

The human cytomegalovirus UL45 gene product is a late, virion-associated protein and influences virus growth at low multiplicities of infection

Marco Patrone,¹ Elena Percivalle,² Massimiliano Secchi,¹ Loretta Fiorina,¹ Guido Pedrali-Noy,¹ Monica Zoppé,^{3†} Fausto Baldanti,² Gabriele Hahn,⁴ Ulrich H. Koszinowski,⁴ Gabriele Milanese^{1,5} and Andrea Gallina^{1,5}

Correspondence

Andrea Gallina
at Istituto di Genetica Molecolare
gallina@igbe.pv.cnr.it

¹Istituto di Genetica Molecolare, CNR, via Abbiategrosso 207, I-27100 Pavia, Italy

²Servizio di Virologia, IRCCS Policlinico San Matteo, Pavia, Italy

³Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

⁴Max von Pettenkofer-Institut, Lehrstuhl für Virologie, LMU-München, München, Germany

⁵Department of Medicine and Surgery, San Paolo Hospital, University of Milano, via A. di Rudini 8, I-20142 Milano, Italy

Human cytomegalovirus (HCMV) encodes a protein related to the large (R1) subunit of ribonucleotide reductase (RR), but does not encode the corresponding small (R2) subunit. The R1 homologue, UL45, lacks many catalytic residues, and its impact on deoxyribonucleotide (dNTP) production remains unknown. Here, UL45 is shown to accumulate at late stages of infection and to be a virion tegument protein. To study UL45 function in its genome context, UL45 was disrupted by transposon insertion. The UL45-knockout (UL45-KO) mutant exhibited a growth defect in fibroblasts at a low m.o.i. and also a cell-to-cell spread defect. This did not result from a reduced dNTP supply because dNTP pools were unchanged in resting cells infected with the mutant virus. Irrespective of UL45 expression, all cellular RR subunits – S-phase RR subunits, and the p53-dependent p53R2 – were induced by infection. p53R2 was targeted to the infected cell nucleus, suggesting that HCMV diverts a mechanism normally activated by DNA damage response. Cells infected with the UL45-KO mutant were moderately sensitized to Fas-induced apoptosis relative to those infected with the parental virus. Together with the report on the UL45-KO endotheliotropic HCMV mutant (Hahn *et al.*, *J Virol* **76**, 9551–9555, 2002), these data suggest that UL45 does not share the prominent antiapoptotic role attributed to the mouse cytomegalovirus homologue M45 (Brune *et al.*, *Science* **291**, 303–305, 2001).

Received 19 June 2003

Accepted 3 September 2003

INTRODUCTION

Human cytomegalovirus (HCMV), a ubiquitous β -herpesvirus that causes severe disease in immunocompromised individuals and in the newborn, replicates in differentiated cells (fibroblasts, microglial, epithelial, endothelial and smooth muscle cells, and monocyte-derived macrophages; Britt & Alford, 1996; Sinzger *et al.*, 1995), and is transported in the bloodstream by abortively infected neutrophils (Gerna *et al.*, 2000). Latent infection is detected in macrophage-granulocyte precursors in the bone marrow and in circulating monocytes (Maciejewski *et al.*, 1992; Soderberg-Naucler *et al.*, 1997; Hahn *et al.*, 1998).

The class I ribonucleoside diphosphate (ribonucleotide) reductases (RR), which catalyse a limiting step in *de novo* deoxyribonucleotide (dNTP) synthesis, are essential for DNA replication of both eukaryotic cells and the DNA viruses that infect them (Jordan & Reichard, 1998; Stubbe *et al.*, 2001). RR holoenzymes comprise a catalytic large (R1) subunit, and a small (R2) subunit required for enzyme activation (Jordan & Reichard, 1998). Like many large DNA viruses, the α - and γ -herpesviruses encode both subunits of a viral RR, which is important for virus replication in resting or post-mitotic cells (Jacobson *et al.*, 1989; Idowu *et al.*, 1992; Heineman & Cohen, 1994; Aurelian, 1998) because these have minute dNTP pools and virtually no endogenous RR (Engstrom *et al.*, 1985; Jordan & Reichard, 1998; Chabes & Thelander, 2000). In addition, the herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) R1 proteins bear an N-terminal extension (see supplementary

[†]Present address: Laboratorio di Terapia Genica e Molecolare, IFC-CNR, Area della Ricerca, Via G. Moruzzi, 56124 Pisa, Italy.

data Fig. 1 at JGV Online, <http://vir.sgmjournals.org>), which has been suggested to contain an intrinsic protein kinase (PK) activity (Chung *et al.*, 1989; Paradis *et al.*, 1991; Cooper *et al.*, 1995), although this view has been challenged (Langelier *et al.*, 1998; Conner, 1999). HSV-2 R1 (ICP10) enhances the function of HSV-2 immediate-early (IE) proteins, plays a role in HSV-2 latency reactivation (Smith *et al.*, 1998) and protects infected cells from chemically induced apoptosis (Perkins *et al.*, 2002; Langelier *et al.*, 2002), in correlation with MEK/MAPK pathway activation (Smith *et al.*, 2000).

HCMV and related β -herpesviruses, such as mouse cytomegalovirus (MCMV) and human herpesvirus 6 and 7, do not carry an R2 gene, so they cannot assemble an all-viral RR. However, they encode an R1 protein (Chee *et al.*, 1990), the RR function of which has been disputed. Human herpesvirus 7 R1 (U28) was enzymatically inactive in combination with α -herpesvirus R2 subunits (Sun & Conner, 1999). However, while investigating the effect of MCMV infection on cellular RR, S. Landolfo and co-workers (Lembo *et al.*, 2000) found an efficient induction of the sole R2, which gave rise to their hypothesis that MCMV R1 (M45), and by inference all β -herpesvirus R1s, assemble with cellular R2 into a mixed viral–cellular enzyme. Implied in this hypothesis is that M45 and its relatives work as atypical R1s, as they have lost redox-active cysteines plus additional residues involved in both capture of tyrosyl radicals from the R2 di-iron centre (Kauppi *et al.*, 1996; Nordlund & Eklund, 1993) and radical reaction at the active site (Uhlin & Eklund, 1994; Eriksson *et al.*, 1997; Stubbe *et al.*, 2001), and as they show no equivalent of the cellular R1 C-terminal stretch that binds R2 (Davis *et al.*, 1994; Lycksell *et al.*, 1994). On the other hand, M45 is essential for MCMV replication in endothelial cells (Brune *et al.*, 2001), which undergo a premature apoptotic death when infected with an M45-knockout (M45-KO) mutant; M45-KO growth in macrophages is also defective. This observation reveals a novel antiapoptotic role of M45, which contributes to MCMV tropism towards cells of the hemangioblast lineage.

The role of HCMV R1 (UL45) has been recently assessed in the infection context. The UL45-KO derivative of an endotheliotropic strain grew in human umbilical vein endothelial cells with normal kinetics and no sign of apoptosis (Hahn *et al.*, 2002). Here, we characterize UL45 expression kinetics in cells infected with the non-endotheliotropic strain AD169, and define UL45 as a late, virion-associated protein. A UL45-KO mutant of the same strain is defective in viral particle accumulation at low multiplicities of infection (m.o.i.), and in spreading in fibroblasts. This is unrelated to dNTP production, as both mutant and parental virus induce cellular RR subunits and accumulate dNTPs with similar efficiency. The protection from Fas-induced apoptosis is minimally reduced in UL45-KO-infected fibroblasts relative to the parental virus-infected cells. UL45 and M45 functions are not identical, a difference

which may be a result of their divergent N-terminal sequences.

