The human cytomegalovirus UL78 gene is highly conserved among clinical isolates, but is dispensable for replication in fibroblasts and a renal artery organ-culture system

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The human cytomegalovirus (HCMV) UL78 ORF is considered to encode a seven-transmembrane receptor. However, neither the gene nor the UL78 protein has been characterized so far. The objective of this study was to investigate the UL78 gene and to clarify whether it is essential for replication. UL78 transcription was activated early after infection, was inhibited by cycloheximide but not by phosphonoacetic acid, and resulted in a 1-7 kb mRNA. Later in the replication cycle, a second mRNA of 4 kb evolved, comprising the UL77 and UL78 ORFs. The 5′ end of the UL78 mRNA initiated 48 bp upstream of the translation start and the polyadenylated tail started 268 bp downstream of the UL78 translation stop codon within the UL79 ORF. By using bacterial artificial chromosome technology, a recombinant HCMV lacking most of the UL78 coding region was constructed. Successful reconstitution of the UL78-deficient virus proved that the gene was not essential for virus replication in fibroblasts. The deletion also did not reduce virus replication in ex vivo-cultured sections of human renal arteries. Analysis of viral proteins at different stages of the replication cycle confirmed these results. Among clinical HCMV isolates, the predicted UL78 protein was highly conserved. However, an accumulation of different single mutations could be found in the N-terminal region and at the very end of the C terminus. Due to the absence of an in vivo HCMV model, the role of UL78 in the pathogenesis of HCMV infection in humans remains unclear.

INTRODUCTION

In human cytomegalovirus (HCMV), the ORFs US27, US28 and UL33 encode putative G protein-coupled receptor (GPCR) homologues (Chee et al., 1990). A fourth ORF, UL78, is also thought to encode a GPCR. However, no experimental data exist concerning this function, so it is currently adequate to consider UL78 as a seven-transmembrane (7TM) protein (Gompels et al., 1995). GPCRs play key roles in transduction of extracellular signals into the cell. Activation of these receptors most often requires binding of ligands, e.g. cytokines. The US28 protein (pUS28) has been shown to be a calcium-mobilizing receptor for several β-chemokines (Gao et al., 1993) and has been suggested to be responsible for β-chemokine sequestration in HCMV-infected fibroblasts in vitro (Bodaghi et al., 1998). It may also induce migration of infected smooth muscle cells (Streblov et al., 1999). It has been shown that pUS28 mediates constitutive, ligand-independent, pertussis toxin-insensitive activation of the phospholipase C Gq/11-dependent signalling pathway (Casarosa et al., 2001), and neither RANTES nor MCP-1 had any effect on this activity. We have shown that infection of fibroblasts with HCMV led to a vigorous, constitutively enhanced formation of inositol phosphates, which was insensitive to pertussis toxin (Minisini et al., 2003). The UL33 protein has been described as non-essential for growth in fibroblasts in vitro (Margulies et al., 1996). However, it has recently been shown that its homologue in rat cytomegalovirus (RCMV), the R33 protein (pR33), is important for viral pathogenesis. Unlike wild-type virus, an RCMV R33-deficient mutant could neither enter into, nor replicate in, the salivary-gland epithelial cells of infected rats (Beisser et al., 1998). A similar observation was made for mouse cytomegalovirus (MCMV) M33. Upon infection of mice with an MCMV M33-deletion mutant, virus could not be recovered from mouse salivary glands (Davis-Poynter et al., 1997). It has also been shown that pM33
exhibits constitutive signalling activity (Waldhoer et al., 2002). We have reported that, like pR33, pUL33 activates multiple signalling pathways in a ligand-independent manner (Casarosa et al., 2003). Although both receptors constitutively activate phospholipase C via Gq/11-mediated, and partially G12/13-mediated, pathways, they exhibit profound differences in the modulation of CAMP-responsive element (CRE) activation – pR33 constitutively inhibits, whereas pUL33 constitutively enhances CRE-mediated transcription. The genomes of the β-herpesviruses human herpesvirus (HHV)-6A, HHV-6B and HHV-7 contain homologues of two 7TM receptors, namely UL12, which is homologous to HCMV UL33, and UL51, which is homologous to HCMV UL78 (Gompels et al., 1995; Nicholas, 1996; Megaw et al., 1998). Interestingly, both the UL33- and UL78-like genes exhibit conservation of position, as well as orientation, within the genomes of β-herpesviruses. Until now, six UL78-like genes had been identified: HCMV UL78, MCMV M78, RCMV R78 and the U51 ORFs of EBV, HHV-6A, HHV-6B and HHV-7 (Gompels et al., 1995; Nicholas, 1996; Rawlinson et al., 1996; Beisser et al., 1999). HHV-6 U51 is known to be expressed on the surface of infected T cells and has been found to bind RANTES/CCL5 with nanomolar affinity, as well as to downregulate the transcription of this chemokine (Menotti et al., 1999; Milne et al., 2000). It has been reported that deletion of the R78 ORF in RCMV confers an attenuated syncytium-inducing phenotype (Beisser et al., 1999). MCMV pM78 led to an accumulation of viral mRNAs and is a constituent of the virion (Oliveira & Shenk, 2001).

