequencing before being used for further applications.

Reconstitution of virus from recombinant BACs. BAC DNA was isolated using the NucleoBond Xtra Midi kit (Macherey-Nagel, Düren, Germany) and transfected into HFF using an MBS transfection kit (Stratagene). Cells were propagated in normal culture medium until viral plaques appeared. If no plaques were detectable within 7 weeks after transfection, the experiment was terminated.

Immunofluorescence. To analyze the kinetics of viral gene expression, monoclonal antibodies (MAbs) against viral proteins from different phases of the replicative cycle of HCMV were used. In detail, MAbs were directed against the immediate-early (IE) proteins IE72 and IE86 (pUL122/123 with MAb E13; Biosoft, Paris, France), the early protein p52 (pUL44 with MAb BS510; Biotest, Dreieich, Germany), and the late major capsid protein (pUL86 with MAb 28-4; kindly provided by W. Britt). For in situ detection of the respective antigens by indirect immunofluorescence, infected cells were grown on 96-well plates, fixed with 80% acetone for 5 min at room temperature, washed with phosphate-buffered saline (PBS), and successively reacted with primary antibodies and Cy3-conjugated goat anti-mouse immunoglobulin G $F(ab)_2$ (Jackson ImmunoResearch) for 60 min at 37°C. Cell nuclei were counterstained with DAPI. To analyze the expression of genes neighboring UL74, indirect immunofluorescence was modified as follows. Cells were fixed with 4% paraformaldehyde for 5 min at room temperature, followed by permeabilization with 0.5% Nonidet P40–1% fetal calf serum in PBS for 10 min. MAbs directed against pUL73 (anti-gN clone 14-16A) and UL75 (anti-gH clone 14-4B) were used as primary antibodies and Alexa488-conjugated goat anti-mouse immunoglobulin G $F(ab)_2$ (Molecular Probes) was used as a secondary antibody.

Analysis of virus growth. For multistep growth curves of HCMV-TB40-BAC4 and HCMV-TB40-BAC4-ΔUL74nt1-37-rev, HFF seeded in six-well plates were infected with cell-free HCMV preparations at an MOI of 0.1 infectious units/cell. After 2 h of incubation, the cultures were washed six times with medium to remove residual virus and were then cultured for 15 days. Starting at day 1 postinfection, supernatant was removed daily from infected cultures and replaced by fresh medium; the supernatant samples were centrifuged at 2,800 × *g*



for 10 min and stored at −80°C prior to determination of the infectious titer by $TCID_{50}$ assay.

For analysis of cell-associated focal growth, infected HFF were cocultured with uninfected HFF at a ratio of 1:3,000 and incubated in the presence of high-titer neutralizing human anti-HCMV serum from a patient during acute infection, intermediate-titer neutralizing serum from latently infected individuals (anti-HCMV hyperimmunoglobulin) (Cytotect; Biotest, Dreieich, Germany), low-titer neutralizing human anti-HCMV serum from latently infected individuals, HCMV-negative serum, or no human serum. After 6 or 7 days, the number of infected cells per focus was determined by immunofluorescence detection of viral IE antigens (pUL122/123). The neutralization capacity of the serum was determined prior to experiments by quantification of IE antigen expression in HFF infected with TB40/E preparations pretreated with serial dilutions of the respective serum.

Electron microscopic observation of infected cells. HFF infected with late-stage HCMV-TB40-BAC4 and HCMV-TB40-BAC4-ΔUL74nt1-37 were seeded in MicroWell Mini Trays (Nunc). Cells were primarily fixed with MEM5 containing 2.5% glutaraldehyde for 15 min at 37°C. The medium was exchanged with PBS containing 2.5% glutaraldehyde and incubated on ice for 30 min. The cells were washed three times with PBS, followed by a secondary fixation with 1% osmium tetroxide for 1 h at room temperature. After cells were washed two times with PBS and five times with double-distilled water, the third fixation was done by incubation with 1% aqueous uranyl acetate for 1 h at room temperature in the darkness. After a final washing step with double-distilled water, samples were dehydrated and embedded in epoxy resin for ultrathin sectioning. After being stained with uranyl acetate and lead citrate, sections were subjected to electron microscopic analysis.