METHODS

Cell cultures, viruses and infections. Human embryonic lung fibroblasts (HELFL) and HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS). Virus stocks were produced by infecting HELFL at an m.o.i. of 0.1. The virus was harvested by sonication when a 100% cytopathic effect was observed and stored at -70°C . The virus titre was determined in HELFL seeded in 96-well microtitre plates; infectious units (IU) were defined as IE72-producing units at 2 days post-infection (p.i.) as previously described (Gallina *et al.*, 1999). Mock extracts were prepared similarly from non-infected cells. For multi-step growth analysis, HELFL monolayers in 24-well dishes were maintained for 1 week after confluence in DMEM/2% FBS. Cells were infected at an m.o.i. of 0.1 IU per cell at 37°C for 1 h. The medium was then replaced with virus-free conditioned medium. At various time points, the monolayers and culture supernatants were frozen at -80°C . Frozen cells were thawed at 37°C , sonicated, and the IU titre of total cell lysate and supernatant from each well determined. Infections for nucleic acid, protein and nucleotide analyses were performed similarly, except that an m.o.i. of 2–4 was used in some cases. For expression kinetics studies, infections were performed in the presence of $50\ \mu\text{g}$ cycloheximide ml^{-1} (CHX; Sigma) or $400\ \mu\text{M}$ phosphonoformic acid (PFA; Sigma). Each drug treatment was initiated 1 h prior to infection, and maintained during virus adsorption and through to the end of the experiment.

Detergent and protease treatment of purified virions. Purified viral particles of HCMV strains AD169 and RV d65 (an AD169 derivative carrying a disrupted UL83; Schmolke *et al.*, 1995) were obtained previously, and tested free from infected cell debris by Western blot analysis with anti-UL44 and anti- α -tubulin antibodies (Gallina *et al.*, 1999). Particles dissolved in 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 were incubated on ice for 30 min. The detergent-insoluble fraction was separated by centrifugation at $100\ 000\ \text{g}$ for 1 h, at 4°C . Alternatively, virions suspended in the same buffer, with/without added Triton X-100, were treated with $50\ \mu\text{g}$ trypsin ml^{-1} at 30°C for 15 min. The reaction was stopped by addition of a protease inhibitor cocktail (Complete, Roche) and incubation on ice for 15 min. Ten μg of virion protein per treatment was analysed by immunoblot.

Nucleic acids purification and analysis. Total DNA from HELFL was purified with the QIAamp DNA mini kit (Qiagen). For Southern blotting, *EcoRI*-digested DNA was electrophoresed on a 0.6% agarose gel and blotted onto Hybond-N+ (Amersham Pharmacia). The membrane was hybridized with an [γ ^{32}P]dCTP-labelled UL45 probe and exposed to a phosphorimager screen.

For viral DNA quantifications, fivefold dilutions of purified DNA (extensively treated at 37°C for 1 h with $40\ \mu\text{g}$ RNaseA ml^{-1} to remove contaminating RNA) were immobilized onto Hybond-N+ membrane. This was hybridized with a 1.5 kbp HCMV-specific probe covering UL83, and the signal quantified by phosphorimaging. The stripped membrane was rehybridized with a probe for human GAPDH to normalize data.

For transcription analysis, RNA from infected/mock-infected HELFL was subjected to two rounds of purification on RNeasy columns (Qiagen), interposing a treatment with 1 U RQ DNase (Promega) (μg RNA) $^{-1}$ at 30°C for 15 min in the supplied buffer. RNA (20 ng) was amplified with primer pairs appropriate for UL45 (primers 2 and 3; these and the other primers mentioned hereafter are listed in

Table 1. Amplification primers

Primer	Sequence	Restriction site
1	5'UL45 CCTCGTGGATCCATGAATCCGGCTGACGCG	<i>Bam</i> HI
2	5'UL45_mer AACTTAGGATCCC CGGCTGACGCGGACGAGGAACAG	<i>Bam</i> HI
3	3'UL45_int CGGCCAGAATTCATAGATCCTCCATGTGACG	<i>Eco</i> RI
4	3'UL45 ATGCGGGAATTCGACTAAGAGGCACAGTAC	<i>Eco</i> RI
9	RTR1_5' GCCTGAATTCTGCTATTATCTATGACCG	<i>Eco</i> RI
10	RTR1_3' CAAGGAATTC AAAGATGTCTAAATGC	<i>Eco</i> RI
11	RTR2_5' TGAATTCAAGAAGAAGGCAGACTG	<i>Eco</i> RI
12	RTR2_3' GCGGATCCTGATACTACCAGCAC	<i>Bam</i> HI
13	p53R2_5' GGCGCGGATCCGCGATGGCGCACC CGGAAAGGC	<i>Bam</i> HI
14	p53R2_3'int CTTTAAGCTTGTTCAGTGAGGGAG	–
15	UL123_5' ACACGATGGAGTCTCTGCGG	–
16	UL123_3' TTCTATGCCGCACCATGTCC	–
17	UL54_5' AAACACCGCCGTGGATCAGG	–
18	UL54_3' CAAACACCGTGCGCCCTGG	–
19	UL32_5' ATCCCTCAGGATGCCTACG	–
20	UL32_3' GACGTGTTGTTGTAATCGG	–
21	GAPDH_5' ACCACAGTCCATGCCATCAC	–
22	GAPDH_3' TCCACCACCTGTTGCTGTA	–

Underlined, restriction sites (names in the column at right); bold face, start codons; bold face-italics: stop codons (antisense).

Table 1), R1, R2 (Pavloff *et al.*, 1992), p53R2 (Tanaka *et al.*, 2000) and other viral or cellular transcripts, with/without previous reverse transcription, according to the Access-RT-PCR kit (Promega) protocol.

dNTP assay. HELF were washed in ice-cold PBS, scraped off in ice-cold 60% methanol and incubated overnight at -20°C . Extracts were centrifuged at 15 000 *g* for 10 min, at 4°C , and supernatants lyophilized and stored at -80°C in aliquots. These were rehydrated just before use in ice-cold water, and immediately assayed for dNTP content using the polymerase assay as described previously (Lindberg & Skoog, 1970; Skoog, 1970).

Plasmid construction and recombinant protein expression. PCR products were generated with *Pfu* polymerase and sequenced after cloning to rule out unwanted mutations. Plasmid pGex-NTD was obtained by cloning into pGex4T2 (Amersham Pharmacia) UL45 codons 2–278, amplified with primers 2 and 3 from AD169 DNA. IPTG-induced *E. coli* DH5 α cells harbouring pGEX-NTD were processed for inclusion body purification (Sambrook *et al.*, 1989). Washed GST-UL45(2–278) inclusion bodies were suspended in PBS and used for chicken immunizations.

Full-length UL45 coding sequence (primers 1–4) was inserted into a pcDNA4/HisMAX-A (Invitrogen) derivative, which lacks an *Nhe*I–*Bam*HI fragment encoding a polyhistidine tract. The final construct was transfected into HEK-293 cells (2 μg DNA per 1×10^6 cells) according to the calcium phosphate protocol.

