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Evidence that the Human Cytomegalovirus 46-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasts

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Abstract

The Human Cytomegalovirus (HCMV) UL72 gene is considered to be the equivalent of the dUTPase gene of the Alpha- and Gamma-herpesviruses. To characterize its function, the expression profiles of UL72 at both the RNA and the protein level were determined. The gene is expressed with a late kinetics and the corresponding UL72 46-kDa protein accumulates late during infection in the cytoplasm of infected cells. The pUL72 was expressed in *E. coli* and the purified recombinant protein did not display a detectable dUTPase activity. The viral yields of reconstituted HCMV RVΔUL72 viruses carrying a deletion within the UL72 ORF demonstrated a moderate growth defect following low MOI infections, whereas their DNA synthesis profiles were not significantly different from those of the parental HCMV RVAD169. These results demonstrate that the UL72 gene product is not a dUTPase and is not essential for replication in human fibroblasts.
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Keywords: Human cytomegalovirus; dUTPase; UL72 gene; Deletion mutant viruses

Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that generally causes benign or asymptomatic infections. However, it is the leading viral cause of congenital infection in humans and a primary cause of morbidity in immunocompromised hosts, such as transplant recipients and HIV-infected patients at risk for life-threatening diseases (Fortunato and Spector, 1999; Griffith, 2000; Landolfo et al., 2003; Mocarski and Courcelle, 2001). Moreover, HCMV infection has been linked to chronic vascular diseases, including transplant vasculopathy, restenosis, and atherosclerosis (Jarvis and Nelson, 2002). The pathogenesis of HCMV-associated diseases largely depends on the ability of the virus to establish latent lifelong infection, counteract host defense mechanisms, and replicate in a wide variety of cells and tissues, including differentiated, post-mitotic cells, such as mesenchymal cells, endothelial cells, epithelial cells, smooth muscle cells, and monocytes/macrophages (Bissinger et al., 2002; Hengel et al., 1998; Lalani et al., 2000; Sinzger et al., 1995). Lysis of permissive infected cells as well as altered cellular gene expression occurring independently of virus replication are thought to contribute to HCMV-mediated pathogenesis (Pass, 2001). Studies from many laboratories have indeed revealed that the reprogrammed gene expression of the HCMV-infected cell is associated with stimulation of genes involved in the generation of pro-inflammatory responses, such as cytokines, chemokines, adhesion molecules, extracellular matrix proteins, and inflammatory mediators (Browne et al., 2001; Fortunato et al., 2000; Simmen et al., 2001). Among the cellular genes whose expression is regulated by HCMV infection, those coding the enzymes of the synthesis of DNA precursors play an important role in virus replication. The absence of functional virus-encoded dNTPs-synthesizing enzymes such as thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthase (TS), and ribonu-

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cleotide reductase (RR) (Chee et al., 1990; Davison et al., 2003; Rigoutsos et al., 2003), in fact, compels HCMV to depend on host cell metabolism to ensure an appropriate supply of dNTPs for its DNA replication. However, these host enzymes are expressed at very low levels in post-mitotic cells. To explain this apparent paradox, it has been hypothesized that HCMV infection of quiescent cells induces biochemical pathways that are required for DNA replication, including enzymes involved in nucleotide metabolism. Subsequently, to gain selective access to the newly synthesized dNTPs, the virus prevents replication of the host cell DNA by blocking cell cycle progression at the beginning of S phase (Fortunato et al., 2000; Wiebusch and Hagemeier, 2001). We have indeed demonstrated that this occurs for some of the enzymes involved in the thymidylate biosynthesis. Infection of quiescent fibroblasts with both murine and human CMV, in fact, increases cellular DHFR and TS content, and inhibition of these activities abrogates virus replication (Gribaudo et al., 2000, 2002; Lembo et al., 1998, 1999). TS catalyzes the de novo biosynthesis of thymidylic acid (dTMP) by reductive transfer of the methylene group from 5,10-methylene-tetrahydrofolate to the 5 position of the substrate, deoxyuridylic acid (dUMP), to form dTMP and dihydrofolate (Maley and Maley, 1990). Therefore, the intracellular availability of dUMP may be the rate-limiting step in dTMP biosynthesis and hence critical for efficient CMV replication in quiescent cells. In mammalian tissues, dUMP may be produced by the deamination of deoxycytidine 5′-monophosphate nucleotide (dCMP) catalyzed by deoxycytidylate aminohydrolase (dCMP deaminase). Alternatively, dUMP generation may involve the reduction of UDP to dUDP by ribonucleotide reductase, followed by phosphorylation of dUDP to dUTP and finally hydrolysis of dUTP to dUMP and pyrophosphate by deoxyuridine triphosphate pyrophosphatase (dUTPase) (Maley and Maley, 1990; McIntosh and Haynes, 1997). More recently, we have observed an increase of dCMP deaminase level, whereas that of dUTPase was undetectable in HCMV-infected fibroblasts (Gribaudo et al., 2003). This differential regulation of the two main dUMP-providing enzymes suggests that dCMP deaminase may be the major contributor of the TS substrate for de novo TTP synthesis. However, the lack of a significant stimulation of cellular dUTPase raises the question of whether this enzymatic activity is required for HCMV replication at all, because in addition to its role in supplying dUMP, it plays a critical role in the maintenance of uracil-free DNA by reducing the availability of dUTP as a substrate for DNA replication. Occurrence of UL72 gene within the HCMV genome, regarded as the evolutionary counterpart of the dUTPase gene in other herpesvirus (Chee et al., 1990; McGeoch and Davison, 1999), suggests that it may compensate for the nonstimulation of the cellular enzyme. These results prompted us to determine whether: (i) UL72 is expressed during HCMV replication; (ii) encodes a functional enzyme, and (iii) is essential during in vitro HCMV replication.

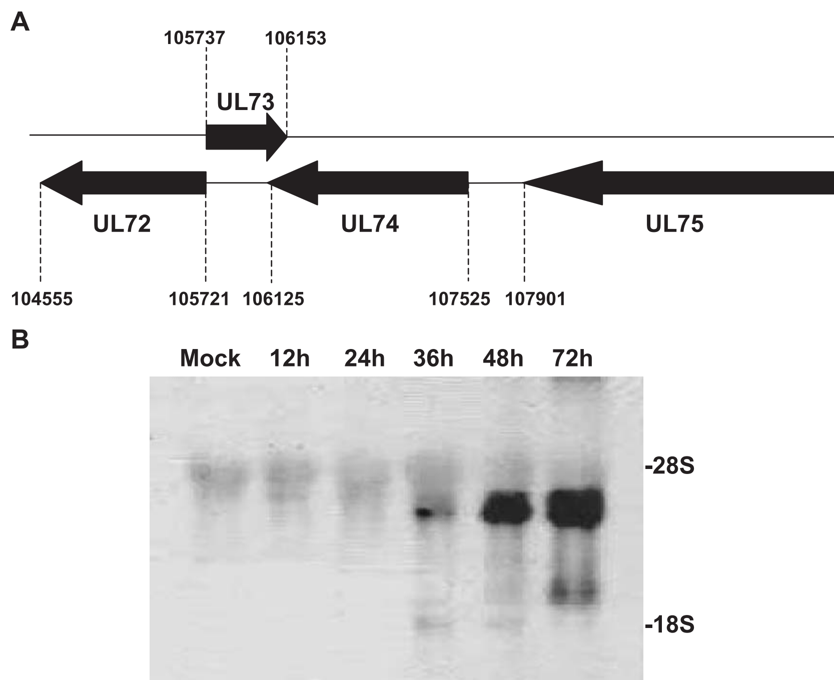

Fig. 1. Characteristics of UL72 RNA expression. (A) Schematic representation of the HCMV AD169 UL72–75 region showing the UL72 location. (B) Expression of UL72 mRNA in HCMV-infected HELF cells. Subconfluent HELF cells were growth-arrested in low-serum medium and then infected with HCMV AD169 (MOI of 5 PFU/cell) or mock-infected (lane 1). Total RNA (30 μg/sample) isolated at 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), 48 h (lane 5), or 72 h (lane 6) p.i. was analyzed by Northern blotting. The filter was hybridized with a radiolabeled single-stranded antisense RNA probe corresponding to UL72 as described in Materials and methods. The positions of 28 and 18 S rRNA are indicated.

In this report, we describe the characteristics of the UL72 gene and protein expression and the lack of an apparent dUTPase activity on the part of the recombinant protein. Furthermore, mutant viruses lacking the UL72 ORF display plaque size and morphology, and viral yield in infected human fibroblasts comparable with those of the parental strain, indicating that the UL72 gene product is not essential for replication in these cells.