To our knowledge, this is the first study of the HCMV UL78 gene. We identified the transcription start point and the start of the polyadenylated tail. We found differences in the regions carrying polyadenylation signals for UL78-like mRNAs in rodent and human CMVs. Specifically, we generated and characterized a UL78-deficient mutant where (Borst et al., 1999), resulting in pSTdelUL78. It has been reported that deletion of the R78 ORF in RCMV confers an attenuated syncytium-inducing phenotype (Beisser et al., 1999). MCMV pM78 led to an accumulation of viral mRNAs and is a constituent of the virion (Oliveira & Shenk, 2001).

**METHODS**

**Cells and viruses.** HCMVs were propagated in human foreskin fibroblasts (HFFs) as described elsewhere (Michel et al., 1996, 1998). Eleven clinical isolates from nine patients from different clinics (one newborn, four children aged 1–5 years and four adults aged 38–77 years) were taken from our diagnostic virus laboratory from the newborn, four children aged 1–5 years and four adults aged 38–77 years) were taken from our diagnostic virus laboratory from the newborn, four children aged 1–5 years and four adults aged 38–77 years.

**Virus titration.** Quantification of viral titres was performed by standard plaque assays with end-point dilutions in triplicate. Virus yield assays (single-cycle replication) were performed on HFFs. Briefly, HFFs were grown in 48-well plates and infected 2 days later. Following adsorption, the monolayers were washed and fresh culture medium was added. At various days post-infection (p.i.), cell-culture supernatants were harvested and titrated in triplicate on HFFs. Cells were fixed and stained with a mAb against the viral pp65 (pUL83). Viral titres were determined by counting the number of antigen-positive cells.

**Plasmid construction and bacterial artificial chromosome (BAC) mutagenesis.** Plasmid cloning was performed by using standard methods (Sambrook et al., 1989). Construction of the shuttle plasmid pSTdelUL78 was done by insertion of DNA fragments amplified with the Expand High Fidelity PCR system (Roche). For generation of the shuttle plasmid, two regions were cloned separately. Firstly, a fragment of 1.9 kbp containing 113 bp of the S′-UL78-coding region was amplified with primers Xba78b (5′-CGGGTGCGCAGAAGGATCCGGTCAC-3′) and Xba78a (5′-TAGCCGATGtcTAGATCCGAGCGG-3′) [nucleotides mutated to create the restriction sites (underlined) are in lowercase]. An internal primer Hind-78 (5′-CGGGTGCGCAGAAGGATCCGGTCAC-3′) and Xba78a (5′-TAGCCGATGtcTAGATCCGAGCGG-3′) [nucleotides mutated to create the restriction sites (underlined) are in lowercase] cut with HindIII and XbaI and inserted into pUC19, resulting in plasmid pUCUL78-5′. Secondly, a 2.1 kbp fragment containing 159 bp of the 3′-UL78-coding region was amplified with primers Xba78b (5′-AACACCTGtcGTAAGAGCCCAGTGTTG-3′) and Eco-78 (5′-GACGGCGGGGATCCTCCGGAGA-3′), cut with XbaI and EcoRI and subcloned into XbaI/EcoRI-digested pUCdel78-5′. From the second plasmid, the entire insert was excised by EcoRI/HindIII digestion and inserted into the shuttle plasmid pST767K (Pósífa et al., 1997), resulting in pSTdelUL78.