RESULTS

Deletion of UL74 blocks virus release and restricts the mutant virus to cell-associated growth. To test our hypothesis that the small-plaque phenotype of gO-null viruses which has previously been reported (8, 13, 33) is due to cell association of the deletion mutant, we attempted to generate a highly specific UL74 deletion mutant of HCMV. Deletions of UL74 reported up to now were complete deletions of the UL74 ORF or destruction of the gene by random transposon insertion mutagenesis. Due to the genomic context of UL74, these deletions could not formally rule out effects on the neighboring genes UL73 and UL75, as UL74 probably contains the poly(A) sites of these genes, and its 3′ part partially overlaps with the 3′ part of UL73. In order to avoid unwanted effects on UL73 and UL75, we constructed a UL74 mutant by ET cloning, in which only the known UL74 start codon and the following 34 bp containing an additional potential start codon were deleted (Fig. 1A). Moreover, the modifications were carried out on the genetic background of the TB40/E-derived BAC TB40-BAC4, which retained the natural broad cell tropism of the clinical HCMV strain and thus allowed us to compare the effects of UL74 on HCMV replication in fibroblasts and endothelial cells. After successful replacement of the target sequence by a kanamycin resistance cassette and subsequent removal of the kanamycin resistance marker, the resulting BAC TB40-BAC4-ΔUL74nt1-37 was transfected into HFF to reconstitute the mutant virus. In addition, using markerless mutagenesis, we constructed a revertant of this mutant, in which the genotype of the parental virus was restored (TB40-BAC4-ΔUL74nt1-37-rev) (Fig. 1B; also see Materials and Methods). By transfection into HFF both the UL74 mutant and its revertant could reconstitute virus, with a clear difference in the phenotype, however. While the revertant BAC formed plaques within 1 week after transfection, it took 3 weeks until plaques were detectable with TB40-BAC4-ΔUL74nt1-37. Moreover, the mutant virus grew with a small-plaque phenotype (Fig. 1C), as previously reported, whereas the revertant formed large, comet-shaped plaques indistinguishable from wild-type virus. Immunofluorescence staining of viral IE antigen corroborated the morphological data (Fig. 1D). Plaques of TB40-BAC4-ΔUL74nt1-37 contained far fewer infected cells per focus than wild-type and revertant viruses, and the foci of IE-antigen-positive cells differed in shape. Wild-type virus and revertant viruses formed large, comet-shaped accumulations of IE-antigen-positive cells whereas the UL74-deletion mutant was restricted to well-demarcated, small round foci without diffuse distribution of infected cells in the surrounding area of the focus. This pattern was reminiscent of the growth characteristics previously reported for cell-associated clinical isolates. Consistent with our cloning strategy, the expression of the neighboring genes UL73 and UL75 was identical to wild-type virus (Fig. 1E), supporting the assumption that the observed phenotype was due to the disruption of UL74.

When the reconstituted TB40-BAC4-ΔUL74nt1-37 virus had grown up to 20% cytopathic effect, the supernatant was tested for the titer of infectious virus released from these cultures. Consistently, in several independent experiments almost no infectivity was detected. For a more detailed quantification, the fraction of late-stage-infected cells expressing major capsid protein was determined. HFF cultures infected with wild-type and revertant viruses were normalized to the same number of late-stage-infected cells (Fig. 2A), and after a complete medium exchange, supernatant of all normalized cultures was sampled for 24 h. Under these conditions, release of infectious virus from TB40-BAC4-ΔUL74nt1-37-infected cultures was repeatedly found to be reduced by 2 to 3 logs compared to wild-type virus (Fig. 2B). Similarly, no release of cell-free infectivity was detected in infected endothelial cells (data not shown). Because of the severe impairment of virus release, classical growth curves were not possible for further

FIG. 1. Generation of the recombinant virus HCMV-TB40-BAC4ΔUL74nt1-37. (A) The deleted sequence of the start codon of UL74 and its downstream 34 bp are indicated. (B) Agarose gel electrophoresis of EcoRI/I-SceI-digested DNAs from TB40-BAC4 (lane 1), TB40-BAC4-ΔUL74nt1-37 (lane 2), TB40-BAC4-ΔUL74nt1-37-rev (lane 3), and TB40-BAC4-ΔUL74nt1-37-ΔUL131kan (lane 4). An open arrow points to the additional fragment which appeared due to the replacement of N-terminal 37 bp of UL74 by a FLP recombination target site containing an additional EcoRI restriction site. A filled arrow points to the fragment which appeared due to the replacement of UL131A by the I-SceI-*aphAI* cassette. (C) Phase-contrast micrographs of virus reconstituted from parental BAC and mutants in HFF by transfection of BAC DNA into HFF. (D) Focal growth of parental and mutant viruses. HFF infected with HCMV-TB40-BAC4, HCMV-TB40-BAC4-ΔUL74nt1-37, or HCMV-TB40-BAC4-ΔUL74nt1-37-rev were cocultured with uninfected HFF at a ratio of 1:3,000 for 7 days, and infected cells were then detected by indirect immunofluorescence against viral IE antigen (pUL122/123; red nuclear signals). Counterstaining was done with DAPI (blue nuclear signals). (E) Detection of pUL73 and pUL75 in fibroblasts infected with HCMV-TB40-BAC4 or HCMV-TB40-BAC4-ΔUL74nt1-37. The respective proteins were visualized by indirect immunofluorescence (green signals). Counterstaining was done with DAPI (blue nuclear signals).