The same UL45 insert was cloned into the retroviral vector pLEGFP-C1 (Clontech) in place of the Neo cassette to create retro-UL45. Retro-UL45 or the void vector (retro-cont) were transfected into 293gp/bsr packaging cells together with plasmid pVG, which expresses vesicular stomatitis virus G protein for envelope pseudotyping (Somia *et al.*, 2000). Retrovirus in transfection supernatants was titrated by infecting 293-HEK monolayers and visualizing enhanced green fluorescence protein (EGFP)-positive cells under an epifluorescence microscope.

Immunoblots and radioimmunoprecipitations. Proteins in infected/mock-infected HELF extracts were quantified by a colorimetric method (BCA, Pierce). Balanced amounts of total protein were analysed by Western blot and radioactive immunoprecipitation (Sambrook *et al.*, 1989), performed according to standard procedures (for details and the list of antibodies employed, see supplementary data on materials at JGV Online, <http://vir.sgmjournals.org>).

Indirect immunofluorescence. HELF seeded on coverslips (1×10^5 cells cm^{-2}) were infected, mock-infected or treated with 0.2 μg doxorubicin ml^{-1} for 48 h to induce DNA damage and p53R2 nuclear localization. Monolayers were fixed in 3.8% (w/v) paraformaldehyde in PBS at 4°C for 30 min. After a 10 min poration in 0.1% (v/v) Triton X-100 in PBS, cells were incubated for 20 min at 37°C in a humid chamber with 6 μg anti-p53R2 ml^{-1} and 2 μg anti-pp72/pp86 ml^{-1} in 0.1% (v/v) Triton X-100/10% (v/v) FBS in PBS. Cells were washed and incubated with secondary antibodies (fluorescein isothiocyanate-conjugated anti-mouse IgG and tetramethylrhodamine-conjugated anti-rabbit IgG; Chemicon), 200 ng 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) ml^{-1} in the same buffer. After a final wash cells were mounted in Prolong (Molecular Probes).

RESULTS

The UL45 protein is a γ -1 product incorporated into the virion

HELF monolayers were infected with HCMV laboratory strain AD169 1 week after confluence. Under these contact inhibition conditions, the cell proliferation indexes before infection (proliferating cell nuclear antigen abundance and mitotic index) were annulled (data not shown). The UL45 mRNA was detected from 3 h p.i. onward using semi-quantitative RT-PCR analysis (see supplementary

data Fig. 2A at JGV Online, <http://vir.sgmjournals.org>). The UL45 transcript, like the γ -1 (early-late) mRNA for UL32 (pp150) and unlike UL123 IE transcript and UL54 (DNA polymerase) β (early) transcript, was found to be reduced by treatment with PFA. This inhibitor of viral DNA polymerase allows differentiation of HCMV products based on their dependence on the onset of DNA replication (see supplementary data Fig. 2B at JGV Online). All transcripts except UL123 mRNA and a cellular control were sensitive to protein synthesis inhibition by CHX (see supplementary data Fig. 2B at JGV Online).

In Western blot analysis, anti-UL45 protein (-pUL45) antibodies recognized a ~ 108 kDa polypeptide in HCMV-infected HELF lysates, which co-migrated with a recombinant pUL45 from transfected HEK-293 human cells (Fig. 1A). The pUL45-specific band was detected at 24 h p.i. and accumulated until 120 h p.i. PFA profoundly inhibited pUL45 accumulation, like that of the bona fide γ -2 (true late) UL99 product pp28 (Depto & Stenberg, 1992) (Fig. 1B). In comparison, in PFA-treated infections a robust synthesis of UL123-122 products (IE1-pp72 and

IE2-pp86 proteins) and a reduced synthesis of UL44 product (pp52, a β protein) were observed at the same time points.

These data assign UL45 to the HCMV γ -1 genes, whose mRNA translation, rather than transcription, is postponed to the onset of genome replication (Mocarski, 1996). The late accumulation kinetics encouraged us to investigate whether pUL45 becomes incorporated into HCMV particles. Significant amounts of pUL45 were indeed found in gradient-purified HCMV particles (Fig. 1C). The UL45 protein remained associated with particles stripped off the envelope by exposure to a non-ionic detergent, but this treatment rendered it completely sensitive to trypsin. A pUL45 band was similarly detected in virions of the RVd65 mutant, an AD169 strain derivative (Schmolke *et al.*, 1995) that lacks a functional UL83 open reading frame and that no longer produces the UL83 protein (pp65)-rich dense bodies. Taking sedimentation with detergent-insoluble components as proof of association with capsid-tegument, protease sensitization in the presence of detergent as proof of association with tegument, and presence in dense body-free particles as proof of incorporation into infectious virions, we infer that, akin to HSV-2 ICP10 (Smith & Aurelian, 1997), pUL45 is exported from the cell as a virion tegument component. As immunofluorescent analysis of infected cells showed that pUL45 is cytoplasmic (data not shown), pUL45 joins the list of tegument proteins, the extra nuclear localization of which proves a re-envelopment step in HCMV virogenesis (Sanchez *et al.*, 2000 a, b).