**Generation and reconstitution of the HCMV recombinant.** Mutagenesis of the HCMV BAC plasmid pHB5 with the shuttle plasmid pSTdelUL78 was performed in Escherichia coli strain CBTS as described previously (Borst et al., 1999; Wagner et al., 2000). Selection and identification of BAC plasmids lacking most of the UL78-coding region were performed as described previously (Wagner et al., 2000). Maxi-preparations of BAC plasmid DNA and reconstitution of infectious virus were performed as described elsewhere (Borst et al., 1999; Casarosa et al., 2003). As an important control, we used the virus RVHB5, obtained from transfection and reconstitution of BACmid pHB5. Cells were passaged 7 days after transfection and cultured until cytopathic effect became visible.

**Viral nucleic acid isolation and analysis.** Fibroblasts were infected at an m.o.i. of 1 and harvested 3 days p.i. Extraction of total DNA from infected cells and Southern blot analysis were performed as described previously (Wagner et al., 2000; Casarosa et al., 2003). The probe used represented nt 112520–112921 of the HCMV genome, comprising the 3′-terminal region of the UL77 ORF and the 5′ region of the UL78 ORF (AD169 strain, GenBank accession no. X17403).

**Primer extension.** Primer extension was performed as described previously (Michel et al., 1993). Briefly, HFFs were infected with HCMV strain AD169 at an m.o.i. of 1. Cells were harvested at 24 h p.i. and total RNA was extracted by using a Midi RNA Extraction kit (Qiagen) as recommended by the manufacturer. Total RNA (20 μg) was annealed for 5 h to 100 ng of a UL78-specific primer: 5′-CGGACATAGAGTACGTGG-3′ (spanning nt 113026–113007, labelled with 32P)ATP by using T4 kinase. The nucleic acid was precipitated and resuspended in 300 μl 50 mM Tris/HCl (pH 8.3), 50 mM KCl, 6 mM MgCl2, 100 μM each dNTP, 5 mM dithiothreitol (DTT), 30 μg actinomycin ml−1, 50 μg BSA ml−1, 1 μg RNasin ml−1 and incubated for 30 min at 42°C with 67 U avian myeloblastosis virus (AMV) reverse transcriptase. The extension products were resuspended in 10 μl double-distilled water and separated on a 6% polyacrylamide gel.

**Identification of the UL78 3′ end.** For cDNA synthesis, total RNA from HCMV-infected fibroblasts was hybridized with the primer mix 5′-GTTAAAAAGCAGGCGCAGGTTTGAATTTTTTTTTTTTTTTT-3′, which contains the sequence for the UL78 gene.
complementary to the M13 universal primer. The reaction mix consisted of 3 μg RNA, 10 μM primer, 1/5 vol. first-strand buffer (Gibco-BRL), 200 μM each dNTP, 50 mM DTT, 100 U RNasin (Promega) and AMV reverse transcriptase. After incubation for 30 min at 37 °C, synthesis was stopped by heating to 94 °C. A 5 μl aliquot of the reaction mix was used for amplification with the M13 universal primer and the UL78-specific primer 1160 (5′-TCTGGTG CGAGAGATGTCAGC-3′) or 1360 (5′-TGTTGGTAACGACAAACC- ACG-3′), hybridizing 355 and 159 bp, upstream of the translation stop codon, respectively. Amplicons were sequenced and the obtained sequences were aligned with the strain AD169.

**Sequencing of UL78 regions from clinical isolates.** Total DNA was extracted from infected fibroblasts by proteinase K digestion as described previously (Michel et al., 2001). UL78 sequences were amplified by PCR. Primers were designed on the basis of data from strain AD169 (GenBank no. CAA35351.1). For amplification, the primers 5′-GGGTTATTTGTCCCGGAG-3′ and 5′-TATCTGC ACTTTTTTCTCCCGG-3′ were used, both hybridizing outside the UL78-coding region. All amplifications were done in duplicate. PCR was performed as follows: 2 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 15 s at 58 °C and 120 s at 72 °C, using a GeneAmp 9700 PCR cycler (Perkin-Elmer) and the Expand High Fidelity PCR system (Roche). Amplification products were purified by using a GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Direct sequencing was performed as described previously (Michel et al., 2001). Briefly, for sequencing, an ABI Prism Cycle Sequencing kit (Applied Biosystems) was used according to the manufacturer’s instructions (Roche Diagnostics) on the GeneAmp 9700 PCR cycler (Perkin-Elmer), performing the program as recommended by the manufacturer and using the sequencing primers UL78-675P (5′-AGACCGCGGAACAGCGG-3′), UL78-1195M (5′-GAGTGCAGCAGCATGTGGG-3′), UL78-250M (5′-CCCTTG GACAAACATGTGGG-3′), UL78-500 (5′-ATAGGATCTGAGCAC AGCTTAGG-3′), UL78-700 (5′-ATAGGGATCTGAGCAC AGCTTAGG-3′), UL78-outP (5′-GGGTTATTTGTCCCGGAG-3′), UL78-outM (5′-TATCTGCACCTTTCTCCCCG-3′), 1160 (5′- TCTGGTGCGAGAGATGTCAGC-3′), 1360 (5′-TGTTGGTAACGAC AAACCAGC-3′) and UL78-970 (5′-CTACTACTGCTGACAGTCT-3′). DNA sequences were separated in a 310 Genetic Analyser (Applied Biosystems).