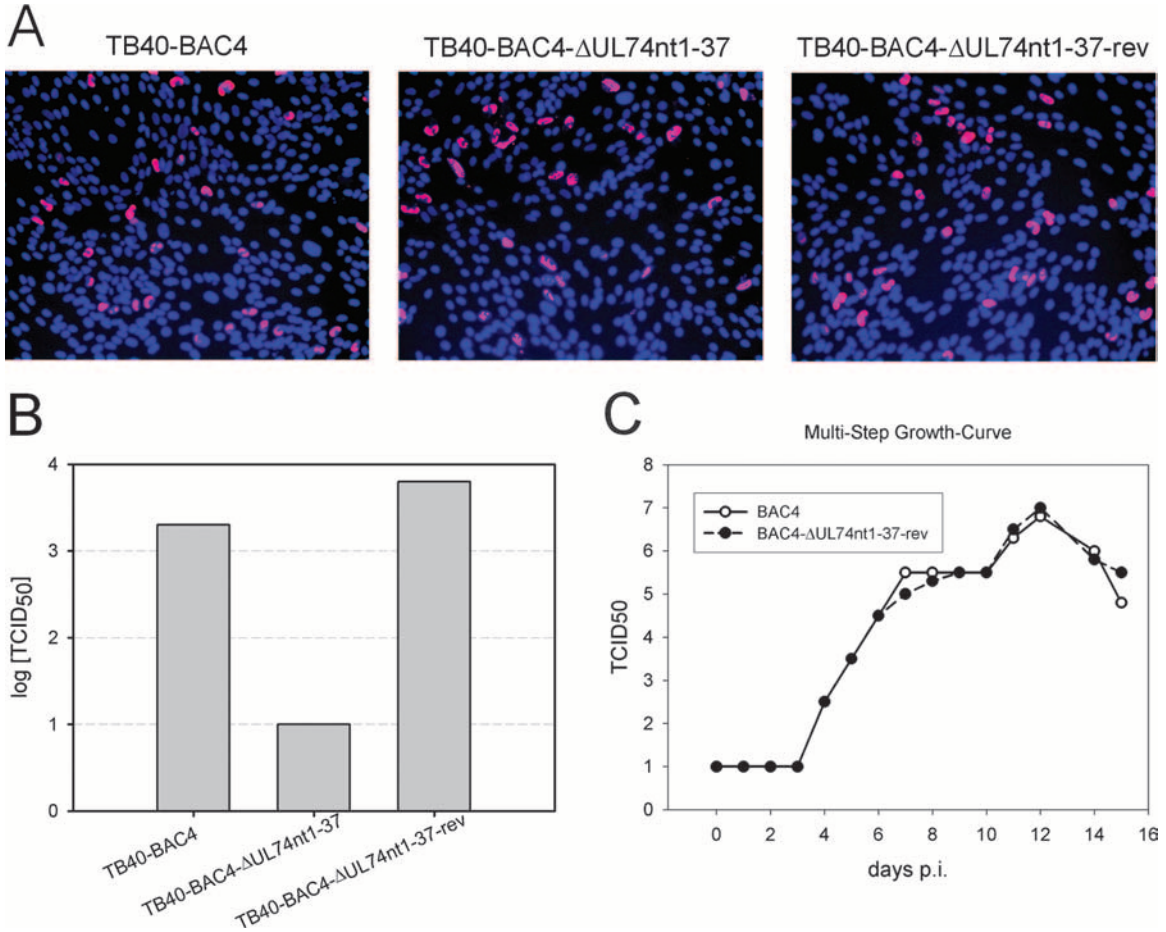


FIG. 2. Quantification of virus release by recombinant virus. (A) Detection of late-stage-infected cells in infected HFF cultures from which supernatant was taken for quantification of infectivity. Late antigen (pUL86) was detected by indirect immunofluorescence (red nuclear signals), and counterstaining was done with DAPI (blue). (B) The amount of infectious progeny released during a 24-h sampling period from cultures shown in panel A was determined by $TCID_{50}$ assays. (C) Multistep growth curves of the HCMV-TB40-BAC4 and HCMV-TB40-BAC4-ΔUL74nt1-37-rev viruses. HFF cultures were infected at an MOI of 0.1. Infectious virus progeny in the supernatants of infected cultures was determined by limiting-dilution analyses.

characterization. In contrast, the growth curve of the revertant virus perfectly resembled the growth curve of wild-type virus (Fig. 2C), supporting the assumption that the defects of the mutant were specific for deletion of UL74 and not due to any second site mutations.

Deletion of UL74 impairs secondary envelopment of progeny virions. Impaired release of infectious virus from cultures infected with the UL74 deletion mutant might have been due to a delay of progression toward the late replication phase. Therefore, we attempted to compare the antigen expression kinetics between mutant and wild-type viruses. The small amount of cell-free virus released by HCMV-TB40-BAC4-ΔUL74nt1-37 was concentrated by ultracentrifugation, which allowed us to harvest sufficient infectious material to infect HFF at an MOI of 0.001. Infections with wild-type virus HCMV-TB40-BAC4 were normalized to the same MOI. Replicates of the infections were stopped by fixation at various time points and then stained for IE, early, or late antigens. Under these conditions, no difference was found regarding the time points of appearance of IE-, early-, and late-antigen-positive cells. IE antigen appeared at 8 h, early antigen appeared at 48, and late antigen appeared at 72 h after infection (Fig. 3A).

Electron microscopic analyses were then performed to find an explanation for the block in virus release from fibroblasts infected with HCMV-TB40-BAC4-ΔUL74nt1-37. Cell cultures in which >5% of cells contained nuclear inclusions were fixed, embedded, cut into sections, and stained for transmission electron microscopy. Late-stage-infected cells characterized by nuclear inclusions but still containing an intact nuclear membrane were analyzed in order to avoid misinterpretation of membrane structures in necrotic cells. There were no obvious differences in nuclear capsid formation between UL74 mutant and wild-type viruses, and the appearance of numerous naked capsids in the cytoplasm of cells infected by either virus indicated that primary envelopment and nuclear egress were also intact. However, there was a striking difference regarding the detection of enveloped cytoplasmic particles. In wild-type-infected cells >50% of cytoplasmic virions were surrounded by a double lipid bilayer whereas not a single enveloped particle was found in 200 cells infected with the deletion mutant, representing several thousands of cytoplasmic capsids (Fig. 3B).