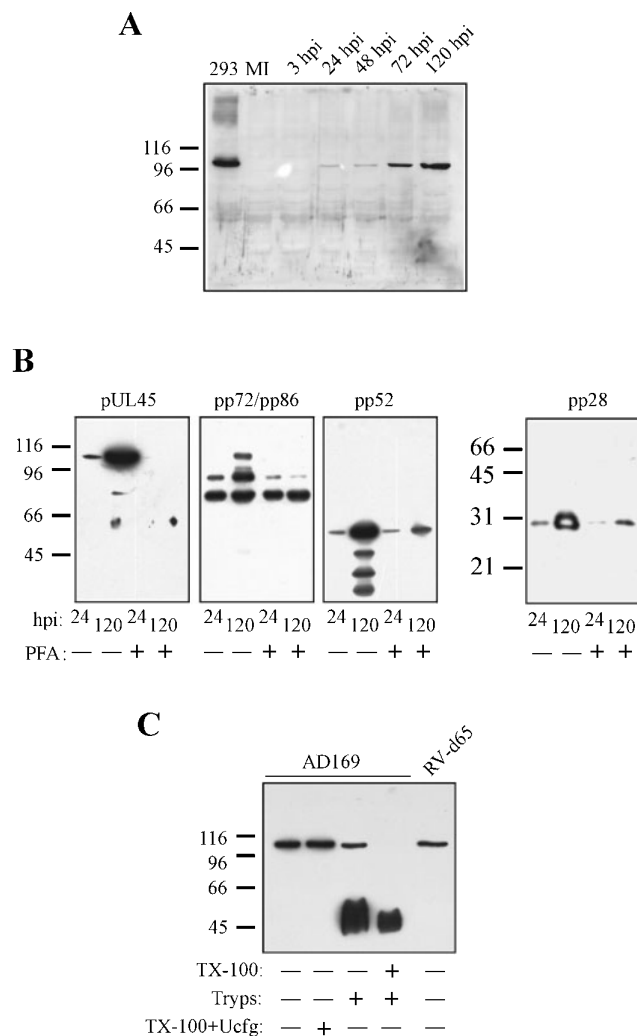


Fig. 1. UL45 protein synthesis in the course of HELF infection with HCMV-AD169. (A) pUL45 detection in HELF (total cell protein from 1×10^4 infected or mock-infected cells), using anti-UL45 polyclonal antibody. 293, total lysate of 5×10^3 HEK-293 cells expressing full-length pUL45; MI, mock infected. Molecular mass marker positions are shown on the left. (B) pUL45 accumulation is suppressed by PFA. AD169-infected HELF were treated or mock-treated with PFA, and cell lysates analysed for content in the indicated proteins; pp72-pp86, UL123-UL122 products; pp52, UL44 product; pp28, UL99 product. The multiple bands seen in the pp52 drug-free samples at 120 h p.i. in this blot (as well as the bands seen in Fig. 2A) are the effect of band contamination and/or spontaneous degradation. Due to the very high sensitivity of the anti-pp28 mAb staining, the pp28 signal at 120 h p.i. without PFA exceeds the film linear dynamic range, and pp28 inhibition by PFA is largely underestimated. (C) pUL45 is incorporated into virion tegument. Purified HCMV virions of either the AD169 strain or the mutant RV d65, which does not produce pp65 lower matrix protein nor dense bodies, were treated or mock-treated with: non ionic detergent (0.5% Triton X-100, TX-100), ultracentrifugation (Ucfcg) and trypsin digestion (Tryps), followed by Western blot analysis with anti-pUL45 antibodies. Partial pUL45 digestion in the absence of detergent is probably due to envelope breakage; the degradation form recognized by antibodies after trypsin exposure has the mobility expected for the pUL45 N-terminal region used as the immunogen, which may indicate a compactly folded domain.

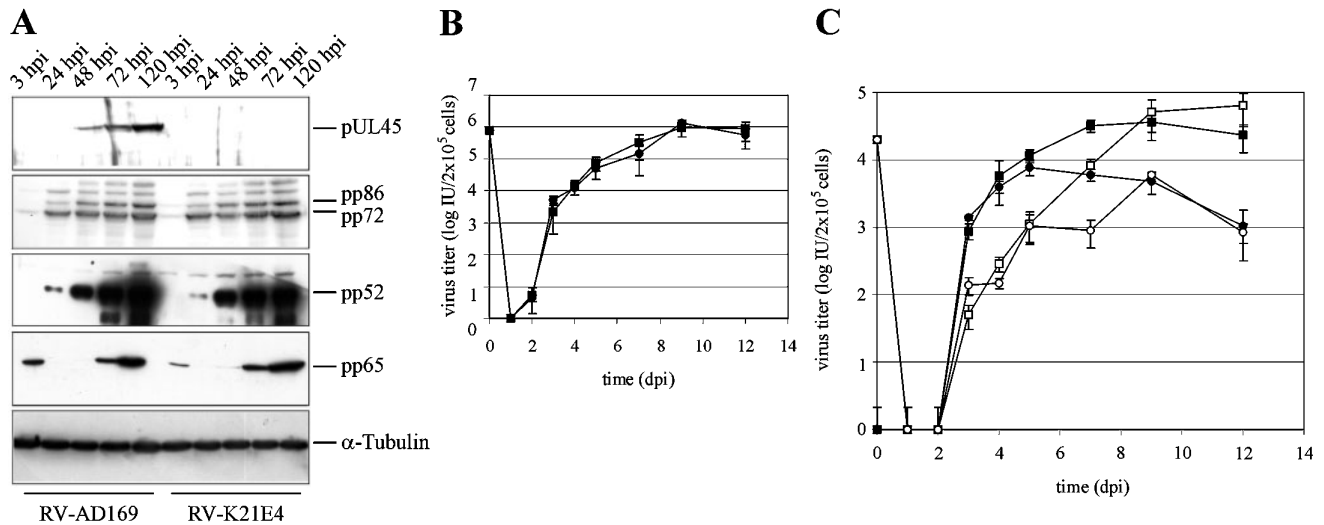


Fig. 2. Growth properties of RV-AD169 and RV-K21E4 in HELF. (A) HELF were infected at an m.o.i. of 4 with either the parental virus or the mutant RV-K21E4 and analysed at the indicated time points for the expression of pUL45, representative IE (pp72–pp86) and β (pp52 and pp65) viral proteins, and of cellular α -tubulin. (B) Virus growth curves at a high and (C) low m.o.i. Resting HELF were infected at an m.o.i. of either 3 or 0.1 IU per cell and titres of the resulting progeny in either whole cell lysates (B) or cell lysates and culture supernatants (C) were determined at the indicated times p.i. The titre at 0 h p.i. represents the input virus (squares, RV-AD169; circles, RV-K21E4; solid symbols, whole cell lysate viral titres; open symbols, culture supernatant viral titres; error bars represent the standard deviation of the geometric mean of three replicate cultures).

The UL45 null mutants are viable but exhibit a replication deficit at a low m.o.i.

Recombinant viruses carrying the UL45 orf interrupted by a transposon (Tn) insertion were isolated by screening a Tn library of the cloned AD169 genome (Borst *et al.*, 1999; Hobom *et al.*, 2000; for details see supplementary data on materials at JGV Online, <http://vir.sgmjournals.org>). Two Tn insertion mutants were identified (K21E4 and K19H1). Direct Bac sequencing mapped Tn insertions at nucleotide 59180 (K21E4) and 58642 (K19H1) (see supplementary data Fig. 3A–C at JGV Online, <http://vir.sgmjournals.org>). Tn insertions truncate UL45 at codon 64 and 244, respectively (see supplementary data Fig. 1 at JGV Online). Reconstituted virus (RV) was recovered by transfection of Bac DNA into fibroblasts. Comparison of RV-AD169 (wild-type), RV-K19H1 and RV-K21E4 by Southern blot (see supplementary data Fig. 3D at JGV Online) and PCR-sequencing (data not shown), confirmed a stable Tn insertion in the virus mutants. We conclude, in agreement with earlier work (Hahn *et al.*, 2002), that the UL45-encoded protein is dispensable for replication of HCMV in primary HELF. Subsequent work focused on RV-K21E4, in which UL45 truncation after a few codons minimizes the risk of a hypomorph variant.

The absence of pUL45 in RV-K21E4 infected HELF was confirmed by Western blot (Fig. 2A). By contrast, the levels of representative IE, β (Fig. 2A) and γ proteins (data not shown) were unchanged in the mutant. This held true in

particular for the UL44 product pp52 (Fig. 2A), an essential viral DNA polymerase processivity factor (Ripalti *et al.*, 1995). UL44 is located downstream from, and is co-oriented with, UL45 (see supplementary data Fig. 3C at JGV Online); thus no position effect is exerted on the downstream gene by Tn insertion within UL45.

Single step (high m.o.i.) HELF infections showed overlapping growth curves for parental and mutant viruses (Fig. 2B), as reported for the UL45-KO mutant of the endotheliotropic HCMV bacvirus (Hahn *et al.*, 2002). Nonetheless, an appreciable reduction of mutant virus accumulation was observed under low-m.o.i. (0.1 IU per cell) conditions. RV-AD169 and RV-K21E4 growth curves diverged at 5 days p.i. and the final titre of the mutant (both cell-associated and secreted) was >1 log lower (Fig. 2C). To test whether the missing UL45 product was the cause of the growth difference, a retrovirus vector was used to *trans*-complement UL45 in HELF. The recombinant retrovirus, here referred to as retro-UL45, expresses the UL45 coding region from the LTR. Additionally, it expresses the EGFP protein from an internal promoter. UL45 protein synthesis following transduction with 5 retrovirus units per cell was monitored by Western blot. UL45 protein was first detected at 24 h p.i.; it accumulated to a plateau level at 48 h p.i. (Fig. 3A) and was stably expressed for at least three subsequent doublings of transduced cells (not shown). EGFP was equally expressed by cells infected with retro-UL45 and with void retroviral vector (retro-cont). EGFP-positive HELF were isolated by

fluorescence-activated cell sorting and used for HCMV infections. RV-AD169 and RV-K21E4 growth curves were reassessed in retro-UL45 and retro-cont transduced cells. While RV-AD169 growth was the same in the two cell hosts, RV-K21E4 replicated more efficiently in retro-UL45 transduced cells, with final titres approaching those of wild-type (Fig. 3B). Thus, the growth defect of UL45 mutant can be ascribed to the disruption of UL45.