Results

Characterization of UL72 gene expression

The reading frame 72 (termed UL72, nt 105721–104555 of the AD169 nucleotide sequence, accession no. X17403.1) (Fig. 1A) in the unique long arm of the HCMV genome codes for a putative protein of 388 amino acids (aa) with a theoretical molecular mass of about 43.5 kDa. To study the characteristics of its expression, UL72 was amplified by PCR from AD169 DNA and cloned. Sequence analysis revealed a perfect match with the previously reported sequence (Chee et al., 1990). To analyze transcripts originating from the UL72 ORF, total RNA from HELF cells infected with HCMV AD169 was isolated at different times postinfection and was analyzed by Northern blots using a UL72 antisense RNA as a probe. A major signal corresponding to an RNA size of about 3.5 kb was only detected in samples obtained at 36, 48, and 72 h p.i. (Fig. 1B), demonstrating that the UL72 RNA was not transcribed abundantly until late times of infection. The signal increased during this time frame and an additional signal corresponding to a 2.1-kb RNA only became detectable at 72 h p.i. These results indicate that UL72 is expressed with a kinetics of a late viral gene. Northern analysis of UL72 transcripts following treatment of infected cells with PFA was consistent with its classification as a true late gene, since PFA abolished the expression of the 3.5 and 2.1 kb RNA signals at 72 h p.i. (data not shown).

To characterize the protein product of ORF UL72, polyclonal rabbit antibodies were raised against a bacterial GST fusion protein containing the UL72 segment from aa 61 to 388. These antibodies were then used to probe immunoblots of total HELF cell protein lysates prepared at different times p.i. A single protein of an apparent molecular mass of about 46 kDa was detected with the antiserum starting from 48 h p.i. (Fig. 2, UL72 panel) and this signal increased considerably until 96 h p.i. pUL72 was not detected in mock-infected cells (Fig. 2, UL72 panel), nor by pre-immune serum (data not shown). Its predicted size (43.5 kDa) corresponds to the band detected by the anti-UL72 antibodies. To further confirm the temporal kinetic class of UL72 expression, HCMV-infected HELF cells were grown in the continuous presence of PFA and protein extracts were prepared at 48 and 72 h p.i. Here,

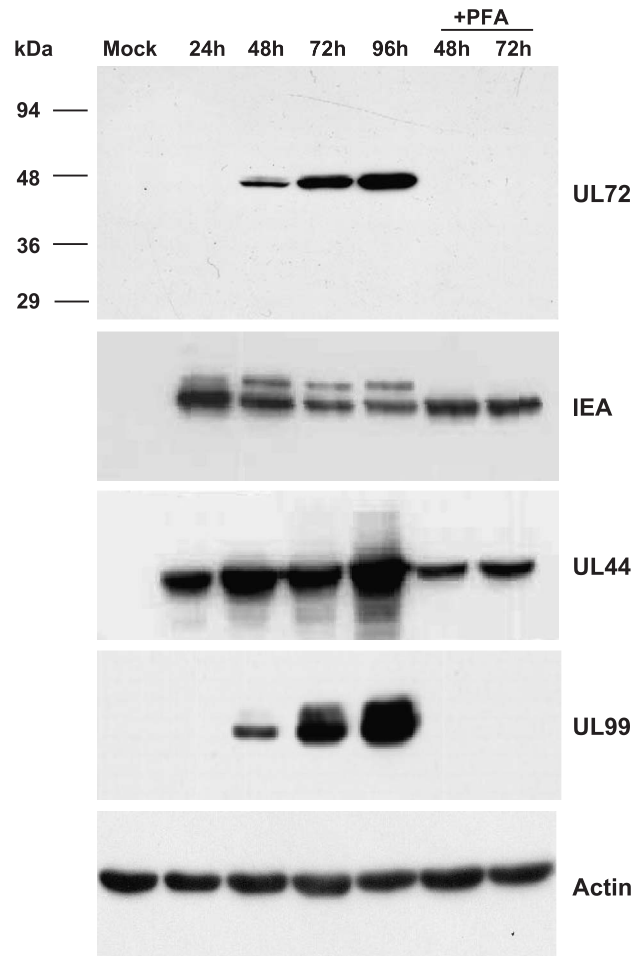

Fig. 2. Characteristics of pUL72 expression. HELF cells were growth-arrested in low-serum medium and then infected with HCMV AD169 (MOI of 5 PFU/cell) or mock-infected. Total cell extracts were prepared at the indicated times after infection, fractionated by SDS-PAGE (50 μg protein/lane), and analyzed by immunoblotting with the anti-UL72 antibodies, with the anti-IEA mAb, the anti-UL44 mAb, or the anti-UL99 mAb described in Materials and methods. Actin immunodetection with a mAb was performed as an internal control. Cell extracts were isolated from mock-infected cells, cells infected with HCMV for 24, 48, 72, and 96 h, or cells infected with HCMV and treated with PFA for 48 and 72 h.

pUL72 was not detected at 48 h nor at 72 h, confirming that it is expressed with a late gene kinetics (Fig. 2, UL72 panel). Expression of IEA (IE1 and IE2), UL44 and UL99 was assessed as a control for IE, E, and L HCMV proteins (Fig. 2).

The intracellular location of pUL72 was then investigated by confocal laser immunofluorescence microscopy using the anti-UL72 and the anti-IEA mAb as probes. Mock-infected cells displayed only background fluorescence, whereas nuclei of infected cells were stained by the anti-IEA mAb throughout infection. In infected cells, pUL72 stained from 48 h p.i. throughout infection and showed a diffuse cytoplasmic staining pattern (Fig. 3). A similar pattern was also observed in HELF cells transiently transfected with a pUL72 expression vector (Fig. 3, right bottom panel).

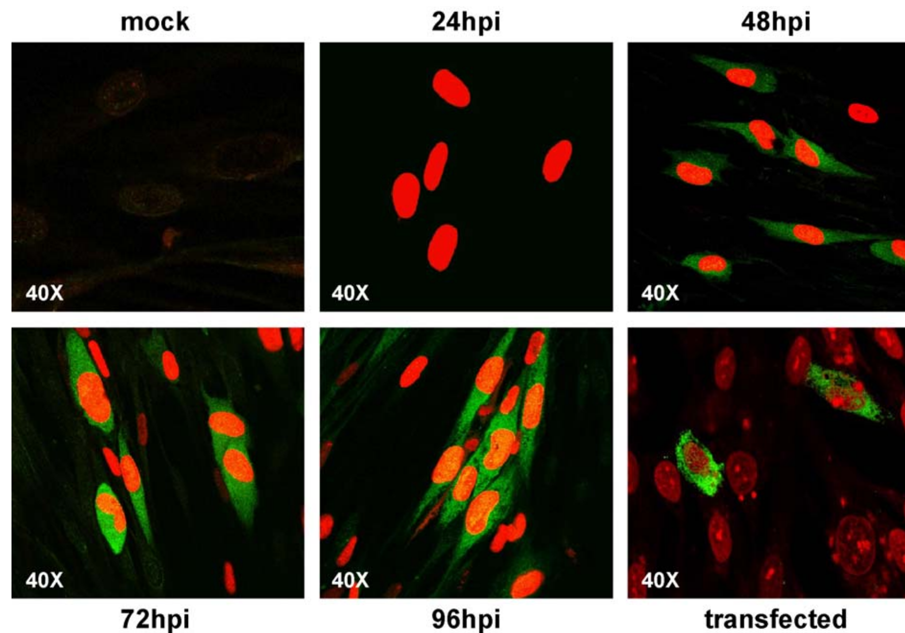

Fig. 3. Localization of UL72 protein in HCMV-infected HELF cells by indirect immunofluorescence. HELF cells grown on coverslips were either transiently transfected with the eukaryotic expression plasmid pcDNA3-UL72 or infected with either HCMV AD169 (MOI of 0.2 PFU/cells) or mock-infected. At 24, 48, 72, 96 h p.i., or 24 h post transfection, cells were washed with PBS, fixed with acetone/methanol, and permeabilized. Infected cells were costained with rabbit anti-UL72 antibodies (green fluorescence) and mouse anti-HCMV IEA mAb (red fluorescence), whereas transfected cells were stained with anti-UL72 antibodies and propidium iodide as described in Materials and methods. The immunolocalization experiments were repeated twice and representative results are presented.