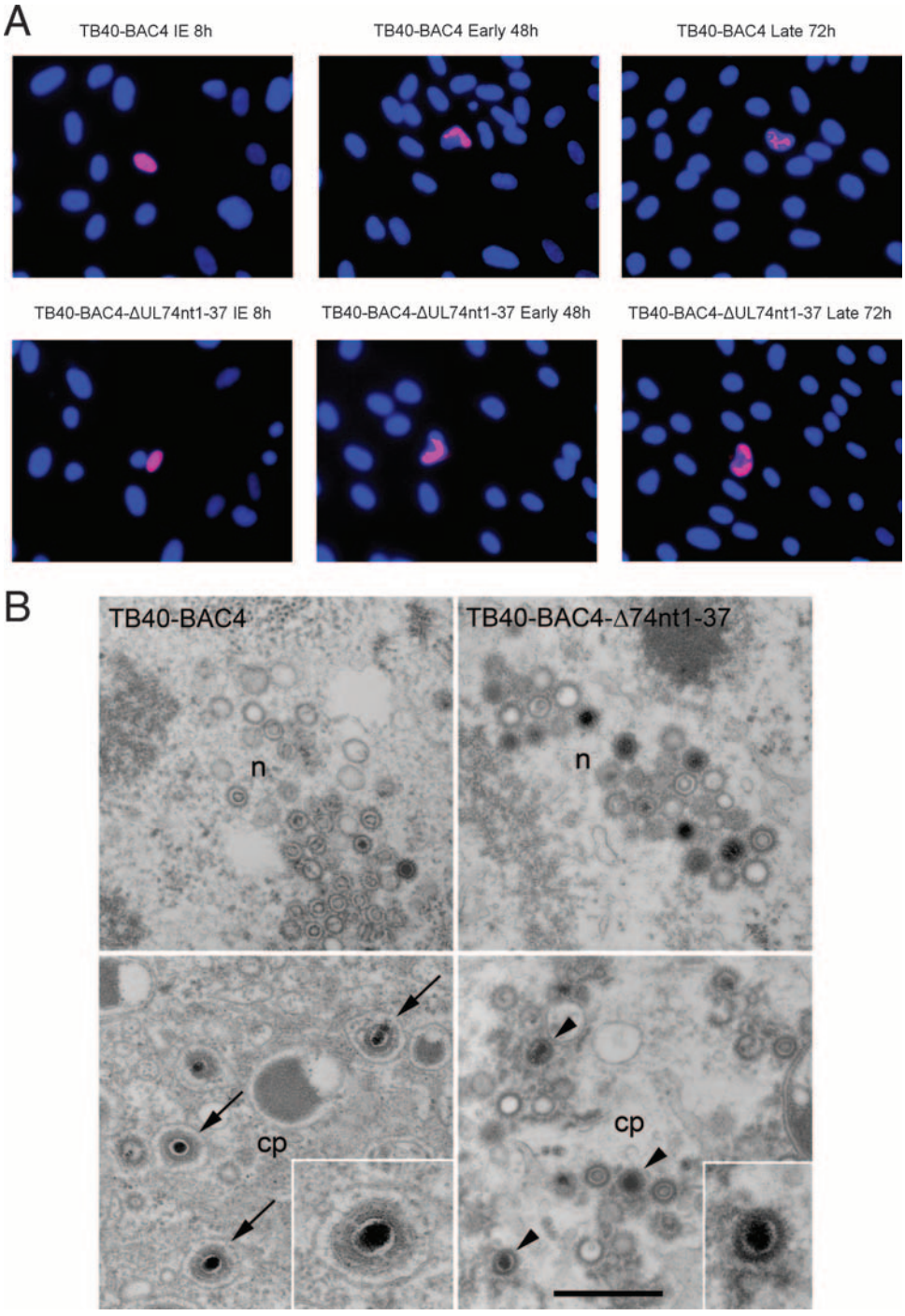


FIG. 3. Viral gene expression kinetics and ultrastructural aspect of fibroblasts infected with wild-type virus and recombinant virus. (A) Expression kinetics of viral IE antigen (pUL122/123), early antigen (pUL44), and late antigen (pUL86) in infected HFF as detected by indirect immunofluorescence. The time point of first appearance is indicated. (B) Representative electron microscopic images of fibroblasts infected with late-stage HCMV-TB40-BAC4 and HCMV-TB40-BAC4-ΔUL74nt1-37. cp, cytoplasm; n, nucleus; arrowheads, virions of HCMV-TB40-BAC4-ΔUL74nt1-37; arrows, virions of HCMV-TB40-BAC4. Scale bar, 500 μm.

Apparently, the mutant virus was severely impaired in terms of secondary envelopment.