The ability of the mutant to form plaques was also investigated. Cells were infected at a low m.o.i., with anti-HCMV antibodies (pooled human sera) added to the medium to prevent secondary infections. Although average plaque extension was similar, the mutant failed to form large plaques (all plaques in the 2–15 cells per plaque

interval at 9 days p.i.), contrary to the parental (>20% of the plaques in the 16–30 cells per plaque interval at 9 days p.i.; data not shown).

UL45 knockout leaves dNTP synthesis unaltered

To test whether the absence of pUL45 was directly hindering HCMV DNA replication due to a dNTP shortage, the kinetics of dNTP accumulation were examined in RV-AD169 and RV-K21E4-infected HELF (Fig. 4A). Infection resulted in the rise of the dATP pool, and a very large expansion of the dTTP pool, in agreement with a previous report (Biron *et al.*, 1986). A peak was detected at 2 days p.i., possibly in relation to a cellular RR peak (see below).

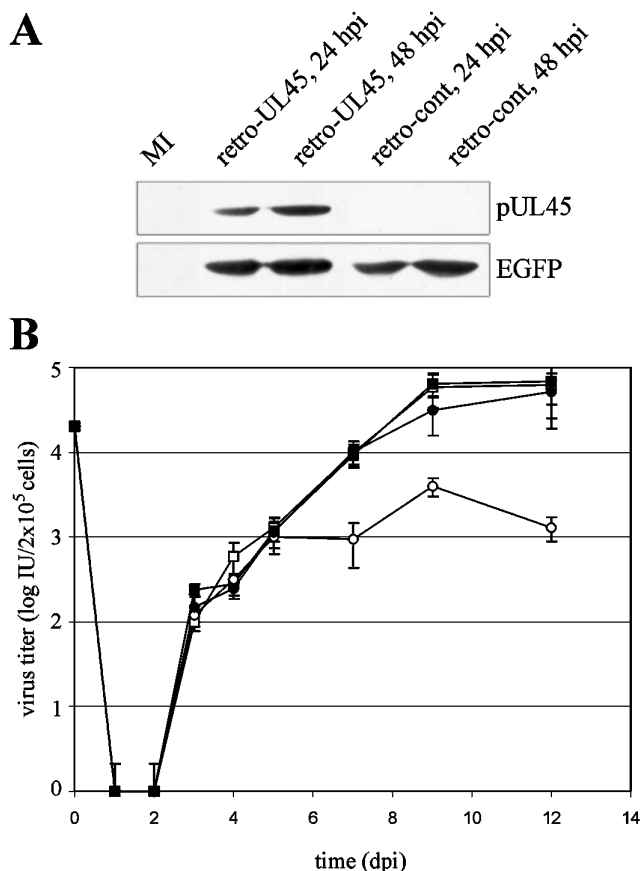


Fig. 3. RV-K21E4 growth deficit is relieved by transcomplementation. (A) HELF were transduced with retroviral vectors expressing UL45 plus EGFP (retro-UL45) or EGFP alone (retro-cont), and found to synthesize the appropriate proteins by Western blot. (B) EGFP-positive cells were isolated by fluorescence-activated cell sorting and infected as above. Only supernatant titres were determined in these experiments. Squares, RV-AD169; circles, RV-K21E4; solid symbols, retro-UL45; open symbols, retro-cont. Each error bar represents the standard deviation of the geometric mean of three replicate cultures.

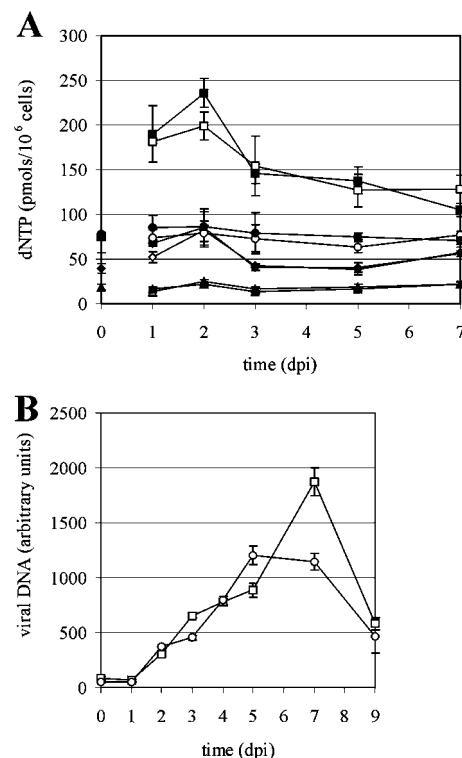


Fig. 4. Deoxyribonucleotide and viral DNA accumulation in RV-AD169 and RV-K21E4 infected cells. (A) Resting HELF were infected at an m.o.i. of 0.1 and low molecular mass cell extracts prepared as described in Methods at each time point. dNTP content was determined with the polymerase assay (Lindberg & Skoog, 1970; Skoog, 1970). Squares, dTTP; circles, dCTP; diamonds, dATP; triangles, dGTP; open symbols, RV-AD169-infected cells; solid symbols, RV-K21E4-infected cells; grey symbols, cells processed immediately after mock-infection. (B) Cells infected as in (A) were harvested at the indicated time points and total DNA purified. Viral DNA was quantified by slot-blot hybridization using a probe for UL83, and subsequent reprobings for GAPDH, to normalize signal for cellular DNA content. Squares, RV-AD169; circles, RV-K21E4. In both (A) and (B), error bars represent the 95% confidence interval of the mean of three replicate cultures.

However, the dNTP pool characteristics remained very similar in RV-AD169 and RV-K21E4-infected cells, throughout the entire course of infection.

Similarly, with both RV-AD169 and RV-K21E4, DNA synthesis was first detectable in total cell DNA at 2 days p.i. and proceeded exponentially until 5 days p.i. (Fig. 4B). Both the wild-type virus and the mutant exhibited DNA doubling times of approximately 24 h. However, mutant DNA accumulated to maximum levels at 5 days p.i., while the wild-type continued accumulating DNA until 7 days p.i. A 1.6-fold decrease in DNA production could be observed at day 7.

HCMV rescues the three cellular RR subunits

The above results indicate that dNTP synthesis and HCMV DNA replication are not primarily affected by UL45 knock out, implying that cellular RR is responsible for the provision of dNTPs to replicating viral DNA. Previous work (Chabes & Thelander, 2000; Engstrom *et al.*, 1985; Jordan & Reichard, 1998) has shown that mammalian RR is activated during the cell cycle. The R2 subunit acts as the limiting factor, restricting RR activity to the S–G₂ phase. By contrast, both R1 and R2 proteins are virtually undetectable in resting cells. Thus, HCMV infection must be able to force the expression of both subunits of human RR, in non-replicating cells. The impact on cellular RR mRNAs was investigated in HCMV infected resting HELF at 3–72 h p.i. By 3 h p.i., the virus had induced a marked host R2 transcription that continued through later times (Fig. 5A), whereas R1 transcripts were already present in non-infected cells. At the protein level (Fig. 5B), both cellular subunits were induced with similar kinetics, from almost undetectable levels to a peak at 48 h p.i., followed by a decrease at later times. A similar pattern was observed in RV-K21E4-infected cells. Cellular R1/R2 synthesis is then induced early following infection, with a post-transcriptional component for R1, and independent of UL45 expression.