pUL72 does not display a dUTPase activity

UL72 of HCMV is regarded as the evolutionary counterpart of the dUTPase gene in other herpesviruses, though its deduced aa sequence lacks some of the conserved dUTPase canonical sequence motifs (McGeoch and Davison, 1999). To determine whether the UL72 protein is functionally active, the UL72-corresponding ORF (1–388) was inserted into pGEX4T3 and expressed as a GST-fusion protein in *E. coli* as described for other herpesvirus dUTPases (Kremmer et al., 1999). The apparent molecular mass of the fusion protein was 72 kDa, as predicted when fusing the 26-kDa GST to the 46-kDa pUL72 protein (Fig. 4A, lane 4). The fusion protein was bound to immobilized glutathione, and after removal of the nonspecifically bound material, pUL72 was released from the GST moiety by thrombin cleavage. The released protein had an apparent molecular weight of about 46 kDa (Fig. 4A, lane 5) identical to that identified by anti-pUL72 antibodies in extracts from HCMV-infected HELF cells (Fig. 2). Analysis on an SDS-PAGE gel demonstrates purity >90% (Fig. 4A). As a control for expression, purification and thrombin release procedures, the human N-dUTPase coding gene (the 22-kDa nuclear form) (Ladner et al., 1996) was cloned and produced as a recombinant protein (Fig. 4A, lanes 2 and 3), respectively). The synthesized pUL72 was then tested for dUTPase activity by incubation with ^{3}H-labeled dUTP and subsequent TLC. As shown in Fig. 4B, 0.5 or 2 μg of purified pUL72 failed to hydrolyze dUTP to dUMP at a rate significantly higher than that of the nonrelated GST protein. By contrast, the same amounts of recombinant human N-dUTPase produced, as expected, a total conversion of the input dUTP to dUMP (Fig. 4B). Moreover, the Murine Herpesvirus 68 ORF54 (the dUTPase gene of MHV68) was expressed and purified using the very same procedure and the recombinant protein shown to be an active dUTPase (Caposio et al., unpublished data). This result demonstrates the reliability of our experimental procedures to produce recombinant herpesvirus dUTPases. The results obtained with the recombinant UL72 protein and those demonstrating the lack of significant dUTPase activity in crude cell extracts or in immunoprecipitates prepared from HCMV-infected quiescent HELF cells (data not shown) suggest that the product of the HCMV AD169 UL72 gene is not an active dUTPase.

Comparison of the UL72 sequence from different HCMV isolates

The AD169 strain may have undergone significant genetic modifications due to the selection pressure imposed by multiple passages on human fibroblasts causing both aa sequence variation and loss of several genes present in wild-type isolates (Cha et al., 1996). Therefore, it was of interest to evaluate the nucleotide and deduced aa sequences of UL72 from wild-type clinical isolates and compare them to the AD169 strain. The entire UL72 ORF from two clinical isolates and the low-passage TB40/E isolate was therefore amplified by PCR and sequenced to determine the extent of sequence variability. The alignment of the four UL72 aa sequences compiled with the Clustal X (1.8) multiple sequence alignment program (Fig. 5) indicates that the UL72

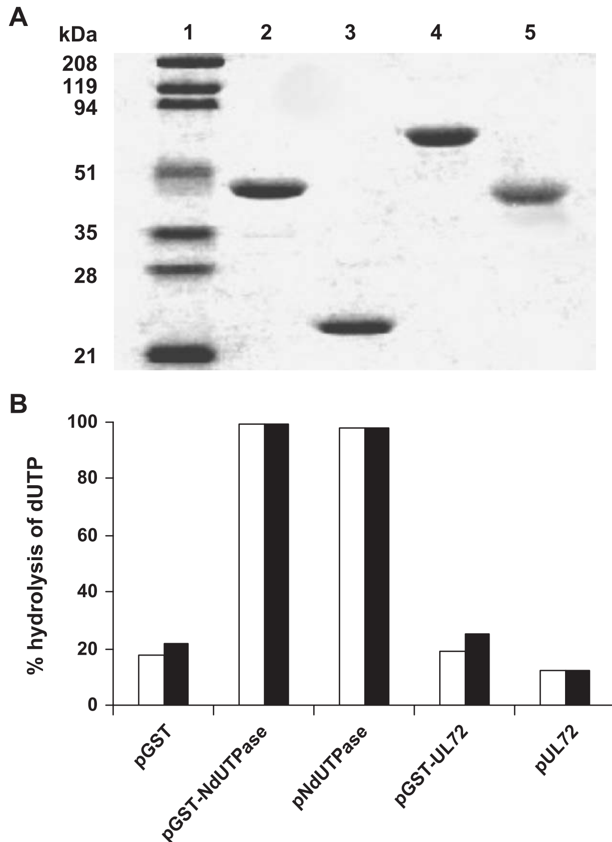

Fig. 4. dUTPase assay of recombinant UL72 and human NdUTPase proteins. The recombinant UL72 protein was expressed in *E. coli* as a GST-UL72 fusion protein, immobilized on a glutathione-sepharose beads and released by thrombin digestion. Purified GST-UL72 and thrombin-released UL72 were then assayed for dUTPase activity as described in Materials and methods. The human nuclear dUTPase isoform (NdUTPase) was expressed and purified as described for UL72. (A) SDS-PAGE pattern of purified recombinant NdUTPase and UL72 proteins. Molecular weight markers (lane 1), pGST-NdUTPase (lane 2), pNdUTPase (lane 3), pGST-UL72 (lane 4), pUL72 (lane 5). (B) dUTPase enzyme assay. The assay was performed as described in Materials and methods with 0.5 (empty bars) and 2 μg (filled bars) of the recombinant proteins. The assay was repeated three times and representative results are shown.

coding sequence is highly conserved in all the analyzed strains and therefore suggests that the UL72 function(s) have to be maintained for virus growth or virus structure in vivo. In addition, the few substitutions were mapped outside the putative dUTPase sequence motifs (1–5) conserved in alpha and gamma-herpesviruses expressing functional enzymes (McGeehan et al., 2001; McGeoch, 1990).

Construction and characterization of mutant viruses lacking the UL72 coding sequence

To investigate the pUL72 role(s) during HCMV infection, we generated substitution mutant viruses carrying a marker gene in the place of the UL72 ORF (Borst et al., 1999). Deletion of UL72 was achieved by linear recombination with a PCR fragment in *E. coli* (DH10B) containing AD169-BAC and expressing λ recombination functions (redαβγ). The PCR fragment was generated using the kanamycin resistance gene and primers containing HCMV-homologous sequences. Structural analysis of two AD169ΔUL72-BAC selected clones (nos. 2 and 3) was performed by restriction mapping and Southern blot hybridization for the presence of the kanamycin resistance cassette. Reconstitution of RVAD169ΔUL72 was obtained by transfection of ΔUL72-BAC into human fibroblasts. Fig. 6 (upper panel) reports the profiles of the AD169-BAC and the clone 3 ΔUL72-BAC DNA bacmid (A + B) and reconstituted virus (C + D). As predicted (Fig. 6, upper panel, A + B), in the *Hin*dIII digest, kan^R insertion cleaves a 22.8-kb band (A + B, lane 3) in AD169-BAC to a 20.4-kb and a 2.4-kb fragment in ΔUL72-BAC clones (A + B, lanes 1–2), respectively. In the *Eco*RI digest, kan^R insertion shifts a 16.4-kb fragment (A, lane 6) in AD169-BAC to a 17.6-kb fragment (A, lanes 4–5) in ΔUL72-BAC clones. Southern blot analysis (Fig. 6, upper panel B + D) confirmed the presence of kanamycin resistance sequences in the ΔUL72 clone 3 bacmid and reconstituted virus. A similar pattern was obtained for the ΔUL72 clone 2 bacmid and reconstituted virus (data not shown). To ascertain whether the site-directed mutagenesis of the UL72 sequences resulted in the loss of pUL72 expression, proliferating HELF cells were infected with reconstituted viruses and protein extracts analyzed for pUL72 expression by immunoblotting. As shown in Fig. 6 (lower panel), pUL72 was absent from RVΔUL72 clones 2 and 3 (lanes 3–4), whereas, as expected, it was produced by RVAD169 (lane 2).

The UL72 protein is dispensable for in vitro growth

To determine if deletion of UL72 had an effect on viral replication, we compared the viral yield and viral DNA content of RVAD169 and two independent RVΔUL72 viruses (clones 2 and 3) in multistep growth curves in both quiescent and exponentially growing HELF cells infected at different multiplicity. Fig. 7A shows the viral yields obtained at 1–4, 6, 8, 10, and 12 days p.i. from quiescent (left panels) or growing (right panels) cells infected at MOIs of 0.1 or 1. While a moderate growth defect (2-fold reduction) was observed for RVΔUL72 mutant viruses at a MOI of 0.1, no significant impairment was measured at a MOI of 1. Additionally, no obvious differences in plaque size and morphology were detected. Evaluation of viral DNA kinetic synthesis in cells infected with RVAD169 or RVΔUL72 mutant viruses did not reveal significant differences (Fig. 7B). Hence, mutations of the UL72 ORF slightly affect virus replication in vitro, indicating that it is not essential for HCMV growth in human fibroblasts.