**Western blot analysis.** Western blot analysis was performed as described previously (Michel et al., 1996, 1998) using mouse mAbs directed against the HCMV proteins IE1, pUL69, pp65 and pp28 (kindly provided by M. Mach, Universität Erlangen, Germany) and a polyclonal antiserum against pUL97 (Michel et al., 1996). Briefly, fibroblasts were infected at an m.o.i. of 1 with the indicated virus strains and total cell lysates were extracted at different time points p.i. SDS-PAGE was performed according to standard protocols. Proteins were visualized with an enhanced chemiluminescence (ECL) reaction according to the manufacturer’s recommendations (Amersham Biosciences).

**Northern blot analysis.** HFFs were infected at an m.o.i. of 1 and harvested at the indicated time points. To arrest virus replication in the immediate-early or early stage, the metabolic inhibitors cycloheximide (CHX, 100 μg ml⁻¹) and phosphonoacetic acid (PAA, 250 μg ml⁻¹) were added to the infected fibroblasts as described previously (Michel et al., 1996). Total RNA was extracted by using a Midi RNA Extraction kit (Qiagen). Total RNA (10 μg) was separated as described by Sambrook et al. (1989). RNA was transferred on to a nylon membrane and immobilized by incubation for 2 h at 60 °C. For subsequent hybridization, DNA probes specific for UL78 and UL77 or for the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were amplified by PCR. Membranes were stripped for 20 min with 0-1 x SSC and 0-1% SDS for rehybridization. Probes were labelled with [³²P]dCTP by using a random priming kit (Pharmacia). Hybridization was carried out at 42 °C for 18 h in the presence of 50 % formamide, 2 × Denhardt’s solution, 5 × SSPE, 0-05 % SDS and 200 μg salmon sperm DNA ml⁻¹. Filters were washed to a stringency of 0-2 × SSC, 0-1 % SDS at 65 °C.

**Renal artery organ-culture system.** Human renal arterial segments were obtained after informed consent of the patients. Segments were cultivated according to Voisard et al. (1999) and Reinhardt et al. (2003) in a mixture of Ham’s F-12/l-glutamine medium and Waymouth medium (1:2) (BioWhittaker) supplemented with 15 % FCS, 1 % penicillin/streptomycin at 37 °C and 5 % CO₂. Segments were inoculated with 5 × 10⁵ cell-free virus particles ml⁻¹ of either AD169 or the recombinant HCMV viruses, or were mock-infected. After 24 h, the arterial segments were washed three times with PBS and further cultivated as described above. Once a week up to day 56 p.i., aliquots of the culture supernatant were quantified for infectious virus. All of the tested supernatant aliquots from mock-infected segments were always negative for HCMV, irrespective of whether the donor was seropositive or seronegative (Reinhardt et al., 2003).

**RESULTS**

**Transcription of HCMV UL78 mRNA in infected fibroblasts**

Northern blot analysis was performed to determine the presence of UL78 mRNA. Fibroblasts were infected and total RNA was extracted at 16 and 36 h p.i. As shown in Fig. 1(a), a RNA band of approximately 1.7 kb was detectable at 16 h p.i. and at the later time point, the UL78-specific probe clearly identified an additional mRNA species migrating at approximately 4 kb. To further elucidate the temporal kinetics of UL78 transcription, infected cells were treated with CHX or PAA. As shown in Fig. 1(b), the appearance of the UL78 1.7 kb mRNA could be detected by 4 h p.i. and was inhibited completely by CHX, but not by PAA, treatment in fibroblasts. On the other hand, the larger transcript of approximately 4 kb was clearly inhibited by PAA. These observations are in line with the recent results of Wang et al. (2004). In conclusion, these results classified UL78 as an early gene that is also transcribed during the late stage of infection. The larger mRNA is a bicistronic transcription unit consisting of UL77 and UL78 coding regions (see Fig. 4).