Deletion of UL74 reduces cell-associated growth in fibroblasts. Growth of HCMV-TB40-BAC4-ΔUL74nt1-37 resembled the growth of antiserum-treated wild-type HCMV, which is commonly assumed to occur by cell-to-cell spread. This suggested that the small-plaque phenotype might be completely explained by the blocked release of viral progeny into the supernatant. Under this presumption, the plaque sizes of both conditions should be about equal. This was quantitatively

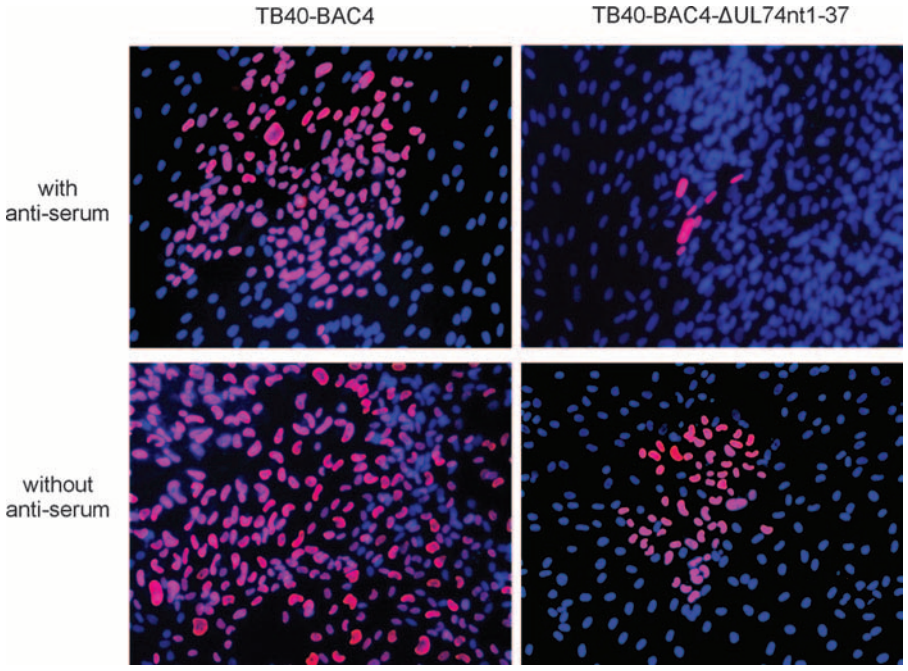


FIG. 4. Reduced focal growth of HCMV TB40-BAC4-ΔUL74nt1-37 compared to serum-treated wild-type virus. Mutant-infected HFF or wild-type-infected HFF were cocultured with uninfected HFF at a ratio of 1:3,000 in the absence or presence of human neutralizing anti-HCMV serum. Seven days after coculture, infected cells were detected by immunofluorescence against viral IE antigens (pUL122/123). Counterstaining was done with DAPI.

tested by focus expansion assays, comparing growth of HCMV-TB40-BAC4-ΔUL74nt1-37 in the absence of serum with growth of HCMV-TB40-BAC4 in the presence of neutralizing serum at concentrations that were sufficient to neutralize even high titers of cell-free virus completely (data not shown). As expected, serum treatment greatly reduced the focus size of HCMV-TB40-BAC4 and changed the appearance from irregularly delimited comet-shaped foci to well-demarcated foci resembling the clearly confined foci of the untreated mutant-infected cell cultures. However, serum-treated wild-type infections still outnumbered untreated HCMV-TB40-BAC4-ΔUL74nt1-37 infections with regard to the number of IE-antigen-positive cells per focus (148 ± 36 versus 46 ± 14) after 7 days of coculture (Fig. 4), indicating that the phenotype of the mutant was more severe than just a restriction to cell-associated spread. Unexpectedly, the focus size of HCMV-TB40-BAC4-ΔUL74nt1-37 was also further reduced by serum treatment (see below). As a major result of these experiments, it became apparent that deletion of gO reduced not only the titer of cell-free progeny virus but also cell-associated expansion in fibroblast cultures.

UL128-131A contributes to the viability of UL74 deletion virus in HFF. The residual cell-to-cell spread might have been due to the fusogenic activity of gH/gL alone or gH/gL/pUL128-131A. In order to test the hypothesis that gH/gL alone is sufficient for cell-associated spread, we generated a BAC carrying a dual deletion of UL74 and UL128-131A. No virus could be reconstituted from this dual mutant after transfection into HFF, while both the UL74 single mutant and the UL128-131A single mutant were viable in the same assay.

Analysis of additional mutants targeting UL74 in combination with either UL128 or UL131A further corroborated this finding (Table 1). Two independent approaches targeting UL74 and UL128 in the genomic background of TB40-derived BACs resulted in mutant genomes that could not reconstitute virus after transfection in HFF. Likewise, dual deletion of UL74 and UL131A also affected the capacity of the respective genomes to reconstitute virus. Only occasionally were plaques

TABLE 1. Reconstitution efficiencies of UL74 null mutants with or without additional deletions

Mutant BAC	Deletion(s)	No. of reconstitutions/no. of transfections
TB40-BAC4-ΔUL74nt1-37	UL74	9/9
TB40-BAC4-ΔUL74nt1-37-ΔUL128-131kan	UL74, UL128-131A	0/3
TB40-BAC1-ΔUL74kan	UL74, UL128	0/2
TB40-BAC4-ΔUL74nt1-37-UL128insA332	UL74, UL128	0/6
TB40-BAC4-ΔUL74nt1-37-ΔUL131kan	UL74, UL131A	1/9
TB40/BAC4-UL131stop-ΔgO-kan	UL74, UL131A	0/6
pHB5-ΔUL74nt1-42 (AD169 based)	UL74, UL131A	0/12