Discussion

Since the release of the complete DNA sequence of the AD169 strain (Chee et al., 1990) and its reanalysis (Davison

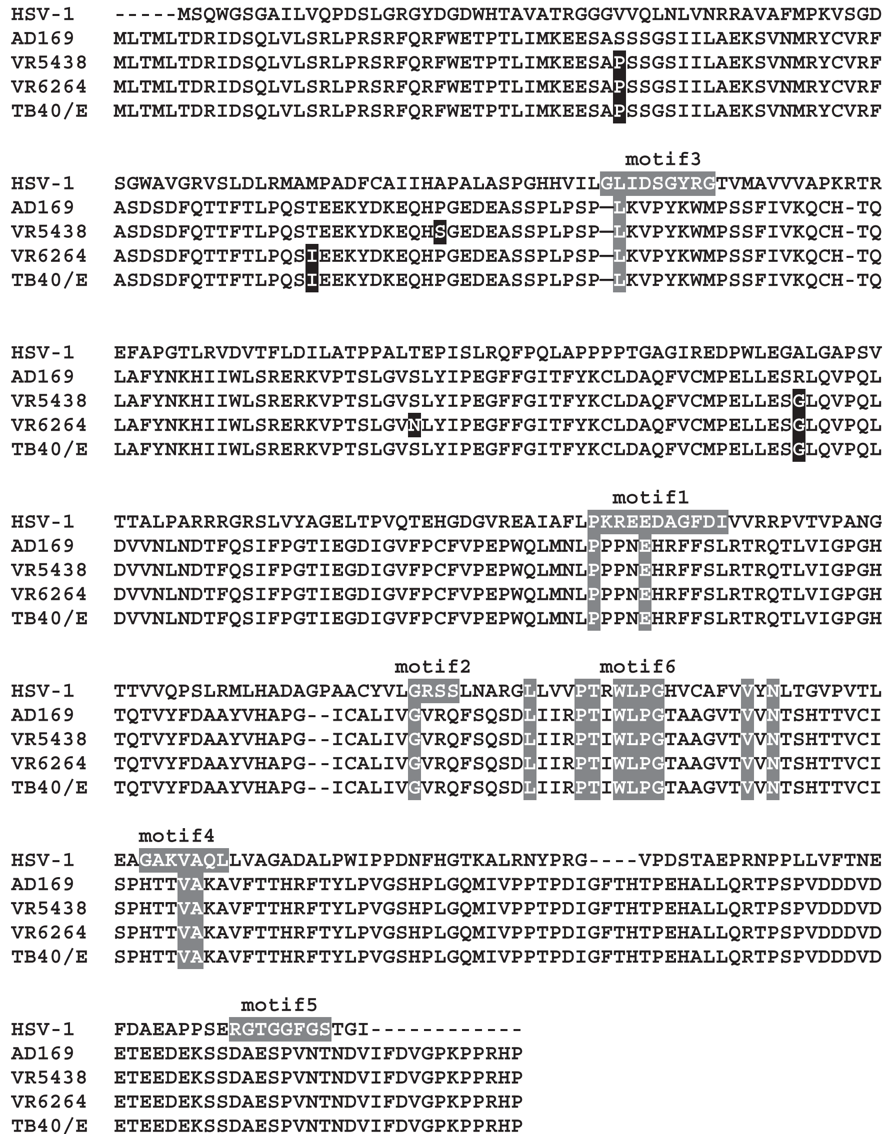

Fig. 5. Alignment of UL72 amino acid sequences from different HCMV strains. The nucleotide sequence of the UL72 gene was determined in two clinical isolate HCMV strains (VR5438, VR6264) and in the endothelial cell tropic TB40/E strain. Their UL72 deduced aa sequences were compared using that of the AD169 laboratory-adapted strain as the arbitrary reference. The substitutions are highlighted. The HSV-1 UL50 aa sequence (GenBank P10234) (McGeoch et al., 1988) is shown as the prototype of functional herpesvirus dUTPase and the five standard conserved motifs (1–5) important for enzymatic activity are shown. Motif 6 is a specific element conserved in the alpha- and betaherpesvirus dUTPases (McGeehan et al., 2001). The sequences were aligned using the Clustal X (1.8) multiple sequence alignment program http://www.ebi.ac.uk).

et al., 2003), the UL72 gene has been considered, from sequence similarities, as the HCMV homolog of the dUTPase genes of *Alpha*- and *Gammaherpesvirinae*. Multiple alignments of cellular and viral dUTPase sequences indicate that despite the variation in the copy number of the characteristic motifs, which are duplicated in the herpesviral dUTPases, most active dUTPases share a common arrangement of five conserved aa motifs (Baldo and McClure, 1999; McClure, 2001; McGeehan et al., 2001; McGeoch, 1990; McIntosh and Haynes, 1997; McIntosh et al., 1992; Mol et al., 1996). Lack of conservation of most of the five motifs in the UL72-deduced sequence (Fig. 5) has led to the suggestion that this protein does not possess a true dUTPase enzymatic activity, though no experimental evidence has been provided. To definitively test this long-standing hypothesis, we have cloned UL72 from the AD169 genome to start its functional characterization. Here, we demonstrate that the UL72 protein product does not elicit any detectable dUTPase activity in our experimental conditions. Although the coding capacity of UL72 is similar to that of alpha and gamma herpesvirus dUTPase genes, the absence of conservation of the five amino acid motifs supports the absence of a dUTPase activity. The present UL72 ORF may thus have arisen due to extensive mutation from an ancestral herpesviral active dUTPase (McGeehan et al., 2001). The lack of UL72 involvement in nucleotide biosynthesis could be