**Transcription start point and start of the polyadenylated tail**

To identify the UL78 transcription start, primer-extension analysis was performed. A UL78-specific oligonucleotide was annealed to total RNA prepared from HCMV-infected fibroblasts and extended by reverse transcriptase. As depicted in Fig. 2(b), the start of the UL78 mRNA was located 48 bp upstream of the translation start codon of the UL78 ORF at nt 112876 (according to the AD169 sequence; GenBank accession no. X17403). For identification of the 5′ end of the UL78 mRNA, RT-PCR was performed. An anchor primer was used with a sequence complementary to polyadenylated tails of mRNA. Next, two different UL78-specific primers were used in order to amplify the 3′ end of the UL78 mRNA. A fragment of approximately 360 bp was
expected when using primer 1160 and a fragment of approximately 160 bp with primer 1360. Independent experiments were done with target RNA from AD169-infected fibroblasts and cells infected with a clinical HCMV isolate. PCR results revealed unexpectedly that both amplified fragments were approximately 250 bp larger than predicted. These results suggested that the site within the UL78 mRNA where the polyadenylated tail is attached comprised sequences of the UL79 ORF. Sequencing of the amplicons revealed that the polyadenylated tail started 268 bp downstream of the UL78 translation stop codon (see Fig. 2b). As shown in Fig. 2, these results suggested that a putative polyadenylation signal (AATAAA) is located 18 bp upstream of the polyadenylation site. A similar configuration can be found in the chimpanzee CMV (ChCMV) genome. As summarized in Fig. 3, to date, several members of a UL78-like gene family have been identified in β-herpesviruses: MCMV M78 (Rawlinson et al., 1996), RCMV R78 (Vink et al., 2000), ChCMV UL78 (Davison et al., 2003), HHV-6 U51 (Gompels et al., 1995), HHV-7 U51 (Nicholas, 1996) and tupaia herpesvirus T78 (Bahr & Darai, 2001). The position and orientation of these genes within the respective herpesvirus genomes are well-conserved. Comparison of the 3’ regions of the UL78-like genes revealed significant differences between CMVs of rodents and primates (see Table 1). Putative polyadenylation signals of RCMV R78 and MCMV M78 were located in regions upstream of the coding regions of R79 and M79, respectively.

Generation of a recombinant HCMV deficient for UL78

To investigate the role of the UL78 gene product during replication, we used the AD169 genome cloned as a BAC in E. coli and constructed a mutant strain (HCMV-delUL78)
in which 1030 bp of the coding region was deleted (Fig. 4a). The BAC DNA was transfected into HFFs and viable virus was isolated, showing that UL78 is not essential for the replication of HCMV in fibroblasts. The deletion was confirmed with DNA extracted from fibroblasts infected with the reconstituted virus and by Southern blot analysis, as shown in Fig. 4(b).

Northern blot analysis was performed to investigate the effect of the deletion on UL78/UL77 transcription. Total RNA was extracted at 36 h p.i. from cells infected with either AD169 or the deletion mutant. Membranes with the separated RNAs were hybridized sequentially with probes specific for the UL78 ORF, UL77 ORF and the cellular GAPDH gene. As shown in Fig. 4(c), different effects on UL78 transcription could be observed. As mentioned above, hybridization of mRNA from AD169-infected fibroblasts showed clearly that the 1.7 kb RNA contained the UL78-coding region, whereas the later-appearing longer RNA consisted of both UL77 and UL78. No signal was detected in RNA samples derived from cells infected with the HCMV UL78-deficient mutant after hybridization with a UL78-specific probe. In the RNA sample from cells infected with HCMV-delUL78, a weak signal at approximately 3 kb was detected after hybridization with the UL77-specific probe, suggesting that the UL77 gene might still be active to some extent. The UL77 mRNA level may have been affected by the absence of the UL78 region, which could result in increased instability of the mRNA molecules.

Absence of UL78 does not modify expression of viral proteins representing the different stages of virus replication

In order to answer the question of whether the absence of the UL78 gene influences viral protein expression at different stages of virus replication, immunoblotting experiments were performed. HFFs were infected with AD169 or HCMV-delUL78. At different time points p.i., cells were harvested. Crude protein extracts were separated by SDS-PAGE and incubated with mAbs directed against IE1, pUL69, pp65 or pp28, or with a polyclonal pUL97 antiserum. These proteins represent different stages of the virus replication cycle. However, as shown in Fig. 5, no significant differences in protein expression could be detected. In individual experiments, there were some differences in band intensities (e.g. pUL69 at 6 h or pp28 at 48 h p.i.), but these were not reproducible in repeated experiments.