A distinct cellular RR small subunit, p53R2, has been described (Tanaka *et al.*, 2000). DNA damaging agents induce the p53R2 gene in a p53-dependent manner, and the protein is targeted to nuclear repair sites; p53R2 can combine with R1 into an alternative holoenzyme (Guittet *et al.*, 2001). We investigated whether p53R2 is also induced by HCMV. Neither RV-AD169 (Fig. 6A) nor RV-K21E4 infection (data not shown) caused a rise in p53R2 transcripts. Rather, p53R2 mRNA was found to decline at late times p.i. (Fig. 6A). Nonetheless, p53R2 protein levels grew through late infection times (Fig. 6B), with an over 10-fold increase at 7 days p.i. We investigated whether also p53R2 subcellular localization was altered by HCMV infection, and this was found to be the case (Fig. 6C). Mock-infected cells displayed a faint cytoplasmic signal when analysed by indirect immunofluorescence with anti-p53R2 antibodies. By contrast, in cells infected with either RV-AD169 or RV-K21E4, p53R2 signal intensified and

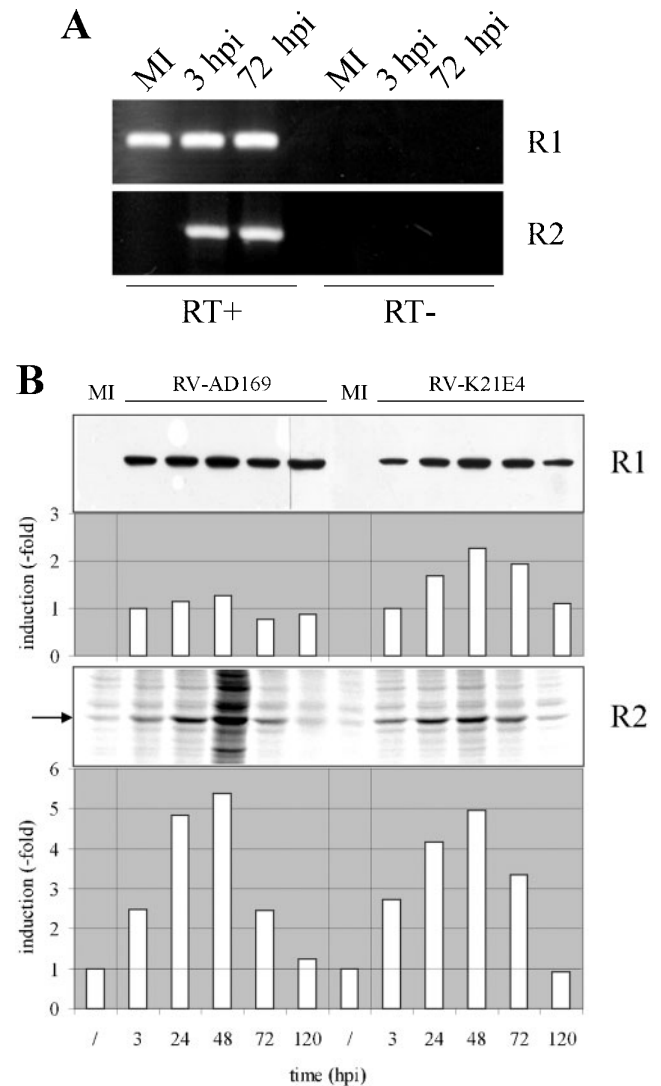


Fig. 5. Cellular S-phase RR subunits are both induced by HCMV infection in a pUL45-independent manner. (A) Total RNA from resting HELF infected with RV AD169 at an m.o.i. of 4, or mock-infected, was reverse transcribed (RT+) or mock-reverse transcribed (RT-) and amplified for 32 cycles with primers for either R1 or R2 gene (Table 1). (B) Resting HELF infected with RV-AD169 or RV-K21E4 as above were analysed at various h p.i. for R1 and R2 expression, in Western blot or by radioactive immunoprecipitation, respectively. Image quantifications are plotted below each panel. The bands at different molecular masses around the R2 band (arrow) indicate a background of non-specifically precipitated proteins; these are particularly intense in the sample at 48 h p.i. using RV-AD169. The values included in the histogram were derived by normalizing the R2 signal by the background signal in each lane.

became in part nuclear, like in cells treated with doxorubicin. HCMV, therefore, not only enhances p53R2 synthesis but also triggers its translocation into the nucleus. The induction-targeting mechanism is not influenced by pUL45.