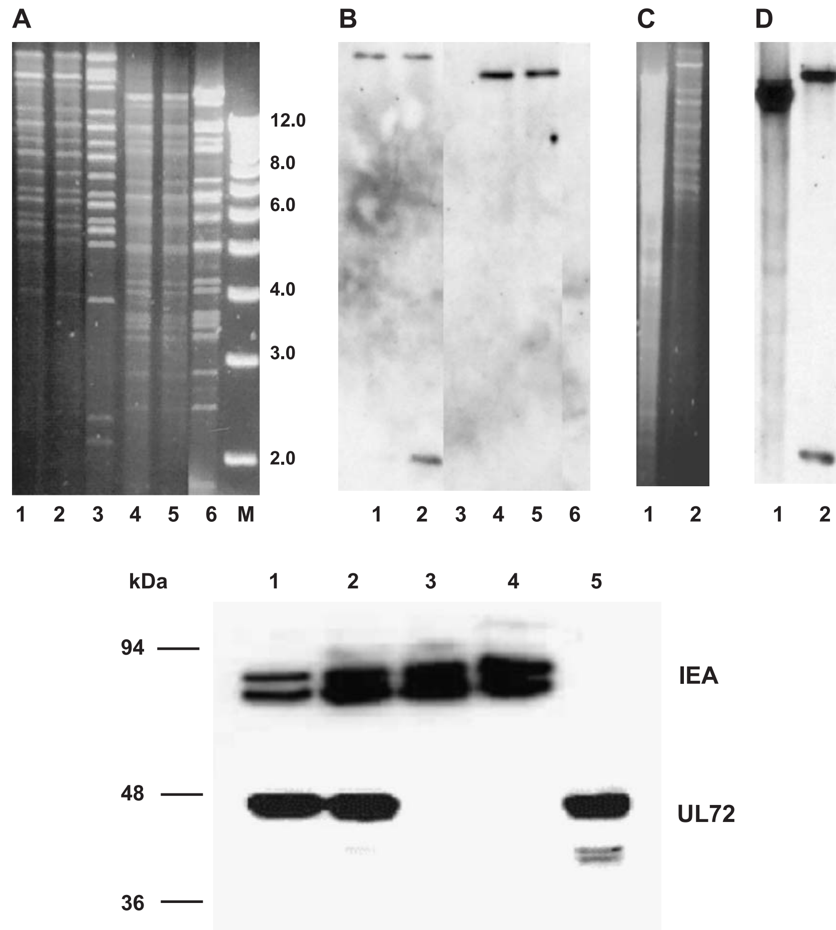

Fig. 6. Characterization of the RVAD169ΔUL72 mutant viruses. (Upper panel) Structural analysis of the AD169ΔUL72-BACs and of RVAD169ΔUL72 reconstituted viruses. DNA from two independently generated clones of AD169ΔUL72-BAC (A + B; lanes 1–2, 4–5) and AD169 reference BAC (A + B; lanes 3, 6) was digested with *Hin*dIII (lanes 1–3) or *Eco*RI (lanes 4–6) and separated on a 0.5 agarose gel, blotted and probed with pACYC to visualize kan^R insertion (B, lanes 1–6). As predicted (A + B), in the *Hin*dIII digest, kan^R insertion cleaves a 22.8-kb band (A + B, lane 3) in AD169-BAC to a 20.4-kb and a 2.4-kb fragment (A + B, lanes 1–2) in ΔUL72-BAC, respectively. In the *Eco*RI digest, kan^R insertion shifts a 16.4-kb fragment (A, lane 6) in AD169-BAC to a 17.6-kb fragment (A, lanes 4–5) in ΔUL72-BAC. DNA of the reconstituted virus RVAD169ΔUL72 (clone 3) (C + D) was digested with either *Hin*dIII (lane 2) or *Eco*RI (lane 1) electrophoresed on an 0.5% agarose gel (C) and hybridized with kanamycin resistance gene (D). M, 1-kb molecular size marker. (Lower panel) Lack of pUL72 expression in reconstituted RVΔUL72 viruses. Proliferating HELF cells were infected with HCMV AD169 (lane 1), RVAD169 (lane 2), RVΔUL72-2 (clone 2) (lane 3), RVΔUL72-3 (clone 3) (lane 4) (MOI of 5 PFU/cell). Total cell extracts were prepared at 72 h p.i., fractionated by SDS-PAGE (50 μg protein/lane), and analyzed by immunoblotting with the anti-UL72 antibodies or with the anti-IEA mAb as described in Materials and methods. As an additional control for UL72 expression, extracts from HELF cells transiently transfected with the pcDNA3-UL72 vector were also included (lane 5).

related to the different strategies exploited by beta, as compared to alpha- and gamma herpesviruses in supplying deoxyribonucleotide to the viral DNA polymerase. Alpha and gamma herpesviruses, in fact, encode functional deoxyribonucleotide-synthesizing enzymes, such as ribonucleotide reductase, thymidine kinase, thymidylate synthase, and dUTPase. Viral dUTPases control dUTP concentrations and provide dUMP as precursor for the TTP biosynthesis pathway (McGeehan et al., 2001; Studebaker et al., 2001). It has been suggested that viral dUTPases facilitate virus replication in differentiated nondividing cells that lack expression of the endogenous cell cycle-regulated enzyme, whereas they are nonessential in dividing tissues (McClure, 2001; McGeehan et al., 2001). Absence of UL72's dUTPase activity raises the question of the requirement of this enzymatic activity for HCMV replication in nondividing cells because cellular and viral dUTPases play a critical role in the maintenance of uracil-free DNA by reducing the availability of dUTP as a substrate for DNA replication. It has been reported that the functional uracil-DNA glycosylase (UNG) encoded by the HCMV UL114 gene is required for efficient viral DNA replication in quiescent cells, where in the absence of viral or cellular UNG expression ΔUNG-mutant viruses failed to proceed to late-phase DNA replication (Courcelle et al., 2001; Prichard et al., 1996). These findings have suggested that by removing uracil residues incorporated early during viral DNA replication, viral UNG introduces nicks serving as substrates for initiation of recombination-dependent replication late in infection. Misincorporation of dUTP and subsequent excision by viral

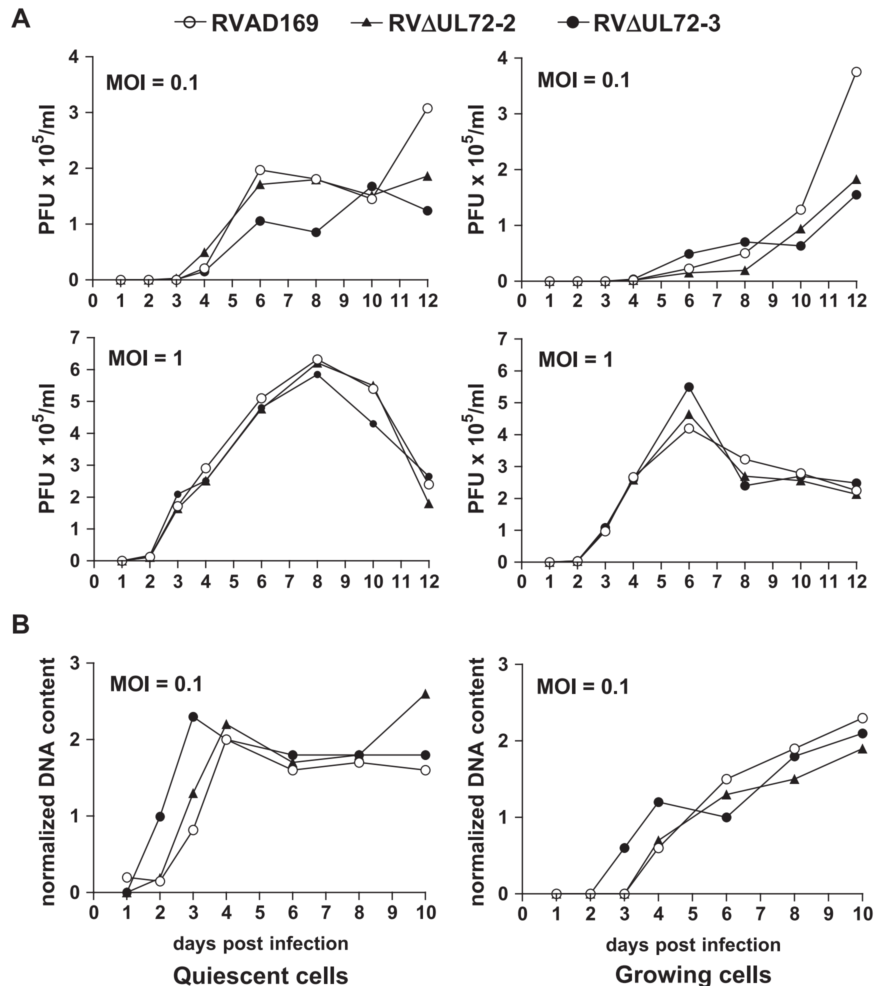

Fig. 7. Multiple-step growth curve analysis. (A) Growth curves of BAC-reconstituted RVAD169 and RVAD169ΔUL72 (designated RVΔUL72) viruses in HELF cultures. Titers were measured at 1–4, 6, 8, 10, and 12 days p.i. by standard plaque assay on HELF cells. Growth curves were determined in both quiescent or proliferating HELF cells infected (MOI of 0.1 or 1 PFU/cell). (B) Viral DNA synthesis of reconstituted RVAD169 and RVΔUL72 viruses. The DNA synthesis rates of RVAD169 and RVΔUL72 viruses were determined during their replication in quiescent or proliferating HELF cells infected (MOI of 0.1 PFU/cell). Total genomic DNA was isolated from infected cells at 1–4, 6, 8, and 10 days p.i. and immobilized on a hybridization membrane by a dot blot apparatus. The filters were sequentially hybridized with ^{32}P-labeled HCMV IE1 and G3PDH probes. The hybridization signals were quantitated with a phosphoimager and adjusted to the differences in G3PDH gene levels.

UNG (in quiescent cells) or by host UNG (in proliferating cells) is therefore though to facilitate the transition from early-phase replication (origin-dependent with a bidirectional theta mechanism) to late-phase amplification (with a rolling-circle form of DNA replication) (Chen et al., 2002; Courcelle et al., 2001). The lack of any HCMV-encoded dUTPase activity and the absence of a significant stimulation of host enzyme expression during infection of quiescent cells (Gribaudo et al., 2003) may contribute to the increase of intracellular dUTP levels. In turn, the subsequent cycles of uracil misincorporation followed by excision promote the transition to efficient late-phase DNA amplification. High dUTP/dTTP ratios, therefore, may not be detrimental to HCMV replication.