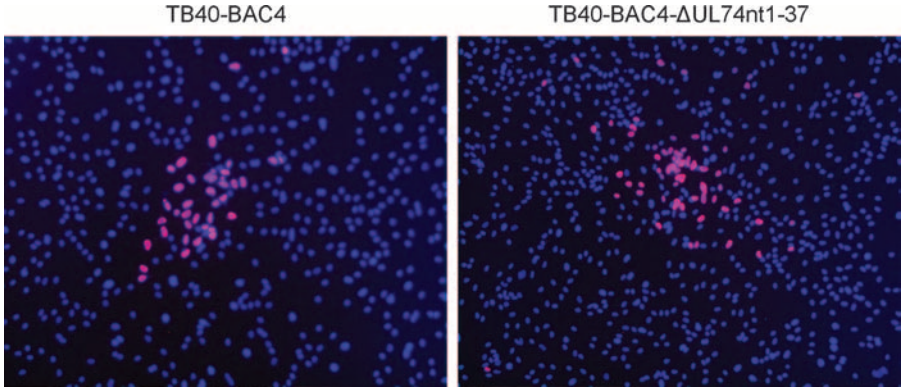


FIG. 5. Growth characteristics of the recombinant virus in HUVEC. Indicated infected HFF were cocultured with uninfected HUVEC at a ratio of 1:3,000 for 6 days, and infected cells were then detected by immunofluorescence against viral IE antigens (pUL122/123). Counterstaining was done with DAPI.

formed after transfection of TB40-BAC4-ΔUL74nt1-37-ΔUL131kan. The virus harvested from this reconstitution carries the expected deletions in UL74 and UL131A but has a slightly different restriction fragment pattern compared to the initially transfected BAC. Deletion of UL74 on the genetic background of HCMV strain AD169, known to be defective in UL131A, resulted in BAC pHB5-ΔUL74nt1-37, from which no virus could be reconstituted. The respective control BACs with a single mutation in either the UL74 or UL128-131A region could regularly reconstitute virus.

From all these experiments it appears that in the absence of UL74, the UL128-131A gene locus also contributes to growth in fibroblasts.

Virus spread in HUVEC is unaffected by UL74 deletion. The effects seen after deletion of UL74, UL131A, or both ORFs suggested a cell-type-specific role of UL74. Our experiments had shown that UL74 affects release of infectious progeny and probably also cell-to-cell spread in fibroblasts. In the presence of UL74, UL131A was completely dispensable for HCMV replication in fibroblasts while it was necessary for efficient spread in HUVEC (1). We now speculated that UL74 in turn might act in a fibroblast-specific way and be dispensable in HUVEC.

Spread of HCMV-TB40-BAC4-ΔUL74nt1-37 in HUVEC cultures was quantitatively analyzed by focus expansion assays. Fibroblasts which were infected with either the UL74-deletion mutant or the wild-type virus were cocultured with an excess of HUVEC; and after 6 days of coculture IE antigen was stained, and the number of infected cells per focus was counted. The focus sizes of wild-type and mutant were identical, indicating that deletion of UL74 had no effect on focal growth in HUVEC (Fig. 5). We then speculated that infected HUVEC might be capable of releasing viral progeny despite the lack of UL74. To test this, supernatants of infected HUVEC were analyzed for the titer of infectious virus, and results were compared to HUVEC cultures with an identical fraction of cells infected by wild-type virus. However, contrary to our speculations, release of virus from HUVEC was also blocked, indicating that focal spread in HUVEC was due to cell-associated transmission (data not shown).

Apparently, the effect of UL74 on virus release is cell type independent, whereas the effect on focal spread applies only to fibroblasts but not HUVEC.

UL74 deletion renders focal spread in fibroblasts sensitive to anti-HCMV serum. The finding that HCMV-TB40-BAC4-ΔUL74nt1-37 displayed a residual cell-associated UL128-131A-dependent growth in fibroblasts prompted us to investigate the sensitivity of this mutant to anti-HCMV serum. We had previously found that focal expansion of clinical isolates in HFF was resistant to neutralizing serum (25), but growth in HUVEC was restricted by the same serum. Based on the consideration that growth of the mutant HCMV-TB40-BAC4-ΔUL74nt1-37 in fibroblasts depended on UL128-131A (like growth of a wild-type isolate in HUVEC), we speculated that the mutant might be sensitive to serum in fibroblasts also. To test this hypothesis, we compared focal expansion of a clinical HCMV isolate and HCMV-TB40-BAC4-ΔUL74nt1-37 in the absence or presence of high-titer-neutralizing human anti-HCMV serum in both cell types. As expected, focal growth of the mutant virus was restricted by high-titer-neutralizing anti-HCMV-serum (Fig. 6) or intermediate titer anti-HCMV serum pool (data not shown) in both cell types, whereas the clinical isolate grew unrestricted in HFF in the presence of either serum. This effect was due to neutralizing antibodies contained in the serum as low-titer-neutralizing human anti-HCMV serum and HCMV-negative serum had no effect on focal growth (data not shown). Obviously, deletion of UL74 not only restricted the resulting HCMV mutant to cell-associated growth but also rendered the mutant more sensitive to restriction by neutralizing anti-HCMV antibodies.