### Table 1. Polyadenylation signals of UL78-like genes

Numbers in parentheses indicate the first nucleotide positions of the putative polyadenylation signals according to the respective published sequences.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Gene</th>
<th>Translation stop codon</th>
<th>Putative polyadenylation signal</th>
<th>Position UL78</th>
<th>Position UL79</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV</td>
<td>UL78</td>
<td>TGA</td>
<td>AATAAA</td>
<td>+247 bp (114466)†</td>
<td>+190 bp</td>
</tr>
<tr>
<td>ChCMV</td>
<td>UL78</td>
<td>TAG</td>
<td>AATAAA</td>
<td>+225 bp (115633)§</td>
<td>+151 bp</td>
</tr>
<tr>
<td>RCMV</td>
<td>R78</td>
<td>TGA</td>
<td>AATAAA</td>
<td>+95 bp (100615)¶</td>
<td>−114 bp</td>
</tr>
<tr>
<td>MCMV</td>
<td>M78</td>
<td>TGA</td>
<td>AATAAA</td>
<td>+1 bp (112405)¶</td>
<td>−236 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AATAAA</td>
<td>+167 bp (112571)¶</td>
<td>−69 bp</td>
</tr>
</tbody>
</table>

*With respect to the stop codon of the UL78-like gene.
†With respect to the translation start codon of the UL79-like gene.
‡GenBank accession no. X17403.
§GenBank accession no. AF480884.
¶GenBank accession no. NC_002512.
§§GenBank accession no. U68299.
The UL78 gene is not important for efficient virus replication

Virus yield assays were performed to analyse whether the UL78 ORF is important for efficient infection and replication in fibroblasts. As shown in Fig. 6(a), no significant differences in virus yield kinetics were observed between supernatants of fibroblasts infected with AD169 and HCMV-delUL78. Infections were performed at an m.o.i. of 0.1. However, the same results were obtained at an even lower m.o.i. of 0.01 (data not shown). This showed that the UL78 gene product is dispensable for replication in fibroblasts in vitro and does not influence the efficiency of replication.

UL78-deficient HCMV can replicate in a renal artery organ-culture system

As no in vivo model for HCMV infection is available, we used an in vitro organ-culture model based on human renal arteries that allowed us to monitor HCMV infection over a long period of time (Reinhardt et al., 2003; Voisard et al., 1999). In order to investigate the replication characteristics of the recombinant viruses in this more complex system, human renal arteries were inoculated in vitro with HCMV wild-type strain AD169, as well as with recombinant HCMV strains. Virus yields were determined from the medium at different times after inoculation. Due to the small size of the artery segments removed from patients, not all viruses could be tested at the same time and on the same artery. Therefore, data were collected from five different independent experiments, as shown in Fig. 6(b–f). Each experiment represents the comparison of the virus yield from artery slices from the same patient infected with different viruses. The recombinant HCMV strain RVHB5 used for generation of the HCMV UL78-deletion mutant virus served as a control. RVHB5, derived from AD169, is the recombinant virus obtained by transfection and reconstitution of Bacmid

Fig. 4. Construction of UL78-deficient HCMV and Northern blot analysis. (a) Schematic representation of HCMV ORFs UL76–UL79. The shaded and open boxes represent the sizes of the ORFs. The top shows the restriction map of AD169 and the bottom shows the organization of the region after disruption of the UL78 ORF. Filled boxes represent the DNA probes used for Southern and Northern blot experiments. Restriction sites: P, PstI; B, BamHI; BX, BstXII; A, AsuII. (b) Southern blot analysis of HCMV DNA extracted from HFFs infected with AD169 or the HCMV UL78-deficient mutant (del 78). DNA was separated on a 0.5 % agarose gel, transferred to a nylon membrane and hybridized with the BamHI–AsuII DNA probe and the entire UL78 coding region indicated in (a). (c) HFFs were infected with the indicated HCMV variants and total RNA was extracted 48 h p.i. Total RNA was separated and transferred to nitrocellulose membranes. Membranes were hybridized with probes specific for UL78 or UL77. Signals representing the 1.7 kb UL78 and the putative 4 kb UL77/UL78 transcripts are indicated. The presumed UL77 mRNA deficient for the UL78 coding region is indicated by a black arrow.