Because the UL72 ORF remained open in all the HCMV strains (Fig. 4), it can be presumed to encode a functional protein. However, its inactivation did not significantly impair in vitro HCMV replication in growing fibroblasts because only a moderate defect was observed at the lowest MOI. We characterized two independently isolated UL72 mutants by a strategy recently employed for the analysis of other HCMV genes (Hahn et al., 2003; Wagner et al., 2002). Plaque size and morphology of each mutant viruses were comparable to those of AD169 under both optimal or suboptimal conditions (such as growing or growth-arrested cells). Insertional mutagenesis procedures may alter adjacent genes. Hence, we verified that insertion of the kan^R cassette into the UL72 ORF did not alter transcription of neighboring UL71 and UL73 genes (data not shown). We conclude that the 46-kDa protein product UL72 is nonessential for in vitro propagation of the virus. This finding is consistent with the functional profilings of the whole HCMV genomes (Dunn et al., 2003; Yu et al., 2003). These functional maps were obtained by global mutational

analysis of all viral ORFs and growth of selected mutants in cultured fibroblasts. In both studies, UL72 was recognized as nonessential for viral replication in fibroblasts and only minor growth defects were observed with the mutant viruses. It is likely, therefore, that UL72, like most HCMV genes whose products have proved to be nonessential for replication in fibroblasts, has evolved to gain a new function presumably related to the virus growth in the natural host, such as dissemination, growth in specific target tissues and pathogenesis, or to counteract host immune reactivity. Relevant to this is the finding that mutations abolishing the glycosylase activity of vaccinia virus UNG without affecting viral DNA replication in cultured cells reduce the virulence of the catalytic-site mutants in a mouse infection model (De Silva and Moss, 2003). However, speculation about the UL72 emergent function cannot be solely based on extrapolation from aa sequences because they did not reveal any significant homology to proteins other than herpesvirus dUTPases. Moreover, the large subunit of ribonucleotide reductase is another example of a CMV-encoded protein presumed to be involved in nucleic acid metabolism that has lost its original activity. Both the human (UL45) and mouse (M45) genes, in fact, encode enzymatically inactive homologs of the large subunit. Furthermore, deletion of the UL45 gene from both the AD169 genome and that of a clinical isolate did not result in any significant growth defect because the mutated viruses grew equally well in fibroblasts and endothelial cells. These finding clearly indicate that UL45's functions are dispensable for HCMV growth in both cell types (Hahn et al., 2002). By contrast, murine M45 has been proved to be essential for MCMV growth in endothelial cell cultures (Brune et al., 2001) and its deletion impairs the in vivo replication of DM45 viruses, indicating that the new function(s) gained by M45 are related to dissemination or growth in the natural host (Lembo et al., 2004). Therefore, it can be envisaged that the generation of murine CMV mutant viruses deleted of the counterpart M72 gene and evaluation of both their growth characteristics in cultures and their ability to disseminate and replicate in the natural host will provide further insights into the 72 ORF's functions.

Materials and methods

Cells and culture conditions

Low-passage human embryonic lung fibroblasts (HELF cells) were grown as monolayers in minimum essential medium (MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin per ml, and 100 μg/ml streptomycin sulfate. Quiescent cells (arrested in G_0/G_1 phase) were obtained by culturing the subconfluent cultures for 48 h in MEM supplemented with 0.5% serum (low-serum medium). Flow cytometry demonstrated that about 90% were growth-arrested.

Viruses

The HCMV strain AD169 was purchased from ATCC (VR-538). Viral stocks were prepared by infecting human embryonic fibroblasts at a virus-to-cell ratio of 0.01. Cells were incubated in MEM supplemented with 1% heat-inactivated fetal bovine serum and cultured until a marked cytopathic effect was seen. Virus stocks were then prepared from sonicated cells and centrifugal clarification and frozen without further processing at −80 °C. Mock-infecting fluid was prepared from uninfected cells by the same procedure. Virus titers were determined by standard plaque assay on HELF cells. HCMV clinical isolates (VR5438 and VR6264) (provided by Prof. G. Gerna, University of Pavia, Italy) were propagated in HELF cells. HCMV strain TB40/E (provided by Dr. C. Sinzger, University of Tübingen, Germany) was propagated in human umbilical vein endothelial cells (HUVEC) to preserve its natural endothelial cytopathogenicity. RVAD169 reconstituted virus and RVAD169ΔUL72 (designated RVΔUL72) mutant viruses were propagated in HELF cells.

Construction of UL72 virus mutant by site-directed mutagenesis

Oligonucleotides with 3′ homology to sequences flanking a kan^R marker and 5′ homology to sequences flanking the viral region to be deleted (UL72) were used to PCR amplify pACYC177 (New England Biolabs) DNA to generate a linear DNA fragment containing the kan^R marker tailed onto short regions of HCMV homology. The PCR fragment was electroporated into cells that contained AD169-BAC and transiently expressed the recombination functions redα/β/γ (Datsenko and Wanner, 2000; Hahn et al., 2003; Muyrers et al., 2000; Wagner et al., 2002). Colonies containing BACs with the kan^R marker inserted were selected on plates containing both kanamycin and chloramphenicol.

The following primer pairs were used to generate the linear PCR recombination fragment for AD169ΔUL72: UL72-for: 5′-**TTG GAA AAC GTA CGT GAT CAC GGA CAC GAC GAG TAC GGG GTT TCT CAT AGA CGT ACT TTA TTA GG**C GAT TTA TTC AAC AAA GCC ACG-3′; UL72-rev: **TCC GCA TCG TCA TCA GGC TCC ATC ATC CTA GCC GAG AAA TCC GTC AAC ATG CGT TAC TGC GTG CGT** GCC AGT GTT ACA ACC AAT TAA CC-3′. HCMV-homologous sequences on the 5′ ends of the oligonucleotides are in boldface.

Nucleic acid analysis

Viral DNA and BAC DNA isolations were performed as previously described (Hahn et al., 2002, 2003). *Hin*dIII- or

*Eco*RI-digested DNA samples were electrophoresed on a 0.5% agar gel, visualized by ethidium bromide, and blotted onto nylon membranes. Hybridization conditions were as previously described (Hahn et al., 2002, 2003). Southern blots were probed with the kanamycin resistance gene contained in the 1.3-kb *Bam*HI–*Xho*I fragment form plasmid pACYC177, labeled with digoxigenin using the DIG DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

For UL72 gene sequence analysis, total DNA was isolated from HELF cells infected with the HCMV AD169, the VR5438, or the VR6264 strains, and from HUVEC cells infected with the TB40/E strain, by resuspending cell pellets in lysis buffer (10 mM Tris–Cl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 μg/ml proteinase K) and incubating the mixtures at 50 °C for 18 h. Digestion was followed by phenol–chloroform extraction, ethanol precipitation, and RNase treatment (1 μg/ml of RNase A for 1 h at 37 °C). The entire UL72 ORF from each HCMV strain was then amplified by PCR, cloned into the pGEM-T-easy plasmid (Promega), and subsequently sequenced by the dideoxy-chain terminator method. Nucleotide sequences were analyzed using the Clustal X (1.8) multiple sequence alignment program (European Bioinformatics Institute).

To evaluate RVAD169 and RVΔUL72 DNA synthesis in infected HELF cells, total DNA was isolated at the indicated times postinfection and its 2-fold dilutions were immobilized on a hybridization membrane and sequentially hybridized with ^{32}P-labeled probes prepared from the *Pst*I–*Bam*HI DNA fragment of the HCMV IE1 gene (exon 4) and mouse G3PDH cDNA. The membranes were autoradiographed and the hybridization signals were quantified with the Bio-Rad Image Analysis System.

For RNA analysis, at the indicated times, infected HELF cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and total cellular RNA was isolated as described (Ausubel et al., 1994). Total RNA (30 μg) was fractionated on a 1% agarose, 2.2 M formaldehyde gel, and then blotted onto nitrocellulose membrane (Hybond C-extra, Amersham). The filters were baked for 2 h at 80 °C and prehybridized for 6 h at 60 °C in 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA. The hybridizations were carried out at 60 °C overnight with 10^6 cpm/ml of ssRNA radiolabeled probes corresponding to the 833-nt *Hin*dIII–*Sma*I segment from the UL72 gene cloned in pBluescript SK plasmid (Stratagene). Antisense RNA transcripts were generated by in vitro transcription with T3 RNA polymerase (Ambion). Filters were washed twice for 30 min at 60 °C with 2× SSC, 0.1% SDS, and twice for 30 min at 60 °C with 0.1× SSC, 0.1% SDS.

Plasmids

pcDNA3-UL72 was constructed by inserting the UL72 ORF (1–388) between the *Bam*HI and *Xho*I sites of the pcDNA3 plasmid (Invitrogen). The pGEX4T3-UL72 and pGEX4T3-NdUTPase plasmids were constructed by inserting between the *Bam*HI and *Xho*I sites of pGEX4T3 the PCR products corresponding, respectively, to the UL72 ORF (1–388) and the nuclear (N) isoform of the human dUTPase (Ladner et al., 1996). pGEX4T3-UL72C was generated by inserting the PCR product corresponding to a UL72 segment comprised between positions 61 and 388 into the *Sma*I site of pGEX4T3.