DISCUSSION

Envelope glycoproteins of herpesviruses play an important role for the initial replication steps like adsorption and penetration and also for maturation of progeny virions and for cell-to-cell spread. Recently, they were also found contributing to nuclear egress (10). In particular, the fusogenic glycoprotein complexes are central for successful replication, with gH and gL as highly conserved constituents of these complexes. gH and gL are associated with additional proteins in some herpesviruses (27), including the β-herpesviruses human herpesvirus

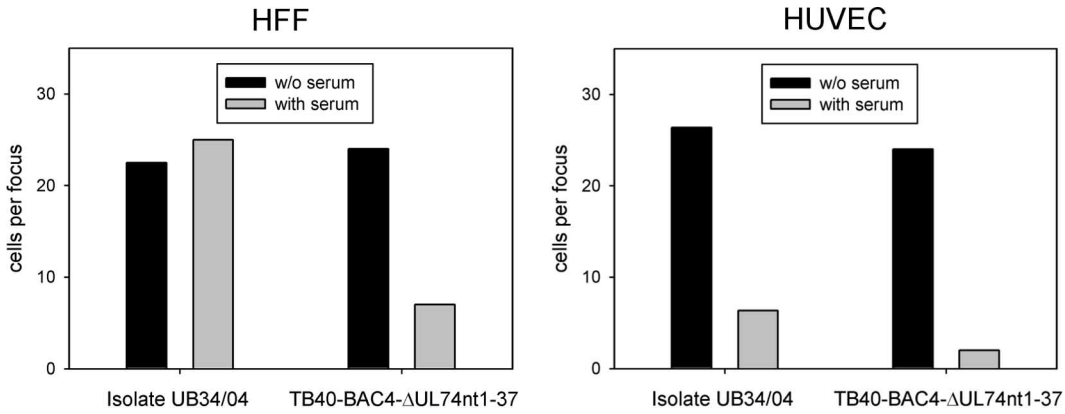


FIG. 6. Comparison of recombinant virus HCMV-TB40-BAC4-ΔUL74nt1-37 and a cell-associated recent clinical isolate regarding serum sensitivity of focal growth in HFF and HUVEC cultures. HFF infected with either HCMV-TB40-BAC4-ΔUL74nt1-37 or a recent clinical isolate were cocultured with uninfected HFF or HUVEC at a ratio of 1:3,000 in the presence or absence of human neutralizing anti-HCMV serum obtained from a patient during acute infection. Six days after coculture, infected cells were detected by immunofluorescence against viral IE antigens (pUL122/123). The number of infected cells/focus was counted to quantify focal expansion. Mean values of three experiments are given.

6 (HHV6) and HHV7, which are closely related to HCMV. In HHV6, gH/gL associates with a gQ1-gQ2 dimer to form a heterotetrameric complex or with gO to form a trimeric complex, and the various complexes bind to different receptors (2, 18, 20). In HHV7 virions pUL47, the positional homologue of HCMV gO, was found associated with gH (22). In Epstein-Barr virus variation in gH/gL complex formation has been linked to cell tropism. A complex of three virus glycoproteins, gH, gL, and gp42, is essential for entry into B cells whereas entry into epithelial cells requires complexes without gp42. In order to accommodate infection of both cell types, the virus can carry both three-part and two-part complexes, but the composition may also vary, depending on the cell type in which the virus was produced (4). In the case of HCMV, gH and gL are complexed either together with gO (14, 29) or with proteins of the UL128-131A gene locus (1, 32). gH and gL are known to be absolutely essential for viral replication (8, 13, 33). UL128-131A proteins are necessary for growth in endothelial and epithelial cell cultures (12, 32). The contribution of gO was less defined. Deletion of the respective gene UL74 was reported to result in a "small-plaque phenotype" (13), but the defects mediating this growth restriction were not known. Therefore, we sought to determine (i) whether gO contributes to release of cell-free virions and/or cell-to-cell spread, (ii) whether gO acts in a cell-type-specific manner like pUL128-131A, and (iii) whether gH/gL alone is also sufficient for replication of HCMV.

The results of our study strongly suggest that deletion of UL74 inhibits release of infectious virus progeny by blocking secondary envelopment. It appears that UL74 deletion mutants are restricted to cell-associated spread and that this mode of transmission greatly depends on the UL128-131A gene locus. In fibroblasts, UL74 contributes to an antiserum-resistant mode of focal spread.

Our finding that mutation of an envelope glycoprotein of HCMV blocks maturation and release of progeny virions while still allowing for cell-associated virus growth is not unprecedented, as a similar phenotype was reported for a mutant virus with a cysteine-to-serine exchange at position 126 of the essential envelope glycoprotein gN (16). Very similar to the reported gN phenotype, our gO deletion led to a cytoplasmic accumulation of nonenveloped particles and reduced the release of infectious progeny by several orders of magnitude. Interestingly, a similar phenotype has also been reported for deletion of the tegument protein pUL99 (23). Obviously, secondary envelopment of HCMV is a multicomponent process in which either of the components is essential for successful maturation. Our mutation strategy with limited deletion only of two possible start codons of UL74 greatly precludes unwanted effects on neighboring reading frames. This was important as both UL73 and UL75 encode essential envelope glycoproteins (gN and gH, respectively). In addition to maintaining the 3′ part of UL74, which overlaps with the 3′ part of UL73, we also took care to preserve possible poly(A) sites of UL73 and UL75, and thus the effects of our deletion are most likely due to the lack of gO. This might include direct as well as indirect effects of this protein. Cultures infected with the mutant virus showed a 100- to 1,000-fold reduction of virus release, and ultrastructural analyses revealed an almost complete lack of secondary envelopment as the likely reason for the previously reported small-plaque phenotype (13). As a consequence of this defect, HCMV-TB40-BAC4-ΔUL74nt1-37 was almost completely restricted to cell-associated growth. An explanation for an effective cell-associated spread despite a profound defect in envelopment is provided by the assumption that nonenveloped particles are transmitted through cell-cell fusions. The potential of gH/gL to induce such cell-cell fusions has been demonstrated in a transient expression system (15). Moreover, using a green fluorescent HCMV mutant, we recently showed that transfer of cytoplasmic material from infected cells to uninfected bystanders actually takes place in the context of an intact virus (7).