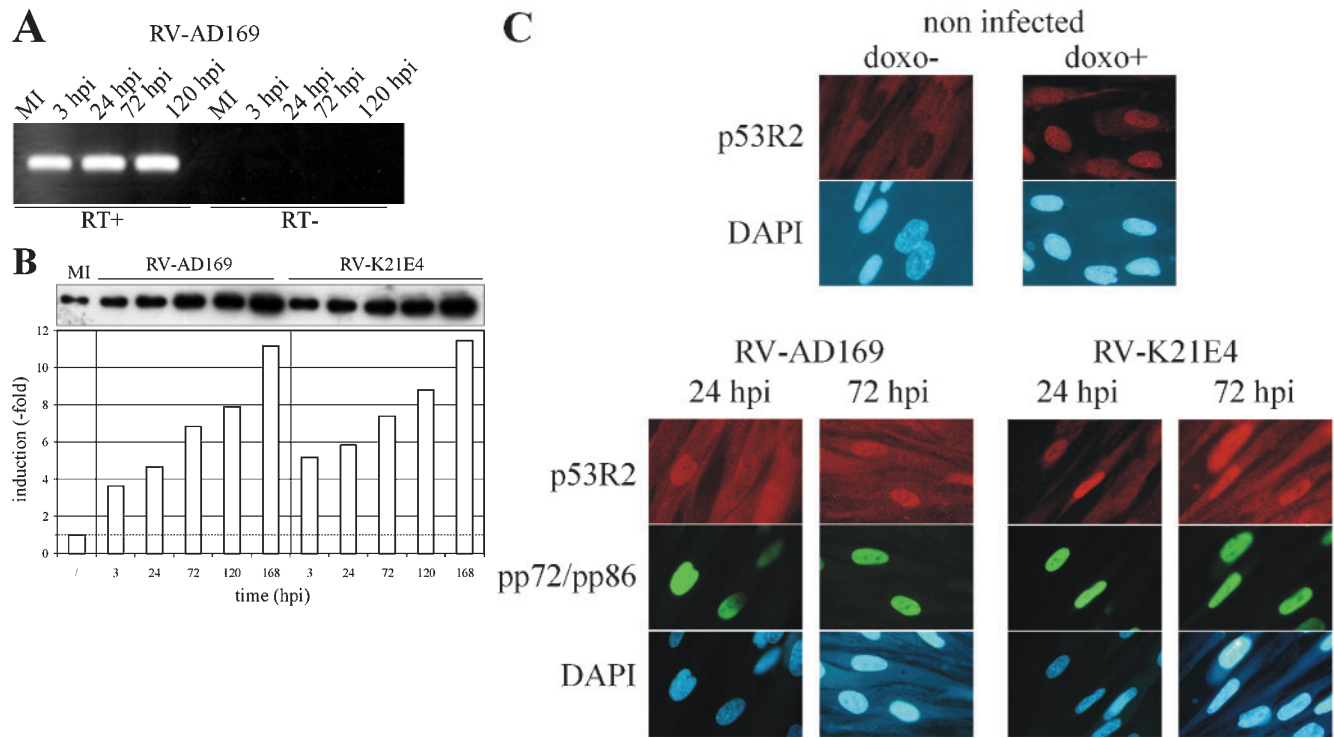


Fig. 6. HCMV infection post-transcriptionally induces p53R2. (A) RT-PCR analysis with p53R2-specific primers (Table 1). Cells were infected as in Fig. 6. (B) Western blot analysis with a p53R2-specific polyclonal antibody, and result quantification. (C) Indirect immunofluorescence analysis. Resting HELF were infected with either RV-AD169 or RV-K21E4, fixed at various time points and double-labelled with anti-p53R2 (red) and anti-pp72/pp86 (green) for immunofluorescence (bottom). Alternatively, non-infected cells were either treated or mock-treated for 48 h with 0.2 μg doxorubicin ml^{-1} to induce DNA damage and p53R2 nuclear localization. Cell nuclei were counterstained with DAPI (light blue). Magnification, $\times 50$.

UL45 is not inherently antiapoptotic

The finding that the UL45 homologue in MCMV, M45, prevents apoptosis in mouse endothelial cells (Brune *et al.*, 2001) prompted the question of whether the growth deficit of the UL45 mutant could be explained by the loss of a proportion of infected cells by apoptotic death. HELF infected with either RV-AD169 or RV-K21E4 were tested at 3, 5 and 7 days p.i. for two markers of apoptosis (nuclear DNA fragmentation and exposure on the cell surface of phosphatidyl serine) as described (Hahn *et al.*, 2002). The parental and mutant viruses did not differ in replicate tests, showing similar, negligible levels of spontaneous apoptosis (<5% apoptotic cells, with both staining procedures).

A difference at the limit of statistical significance did emerge only when apoptosis was provoked by exposing the infected HELF to anti-Fas antibody plus CHX. Cell survival amounted to approximately 50% of the RV-AD169 infected monolayer and 26% of RV-K21E4 infected cells (Fig. 7). To confirm that the viral protein was responsible for the parental virus advantage, and to test whether UL45 could trigger the antiapoptotic effect in the absence of infection, the experiment was repeated in retro-UL45-transduced cells. In this setting, survival of

parental- and mutant-infected HELF was indistinguishable, but pUL45 was unable to protect uninfected cells (Fig. 7). Thus, pUL45 confers a marginal but specific protection against death receptor signalling in the context of virus infection, but does not display the properties of a dominant antiapoptotic factor.

DISCUSSION

The large DNA virus RRs originated from ancestrally pirated cellular genes, enabling those viruses to uncouple their replication from the availability of cellular RR, and therewith to expand their tissue tropism in the host. Such a function is still operating in α - and γ -herpesviruses. Some of the α -herpesvirus R1 genes, however, have been made bifunctional by acquisition of a signalling-antiapoptotic function (Perkins *et al.*, 2002; Langelier *et al.*, 2002). The results described here, based on an HCMV laboratory strain, together with the recent reports on an HCMV endotheliotropic strain and on MCMV, make a strong case that β -herpesviruses have taken an additional evolutionary step, wherein the hijacked gene loses the original enzyme function. Our results, in fact, argue against any residual function of pUL45 as an active R1. Two independent UL45

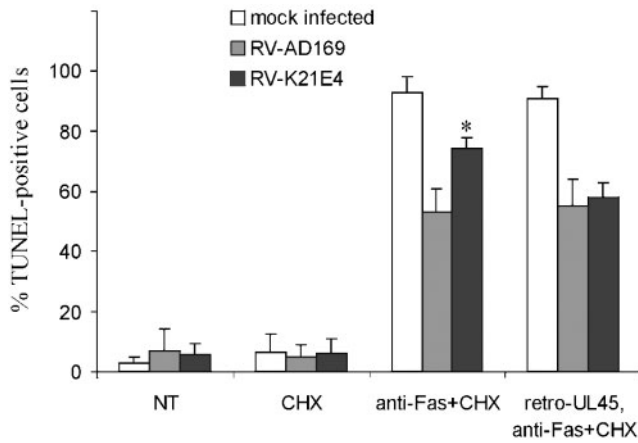


Fig. 7. Effect of RV-AD169 and RV-K21E4 infection on HELF apoptosis induced by anti-Fas. Resting HELF seeded on coverslips were infected (m.o.i. of 4) or mock-infected with either RV-AD169 or RV-K21E4. In one case, cells were transduced with retro-UL45 to provide pUL45 *in trans* before HCMV infections. Cells were treated at 84 h p.i. for 12 h with 0.2 μg anti-Fas mAb 7C11 (Roche) ml^{-1} in the presence of 10 μg CHX ml^{-1} , with CHX alone, or mock-treated. Paraformaldehyde-fixed cells were processed for terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labelling (TUNEL; Gavrieli *et al.*, 1992). Cell death (% TUNEL-positive cells) was determined by visual observation of cells under a phase microscope. Ten $\times 25$ fields (>700 nuclei) per infection were analysed. Error bars are the 95% confidence interval of the mean of three replicate cultures. *, $P < 0.05$.

null mutants of HCMV could be readily propagated in resting fibroblasts. A single mutant, RV-K21E4, was more extensively analysed. Mutant replication at a high m.o.i. was indistinguishable from that of the parent, as was the accumulation of representative β/γ products requiring DNA synthesis for optimal expression. Thus, the absence of pUL45 does not dramatically affect the replication cycle of HCMV. Above all, a direct assay of dNTP and viral DNA accumulation in infected cells failed to demonstrate significant differences between parental and mutant virus, giving support to the view that HCMV R1 is not functionally related to dNTP production. We have directly assessed the ability of recombinant pUL45 from *E. coli* to associate with cellular R2 or p53R2 into an active RR. Unpublished data, (M. Patrone) demonstrate that pUL45 is devoid of RR activity.

To offset the loss of a virus-encoded RR, HCMV effectively rescues cellular RR activity in resting HELF, by upregulating all known cellular RR subunits, according to our data. In the case of S–G2 phase RR subunits, our data extend the evidence from a DNA microarray study to the infection context, showing that both R1 and R2 genes are induced, together with many other S-phase genes under E2F1 transcriptional control, in human fibroblasts expressing HCMV IE2–pp86 (Song & Stinski, 2002). While R1–R2 induction

fits into the general picture of cell cycle subversion operated by HCMV IE proteins, conducive to the expression of S phase replication proteins (reviewed by Fortunato *et al.*, 2000; Femington, 2001; Kalejta & Shenk, 2002), our finding that p53R2 is also induced and targeted to the nucleus of infected HELF is intriguing because p53R2 is related to DNA repair, not replication; the p53R2 gene is indeed the first p53 transcriptional target directly linking p53 to repair (Tanaka *et al.*, 2000; Yamaguchi *et al.*, 2001). In HCMV infected fibroblasts p53 protein is stabilized, but its ability to activate transcription is blocked (Kalejta & Shenk, 2002). Our data do not contradict this point, as p53R2 transcript levels do not increase during infection. Nevertheless, HCMV is capable of circumventing p53 blockade by post-transcriptionally inducing p53R2 – whether this is by enhancing mRNA translation or by stabilizing the protein remains to be established. A related question is whether p53R2 nuclear targeting is a consequence of the activation of a DNA damage signal in the infected cell, or is obtained ad hoc by virus encoded factor(s). In either case, the concentration of an RR activating subunit in the proximity of HCMV replication factories may help to boost the supply of precursors to the viral DNA synthesis and repair machinery.