Expression and purification of UL72 and NdUTPase proteins

Synthesis of recombinant dUTPases and their purification were performed according to Kremmer et al., 1999. pGEX4T3-UL72 and pGEX4T3-NdUTPase proteins were expressed in *E. coli* AD202. Flasks containing Luria–Bertani (LB) broth were inoculated with 1/10 vol of an overnight culture and incubated in a rotatory shaker at 37 °C until the adsorbance at 600 nm reached 0.7. Then, IPTG (Sigma) was added to a final concentration of 1 mM and incubation was continued for 2 h at 37 °C. Cells were collected by centrifugation for 15 min at 6000 rpm, washed once in PBS, and resuspended in lysis buffer [150 mM NaCl, 100 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton-X, protease inhibitor cocktail (Sigma), lysozyme 1 mg/ml]. Cells were then sonicated on ice and the suspension was cleared by centrifugation for 20 min at 10 000 rpm. The recombinant proteins, soluble under these conditions, were analyzed by SDS-PAGE and visualized by Coomassie Blue staining. The UL72 and NdUTPase proteins were then affinity-purified on glutathione-sepharose 4B (Pharmacia) beads as described (Ausubel et al., 1994) and further purified by enzymatic release from the GST moiety by digestion with thrombin) (Pharmacia). The released proteins were dialyzed against 50 mM Tris–Cl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 20% glycerol, and stored at −80 °C until use.

To generate an anti-UL72 serum, GST-UL72C (61–388) was expressed in *E. coli* AD202 and affinity-purified on glutathione-sepharose 4B beads (Pharmacia) as described (Ausubel et al., 1994). The C-terminal part of the UL72 protein was used for rabbit immunization to produce antibodies against pUL72. The serum obtained by bleeding at 1 week after the fourth immunization was precipitated with ammonium sulphate at 45% of saturation and IgG were then purified on a protein A affinity column (Pharmacia) according to the manufacturer's specifications.

Enzyme assay

dUTPase activity was measured by evaluating the hydrolysis of dUTP to dUMP. The 20-μl reaction mixture contained 50 mM Tris–HCl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 1mg/ml BSA, 25 mM NaF, 2 mM PNPP, 1

μl [^{3}H]dUTP (18.6 Ci/mmol) (Amersham), and the purified recombinant proteins (100 ng to 2 μg). All the assays in duplicate were incubated for 30 min at 37 °C and reactions were stopped by boiling for 3 min. An aliquot of the reaction mixture (2 μl) was loaded on a TLC PEI-cellulose plate together with 2 μl of a solution containing dUTP and dUMP (each at 100 mM) to provide an optical marker under UV irradiation. PEI-cellulose plates were then developed in 0.5 M LiCl and 2 M acetic acid and, dried and examined under UV light. The marker spots corresponding to dUTP and dUMP were then cut out and the radioactivity associated was quantified by liquid scintillation counting.

Immunofluorescence microscopy

HELF cells grown on coverslips were transiently transfected with the pcDNA3-UL72 vector using the Lipofectamine plus reagent (Invitrogen) or infected with HCMV AD169 (MOI of 0.2 PFU/cell). At 24, 48, 72, 96 h p.i., or 24 h post transfection, cells were washed with PBS, fixed with cold acetone and methanol for 5 min at −20 °C and then washed again with PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min at 4 °C, washed with PBS, and then incubated at RT for 1 h with normal human serum to block HCMV-induced Fc receptors. Thereafter, infected cells were incubated with the rabbit anti-UL72 antibody (diluted 100-fold) and mouse anti-HCMV IEA mAb (clone E13, Argene Biosoft) (diluted 100-fold) in PBS containing 10% of normal human serum for 1 h at RT, while coverslips of transfected cells were incubated only with the rabbit anti-UL72 antibody (diluted 100-fold). After washing with PBS−1% BSA, 0.05% Tween 20, coverslips of infected cells were incubated with FITC-conjugated anti-rabbit Ig (Sigma) and Texas Red-conjugated goat anti-mouse Ig (Molecular Probes, Eugene, OR) antibodies in PBS−1% BSA for 1 h at RT, while coverslips of transfected cells were incubated with the FITC-conjugated anti-rabbit Ig only and then stained with propidium iodide (0.1 μg/ml). Finally, coverslips were washed with PBS and mounted in 90% glycerol. Immunofluorescence microscopy was performed on an Olympus IX70 inverted confocal laser scanning microscope, equipped with a krypton−argon ion laser (488/568). Images derived from both channels (fluorescein and Texas Red) were simultaneously recorded at identical apertures. The fluoroscein-derived images were assessed as green and the Texas Red-derived images as red.

Preparation of protein extracts and immunoblotting

Whole-cell extracts were prepared by resuspending pelleted cells in lysis buffer containing 125 mM Tris−Cl, pH 6.8, 1% SDS, 20 mM dithiotreitol, 1 mM phenylmethylsulphonyl fluoride, 4 μg/ml leupeptin, 4 μg/ml aprotinin, 1 μg/ml pepstatin. After a brief sonication, soluble proteins were collected by centrifugation at 15 000 × *g*. Supernatants were analyzed for protein concentration with a Bio-Rad D_c protein assay kit (Bio Rad Laboratories) and stored at −80 °C in 10% glycerol. Proteins were separated by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). Filters were blocked in 5% nonfat dry milk in 10 mM Tris−Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and immunostained with the rabbit anti-UL72 antibody (diluted 1000-fold), the mouse anti-HCMV IEA mAb (clone E13, Argene Biosoft) (diluted 200-fold), or the mouse anti-HCMV UL44 mAb (purchased as antibody 1202 from Goodwin Institute, Plantation, Fl) (1:1000), the mouse anti-HCMV UL99 mAb (purchased as antibody 1207 from Goodwin Institute, Plantation) (1:1000), or the mouse anti-actin mAb (Chemicon International) (diluted 2000-fold) at RT for 1 h. Immune complexes were detected with either sheep anti-mouse Ig or goat anti-rabbit Ig antibodies conjugated to horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence (Super Signal, Pierce).

Analysis of mutant virus growth and DNA synthesis

To determine the extent of viral replication, multiple-step growth analyses were performed in HELF cells incubated in low-serum medium for 48 h (quiescent cells) or plated the day before the infection (growing cells) and then infected with RVAD169 or RVΔUL72 (clones 2 and 3) (MOI of 1 or 0.1 PFU/cell). One well per plate was mock-infected and served as cell control. Virus absorption medium was then replaced with the same low-serum medium or with fresh growth medium. At the indicated time postinfection, cells and supernatants were harvested and disrupted by sonication. The disrupted cells were centrifuged at 500 × *g* for 10 min, and supernatants were assayed for infectivity by a standard plaque assay.

To evaluate RVAD169 or RVΔUL72 DNA synthesis, HELF cells were grown to subconfluence in 24-well plates, incubated in low-serum medium for 48 h (quiescent cells), or plated the day before infection (growing cells) and then infected with RVAD169 or RVΔUL72 (MOI of 0.1 PFU/cell). One well per plate was mock-infected and served as cell control. Total DNA was then isolated at the indicated times postinfection as described above.

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), 1994. Curr. Protoc. Mol. Biol., vol. 1. Wiley, New York.

Baldo, A.M., McClure, M.A., 1999. Evolution and horizontal transfer of dUTPase-encoding genes in viruses and their hosts. J. Virol. 73, 7710–7721.

Bissinger, A.L., Sinzger, C., Kaiserling, E., Jahn, G., 2002. Human cytomegalovirus as a direct pathogen: correlation of multiorgan involvement and cell distribution with clinical and pathological findings in a case of congenital inclusion disease. J. Med. Virol. 67, 200–206.

Borst, E.M., Hahn, G., Koszinowski, U.H., Messerle, M., 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. J. Virol. 73, 8320–9329.

Browne, E.P., Wing, B., Coleman, D., Shenk, T., 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J. Virol. 75, 12319–12330.

Brune, W., Menard, C., Heesemann, J., Koszinowski, U.H., 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science 291, 303–305.

Cha, T.A., Tom, E., Kemble, G.W., Duke, G.M., Mocarski, E.S., Spaete, R.R., 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J. Virol. 70, 78–83.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchison III, C.A., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M., Barrell, B.G., 1990. Analysis of the protein-coding content of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. 154, 125–169.

Chen, R., Wang, H., Mansky, L.M., 2002. Roles of uracil-DNA glycosylase and dUTPase in virus replication. J. Gen. Virol. 83, 2339–2345.

Courcelle, C.T., Courcelle, J., Prichard, M., Mocarski, E.S., 2001. Requirement for uracil-DNA glycosylase during the transition to late phase cytomegalovirus DNA replication. J. Virol. 75, 7592–7601.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97, 6640–6645.