Although it is generally accepted that complexes containing gH and gL mediate fusion events during HCMV infection, the requirements for additional complex partners were unclear. By using transient cotransfection of glycoproteins, it has been shown that gH/gL alone can be sufficient to induce cell-cell fusions (15), but this effect was restricted to certain immortal-

ized cell lines. In primary human endothelial and epithelial cells, pUL128-131A is necessary for infection (1, 12, 32). The situation in primary fibroblasts appears to be more complex, i.e., both gO and pUL128-131A contribute to successful replication. In the absence of gO, HCMV can grow in a cell-associated manner in fibroblasts, and the UL128-131A gene region apparently contributes to this residual growth, as indicated by our results with double mutants. The efficiency of reconstitution was dramatically reduced when UL128-UL131A or parts thereof were deleted in addition to disruption of UL74. Only occasionally could viable virus be reconstituted with TB40-BAC4-ΔUL74nt1-37-ΔUL131kan, whereas no other double mutants could reconstitute virus. While this strongly suggests a contribution of UL128-131A to growth in fibroblasts in the absence of UL74, it also indicates that this double deletion is not absolutely lethal. Given the changes in the restriction fragment pattern which occurred during reconstitution, it is tempting to speculate that compensating second site mutations have been acquired after transfection of the mutant BAC into fibroblasts during replication of the transfected genomes. This appears possible as the block of UL74 mutants occurred during the late replication phase. Transfected BACs can therefore express IE and early genes and probably replicate their genome, allowing for the accumulation of second site mutations, which might occasionally rescue the function of the gH/gL complex. We are currently investigating the reconstituted TB40-BAC4-ΔUL74nt1-37-ΔUL131kan virus for such mutations. Although this is highly speculative at this moment, it may also explain previous reports where UL74 mutants on the background of AD169 or Towne were viable (8, 13, 33). AD169 has a defect in UL131A, and Towne has a defect in UL130; therefore, UL74 deletions from these genomes might be expected to behave in the manner of our dual mutant. Considering possible alternative explanations, it is noteworthy that both AD169 mutants were generated by transposon insertion mutagenesis, perhaps allowing for a residual UL74 function. The UL130 defect in Towne concerns only the very C-terminal part of the protein and also may allow for a residual function of this protein in infected fibroblasts, whereas the UL131A deletion tested here has been reported to abolish expression of the complete UL128-131A gene region. To further corroborate our negative results, we additionally tested a UL74atg mutant on the background of the AD169-BAC pBH5. In repeated attempts, four different clones of pHB5-ΔUL74nt1-42 could not reconstitute viable virus. Taken together, our experiments with various UL74/UL128-131A mutants indicate that UL128-UL131A not only is essential for growth in endothelial cells (1, 12) but also contributes to cell-associated growth in fibroblasts. Our results also suggest that gH and gL cannot efficiently mediate cell-free or cell-associated infection in primary fibroblasts without their known viral complex partners.

Our finding that UL128-131A contributes to replication in fibroblasts in the absence of gO also has implications with regard to cell type specificity of gH/gL-containing complexes. It means that gO rather than UL128-131A acts in a cell-type-dependent way. In particular, this scenario suggests the following: (i) gH/gL together with proteins of the UL128-131A gene region can mediate cell-associated spread, irrespective of the cell type as the effect of UL128-131A deletions on the background of the UL74 mutant on growth in fibroblasts resembles the effect of a single UL131A deletion in endothelial cells; (ii) gH/gL/gO contributes to HCMV spread specifically in fibroblasts as focal growth of HCMV-TB40-BAC4-ΔUL74nt1-37 was unaltered in endothelial cell cultures but reduced in fibroblast cultures; (iii) in contrast, the contribution of gO to release of cell-free virus is cell type independent.

In the search for a biological role of the gO-mediated viral spread in fibroblasts, the differential sensitivity to antibody-mediated neutralization of the UL131A-mediated spread and the gO-mediated spread might provide an answer. While cell-associated growth of recent clinical HCMV isolates in fibroblasts has previously been shown to resist neutralizing human anti-HCMV serum (25), growth of wild-type virus in HUVEC, which depends on the UL128-131A gene products, was sensitive to human antiserum. The residual cell-associated growth of HCMV-TB40-BAC4-ΔUL74nt1-37 in fibroblasts was also inhibited by human antiserum, suggesting that pUL128-131A-mediated cell-to-cell spread can be neutralized by anti-HCMV-serum. Consistent with this assumption, focal growth of HCMV-TB40-BAC4-ΔUL74nt1-37 could be specifically inhibited by pUL131A antiserum (data not shown). It appears that UL74 renders cell-associated spread in fibroblasts insensitive to neutralizing antisera and thus may give an edge to HCMV for survival in the host.

Taken together, our findings indicate that pUL74 contributes to release of cell-free virus progeny from fibroblasts and endothelial cells by blocking secondary envelopment, whereas pUL128-131A contributes to cell-associated growth in both cell types. The additional contribution of pUL74 to antiserum-resistant cell-associated growth may provide an important advantage for survival of HCMV in its host.

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