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pHB5 (Borst et al., 1999), which is lacking the region between ORFs US2 and US6. This region is substituted by the BAC cassette. Virus titres in the culture medium of the arteries were determined up to a maximum of 56 days p.i. As shown in Fig. 6, the growth kinetics of the different viruses were comparable, although the amount of virus produced by the recombinants was slightly lower than that of wild-type AD169 during the first weeks after infection (Fig. 6b, d and f). However, approaching day 17 p.i., virus yields were fairly similar. Comparing the UL78-deficient strain and the parental RVHB5 recombinant (Fig. 6c and e), no effect on overall replication in the arteries could be observed. HCMV reactivations were never observed in the segments of seropositive donors. Thus, these results further strengthened the assumption that the HCMV UL78 gene is not essential for virus replication. The cell types in the arteries supporting virus replication were not determined in this study.

**UL78 is conserved among clinical isolates**

In order to elucidate whether sequence variations in UL78 existed, as is observed in the US28 protein, 11 low-passaged clinical isolates from nine patients were investigated. The
isolates were derived from different specimens (urine, throat wash and leukocytes). As summarized in Fig. 7, multiple sequence alignments of the predicted amino acid sequences revealed that the UL78 protein was highly conserved among the clinical isolates. However, several variations could be found in the N-terminal region and at the very end of the C-terminal region. Overall, when compared with AD169, the identity was 98.5–99.7% at the nucleotide level and 98.2–100% at the amino acid level. On the basis of the secondary structure as predicted by the SWISS-PROT program on the ExPASy Proteomics Server (last release 1999) (Bairoch & Boeckmann, 1992), the first 41 aa of UL78 might constitute the first extracellular part of the protein (Fig. 7). Isolates from the same patient, but isolated from different specimens, exhibited no sequence variations.

**DISCUSSION**

β-Herpesviruses of different hosts contain genes that are considered to be homologous in function. In this study, we characterized the UL78 gene, which is thought to be a 7TM protein. In contrast to ORFs US27, US28 and UL33, the predicted amino acid sequence of pUL78 does not exhibit stringent homology to GPCRs. This assumption results from the comparison with HHV-6 U51, which is related closely to UL78 (Gompels et al., 1995). However, in contrast to U51, which can bind RANTES/CCL5 with nanomolar affinity, no pharmacological data exist for pUL78. The protein is only suspected to be a homologue of GPCRs because of structural properties, which include seven putative hydrophobic regions, two cysteine residues that are predicted to be required for correct folding of GPCRs and a region that exhibits similarity to a domain known to be required for G-protein coupling (Gompels et al., 1995; Vink et al., 2001). When compared with other UL78-like proteins of animal CMVs, only one conserved cysteine residue (aa C109) could be identified. The second cysteine residue in the second intracellular loop, which would be necessary to form a disulfide bridge, might be located at aa C187. The conserved motif DRL (aa 133–135) required for G-protein binding can be found in the second extracellular loop. It is unclear why most of the amino acid substitutions in different virus isolates are located in the N-terminal region. The higher variation of an extracellularly exposed N-terminal region indicates that this part of the molecule is not involved in binding of a specific ligand and may be helpful in immune evasion. This observation was also made in US28 proteins from different patient isolates but, until now, no functional relevance has been linked to this phenomenon (R. Minisini, B. Möpps, T. Mertens & P. Gierschick, unpublished data).

Here, we showed that the UL78 mRNA has an early/late transcription pattern similar to those of a number of CMV GPCR homologues, including US28 (Bodaghi et al., 1998; Zipeto et al., 1999), RCMV R78 (Beisser et al., 1999) and MCMV M78 (Oliveira & Shenk, 2001), and is not suppressed by PAA. All of these mRNAs are transcribed early in the replication cycle and the transcripts are suppressed by CHX, but not by PAA. These findings are in accordance with the recently reported results of Wang et al. (2004) concerning the function of HCMV UL76. These authors also found that the UL78 transcript was not detected under immediate-early conditions and was not inhibited by PAA. In early/late and late stages, these ORFs were part of longer transcripts consisting of US27/US28, R77/R78 or M77/M78, respectively. Our Northern blot analyses showed clearly that the longer mRNA, which is expressed preferentially during the late stage of HCMV replication, included UL77/UL78-coding sequences.