Davison, A.J., Dolan, A., Akter, P., Addison, C., Dargan, D.J., Alcendor, D.J., McGeoch, D.J., Hayward, G.S., 2003. The human cytomegalovirus genome revised: comparison with the chimpanzee cytomegalovirus genome. J. Gen. Virol. 84, 17–28.

De Silva, F.S., Moss, B., 2003. Vaccinia virus uracil DNA glycosylase has an essential role in DNA synthesis that is independent of its glycosylase activity: catalytic site mutations reduce virulence but not virus replication in cultured cells. J. Virol. 77, 159–166.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Liu, F., 2003. Functional profiling of a human cytomegalovirus genome. Proc. Natl. Acad. Sci. U.S.A. 100, 14223–14228.

Fortunato, E.A., Spector, D.H., 1999. Regulation of human cytomegalovirus gene expression. Adv. Virus Res. 54, 61–128.

Fortunato, E.A., McElroy, A.K., Sanchez, V., Spector, D.H., 2000. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. Trends Microbiol. 8, 111–119.

Gribaudo, G., Riera, L., Lembo, D., De Andrea, M., Gariglio, M., Rudge, T.L., Johnson, L.F., Landolfo, S., 2000. Murine cytomegalovirus stimulates cellular thymidylate synthase gene expression in quiescent cells and requires the enzyme for replication. J. Virol. 74, 4979–4987.

Gribaudo, G., Riera, L., Rudge, T.L., Caposio, P., Johnson, L.F., Landolfo, S., 2002. Human cytomegalovirus infection induces cellular thymidylate synthase gene expression in quiescent fibroblasts. J. Gen. Virol. 83, 2983–2993.

Gribaudo, G., Riera, L., Caposio, P., Maley, F., Landolfo, S., 2003. Human cytomegalovirus requires cellular deoxycytidylate deaminase for replication in quiescent cells. J. Gen. Virol. 84, 1437–1441.

Griffith, P.D., 2000. Cytomegalovirus. In: Zuckerman, A.J., Banatvala, J.E., Pattison, J.R. (Eds.), Principles and Practice of Clinical Virology, 4th ed. Wiley, New York, pp. 79–115.

Hahn, G., Khan, H., Baldanti, F., Koszinowski, U.H., Revello, M.G., Gerna, G., 2002. The human cytomegalovirus ribonucleotide reductase homolog UL45 is dispensable for growth in endothelial cells, as determined by a BAC-Cloned clinical isolate of human cytomegalovirus with preserved wild-type characteristics. J. Virol. 76, 9551–9555.

Hahn, G., Rose, D., Wagner, M., Rhiel, S., McVoy, M.A., 2003. Cloning of the genomes of human cytomegalovirus strains Toledo, TownevarRIT3, and $Towne_{long}$ as BACs and site-directed mutagenesis using a PCR-based technique. Virology 307, 164–177.

Hengel, H., Brune, W., Koszinowski, U.H., 1998. Immune evasion by cytomegalovirus: survival strategies of a highly adapted opportunist. Trends Microbiol. 6, 190–197.

Jarvis, M.A., Nelson, J.A., 2002. Mechanisms of human cytomegalovirus persistence and latency. Front. Biosci. 7, 1575–1582.

Kremmer, E., Sommer, P., Holzer, D., Galetsky, S.A., Molochkov, V.A., Gurtsevitch, V., Winkelmann, C., Lisner, R., Niedobitek, G., Grasser, F.A., 1999. Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) ORF54 encodes a functional dUTPase expressed in the lytic replication cycle. J. Gen. Virol. 80, 1305–1310.

Ladner, R.D., McNulty, D.E., Carr, S.A., Roberts, G.D., Caradonna, S.J., 1996. Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. J. Biol. Chem. 271, 7745–7751.

Lalani, A.S., Barrett, J.W., McFadden, G., 2000. Modulating chemokines: more lessons from viruses. Immunol. Today 21, 100–106.

Landolfo, S., Gariglio, M., Gribaudo, G., Lembo, D., 2003. The human cytomegalovirus. Pharmacol. Ther. 98, 269–297.

Lembo, D., Angeretti, A., Gariglio, M., Landolfo, S., 1998. Murine cytomegalovirus induces expression and enzyme activity of dihydrofolate reductase in quiescent cells. J. Gen. Virol. 78, 2803–2808.

Lembo, D., Gribaudo, G., Cavallo, R., Riera, L., Angeretti, A., Hertel, L., Landolfo, S., 1999. Human cytomegalovirus stimulates cellular dihydrofolate reductase activity in quiescent cells. Intervirology 42, 30–36.

Lembo, D., Donalisio, M., Hofer, A., Cornaglia, M., Brune, W., Koszinowski, U.H., Thelander, L., Landolfo, S., 2004. The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J. Virol. 78, 4278–4288.

Maley, F., Maley, G.F., 1990. A tale of two enzymes, deoxycytidylate deaminase and thymidylate synthase. Progr. Nucleic Acid Res. Mol. Biol. 39, 49–80.

McClure, M.A., 2001. Evolution of the DUT gene: horizontal transfer between host and pathogen in all three domains of life. Curr. Protein Pept. Sci. 2, 313–324.

McGeehan, J.E., Depledge, N.W., McGeoch, D.J., 2001. Evolution of the dUTPase gene of mammalian and avian herpesviruses. Curr. Protein Pept. Sci. 2, 325–333.

McGeoch, D.J., 1990. Protein sequences show that the 'pseudoproteases' encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. Nucleic Acids Res. 18, 4105–4110.

McGeoch, D.J., Davison, A.J., 1999. The molecular evolutionary history of the herpesviruses. In: Domingo, E., Webster, R., Holland, J. (Eds.), Origin and Evolution of Viruses. Academic Press, London, pp. 441–465.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E., Taylor, P., 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69, 1531–1574.

McIntosh, E.M., Haynes, R.H., 1997. dUTP pyrophosphatase as a potential target for chemotherapeutic drug development. Acta Biochim. Pol. 44, 159–172.

McIntosh, E.M., Ager, D.D., Gadsen, M.H., Haynes, R.H., 1992. Human dUTP pyrophosphatase cDNA sequence and potential biological importance of the enzyme. Proc. Natl. Acad. Sci. U.S.A. 89, 8020–8024.

Mocarski, E.S., Courcelle, C.T., 2001. Cytomegalovirus and their replica-

tion. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincot-Raven, New York, pp. 2629–2673.

Mol, C.D., Harris, J.M., McIntosh, E.M., Tainer, J.A., 1996. Human dUTP pyrophosphatase: uracil recognition by a β hairpin and active sites formed by three separate subunits. Structure 4, 1077–1092.

Muyrers, J.P., Zhang, Y., Stewart, A.F., 2000. ET-cloning: think recombination first. Genet. Eng. (N. Y.) 22, 77–98.

Pass, R.F., 2001. Cytomegalovirus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincot-Raven, New York, pp. 2675–2705.

Prichard, M.N., Duke, G.M., Mocarski, E.S., 1996. Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. J. Virol. 70, 3018–3025.

Rigoutsos, I., Novotny, J., Huynh, T., Chin-Bow, S.T., Parida, L., Platt, D., Coleman, D., Shenk, T., 2003. In silico pattern-based analysis of the human cytomegalovirus genome. J. Virol. 77, 4326–4344.

Simmen, K.A., Singh, J., Luukkonen, B.G., Lopper, M., Bittner, A., Miller, N.E., Jackson, M.R., Compton, T., Fruh, K., 2001. Global modulation of cellular transcription by human cytomegalovirus is initiated by viral glycoprotein B. Proc. Natl. Acad. Sci. U.S.A. 98, 7140–7145.

Sinzger, C., Grefte, A., Plachter, B., Gouw, A.S., The, T.H., Jahn, G., 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J. Gen. Virol. 76, 741–750.

Studebaker, A.W., Balendiram, G.K., Williams, M.V., 2001. The herpesvirus encoded dUTPAse as a potential chemotherapeutic target. Curr. Protein Pept. Sci. 2, 371–379.

Wagner, M., Gutermann, A., Podlech, J., Reddehase, M.J., Koszinowski, U.H., 2002. Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J. Exp. Med. 196, 805–816.

Wiebusch, L., Hagemeier, C., 2001. The human cytomegalovirus immediate early 2 protein dissociates cellular DNA synthesis from cyclin-dependent kinase activation. EMBO J. 20, 1086–1098.

Yu, D., Silva, M.C., Shenk, T., 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc. Natl. Acad. Sci. U.S.A. 100, 12396–12401.