![Fig. 7. The predicted secondary structure of the putative UL78 protein (according to the SWISS-PROT program on the ExPASy Proteomics Server; Bairoch & Boeckmann, 1992) is shown. Amino acids located in the membrane are depicted as open circles and those that are located extra- or intracellularly are indicated in grey. Conserved amino acids are shown as dotted circles. Mutations that exhibited differences among the various UL78 proteins are represented by black circles.](image-url)
Detection of the polyadenylated tail of the UL78 mRNA and sequence alignments revealed significant differences among the CMVs of different species. Although the functional relevance of the putative polyadenylation signals in the CMVs of humans, chimpanzee, rat and mouse is unknown, this observation might reflect evolution in different hosts. In the T78–T79 region of the tupaia herpesvirus, for example, no polyadenylation signal could be found, even though T78 has 25 and 26% similarity to ChCMV UL78 and HCMV UL78, respectively. It should be noted that the region between T78 and T79 encodes a putative protein with homology to MCMV M59. Such a configuration has not been observed in the other CMV genomes.

Due to the fact that UL78-like genes are present in all known β-herpesviruses, it has been speculated that these genes may play a crucial role in the pathogenesis of infection. Despite the relatively low level of sequence similarity to known chemokine receptors, HHV-6A U51 was reported to bind several chemokines, e.g. CCL2 and CCL5, as well as vMIP-II, a chemokine encoded by HHV-8 (Milhe et al., 2000). Concerning HHV-6 U12, it has been reported that RANTES/CCL5 elicits calcium release (Isegawa et al., 1998). However, to date, no ligand binding or signalling has been shown for HCMV pUL78. Some interesting differences have been observed following infection with mutant viruses deficient for UL78-like genes of CMVs. It has been shown that RCMV pR78 and MCMV pM78 serve important functions during in vivo infection (Beisser et al., 1999; Oliveira & Shenk, 2001). R78-deficient mutants are associated with a lower mortality rate in immunocompromised rats than in animals infected with wild-type virus, although no significant differences in virus yield could be found between strains with deletions and wild-type virus in vivo (Beisser et al., 1999). Furthermore, pR78 of RCMV seems to influence virus replication in fibroblasts and smooth muscle cells, where the R78-deficient mutants were found to replicate 10- to 100-fold less efficiently than wild-type RCMV. The M78-deficient mutant of MCMV only exhibited slightly reduced growth kinetics in vitro, but the authors suggested that pM78 facilitated accumulation of immediate-early viral mRNA (Oliveira & Shenk, 2001). The deletion of R78 led to an attenuated, syncytium-inducing phenotype, whereas no phenotype has been described after infection with an M78-deficient mutant (Oliveira & Shenk, 2001). As shown in the present study, the UL78-deficient HCMV mutant also exhibited no phenotype either in fibroblasts or in the in vitro renal-artery model system. Again, these results lead to the assumption of functional differences of the UL78-like proteins in different CMV species. Our results showed that the UL78 gene product is not as important for virus replication as pR78 for RCMV or pM78 for MCMV, at least in vitro. Recently, we showed that, although MCMV pM97 and HCMV pUL97 shared strong homologies in their amino acid sequences, they exhibited different functional properties (Wagner et al., 2000). Differences between in vitro and in vivo results were also obtained with other genes. Viruses deficient for pR33 or pM33 were found to replicate in vitro with efficiencies similar to those of the corresponding wild-type viruses (Beisser et al., 1998; Davis-Poynter et al., 1997). However, the replication of both pR33 and pM33 deletion mutants was found to be impaired in the salivary glands of infected animals. Functional differences among the homologous proteins R33 and UL33 have also been found (Casarosa et al., 2003). Although both receptors constitutively activate phospholipase C via Gq/11, and partially via Gi/o-mediated pathways, they exhibited profound differences in the modulation of CRE activation – pR33 constitutively inhibited, whereas pUL33 constitutively enhanced CRE-mediated transcription.

The lack of an HCMV in vivo model still represents a great obstacle for investigating the influence of particular genes on HCMV pathogenesis. Thus, it is still questionable whether pUL78 can be considered as a potential target for future development of novel antiviral strategies as suggested (Beisser et al., 1999).

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REFERENCES


Casarosa, P., Grujthuijsen, Y. K., Michel, D. & 9 other authors (2003). Constitutive signaling of the human cytomegalovirus-encoded...

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