Three phenotypes could be associated with the UL45-KO mutant RV-K21E4. A multi-step growth analysis highlighted a 1 log (50-fold) growth deficit. To formally assign the growth defect to UL45 inactivation, the viral protein was complemented *in trans* via a retrovirus vector. The *trans*-complemented mutant grew like the parental, showing that the phenotype can be safely ascribed to pUL45 absence and not to polar effects on the surrounding genes, UL44 and UL46 (minor capsid protein), both of which are essential for virus replication (Mocarski, 1996; Pari & Anders, 1993; Pari *et al.*, 1993; Ripalti *et al.*, 1995). This also rules out adventitious mutations introduced during bacvirus manipulations in the prokaryotic host. A possibly related feature is that UL45-KO mutant forms plaques less efficiently than the parent. As both phenotypes are typical of defects in virus egress and/or cell-to-cell spread, pUL45 might be involved in some aspect of virion assembly and/or penetration. In agreement with this hypothesis, pUL45 is a late phase product and is present in purified virions as a tegument component, with the potential to enhance virion infectivity or to act as a factor delivered to the host cell upon infection.

An additional feature of the mutant is that it confers on the infected cells a slightly (<2-fold) reduced protection from anti-Fas-induced apoptosis relative to the parental. This difference was not observed with agents acting via the mitochondrial pathway (etoposide and menadione; data not shown). Also, non-infected fibroblasts synthesizing pUL45 were not protected from Fas activation. Four HCMV products have been identified that counter experimentally induced apoptosis: IE1–IE2 proteins, which interfere with p53 function and activate the phosphatidylinositol 3'-OH

kinase-Akt pathway (Zhu *et al.*, 1995; Yu & Alwine, 2002); vMIA (UL37x1), which acts at the mitochondrial level (Goldmacher *et al.*, 1999); and the caspase-8 inhibitor vICA (UL36) (Skaletskaya *et al.*, 2001). vICA is inactivated by a mutation in the HCMV strain AD169*var*ATCC, which explains the incomplete protection imparted by the parental virus in our study (Skaletskaya *et al.*, 2001; this study). All these proteins confer a substantial protection when assayed independent of virus infection; that is, they are strictly speaking antiapoptotic. By contrast, the effect of pUL45 is weak, selective for Fas-induced apoptosis among the tested stimuli, and restricted to infected fibroblasts. Perhaps, this mild pro-survival activity constitutes a side-effect of a physiological role. MAPK/ERK and p38 MAPK are activated by HCMV (Rodems & Spector 1998; Johnson *et al.*, 2000, 2001), and the impact of pUL45 on this and other transduction pathways warrants exploration.

A relevant question is to what extent does pUL45 differ from M45. A reduction in progeny virus titre similar to that described here is apparent in MCMV M45-KO mutants grown in murine fibroblasts (Brune *et al.*, 2001). Thus, the loss of RR activity and a measurable impact on virus propagation in fibroblasts are seemingly general traits of β -herpesvirus R1 proteins. The report by Lembo *et al.* (2000) that dNTP pools in MCMV-infected fibroblasts resist the shift induced by exogenous thymidine has been taken as an indication that MCMV synthesizes an active, allosterically unregulated, R1. However, the effect might involve other levels of dNTP homeostasis, e.g. nucleotide excretion.

On the other hand, M45 has been recognized as a factor preventing cell-autonomous apoptosis in MCMV-infected endothelial cells (Brune *et al.*, 2001), while its antiapoptotic potential when expressed alone has not been analysed. Growth in endothelial cells could not be checked for the mutants described here (AD169 strain is non-endotheliotropic), but the study of an UL45-KO mutant of an endotheliotropic strain failed to show apoptosis of infected human umbilical vein endothelial cells (Hahn *et al.*, 2002). In β -herpesvirus R1 proteins, the RR region of homology (a central all-alpha and distal barrel domain; see supplementary data Fig. 1 at JGV Online, <http://vir.sgmjournals.org>), common to the entire R1 family, is preceded by a unique N-terminal region subdivided into an N-terminal stretch heterogeneous in both size and sequence, followed by a conserved ~110 aa homology box (BRH1). The long (385 amino acids) M45 N-terminal stretch has interspersed tracts of homology to HSV-2 ICP10 that might account for the antiapoptotic function. These are not detected in the 167 amino acid pUL45 N-terminal stretch. It is thus conceivable that distinct, specialized functions reside in pUL45 and pM45 N termini, while the BRH1 and RR homology could exert conserved function(s). In transfected HEK-293 cells, full-length pUL45 is targeted to the insoluble, cytoplasmic cytoskeleton, whereas a variant devoid of the RR region is cytosolic (M. Patrone, unpublished data).

UL45 RR domains might then exert a targeting function, possibly to permit incorporation into virion tegument.

ACKNOWLEDGEMENTS

The authors are indebted to Lorena Zentilin and Mauro Giacca for the gift of anti-R2 monoclonal antibody. This work was supported by the Italian Ministero della Salute, Istituto Superiore di Sanità Progetto Nazionale AIDS (grant no. 40B.66 to G.M. and 50D.12 to F.B.), by the Italian Health Ministry–Target Research 2001 (grant 08920401 to G.M. and F.B.), by the CNR-Biotechnology Target Project (to A.G. and F.B.), by the MIUR COFIN 2001 (grant 2001055821_005 to G.M. and F.B.), and by the Deutsche Forschungsgemeinschaft SFB 455 (Project A7/U. H. K. and B7/G. H.).

REFERENCES

- Aurelian, L. (1998).** Herpes simplex virus type 2: unique biological properties include neoplastic potential mediated by the PK domain of the large subunit of ribonucleotide reductase. *Front Biosci* **3**, D237–249.
- Biron, K. K., Fyfe, J. A., Stanat, S. C., Leslie, L. K., Sorrell, J. B., Lambe, C. U. & Coen, D. M. (1986).** A human cytomegalovirus mutant resistant to the nucleoside analog 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine (BW B759U) induces reduced levels of BW B759U triphosphate. *Proc Natl Acad Sci U S A* **83**, 8769–8773.
- 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**, 8320–8329.
- Britt, W. J. & Alford, C. A. (1996).** Cytomegalovirus. In *Fields Virology*, 3rd edn, vol. 2, pp. 2493–2523. Edited by B. N. Fields, D. M. Knipe, P. M. Howley. Philadelphia: Lippincott–Raven.
- Brune, W., Menard, C., Heesemann, J. & Koszinowski, U. H. (2001).** A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. *Science* **291**, 303–305.
- Chabes, A. & Thelander, L. (2000).** Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. *J Biol Chem* **275**, 17747–17753.
- Chee, M. S., Bankier, A. T., Beck, S. & 12 other authors (1990).** Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol* **154**, 125–169.
- Chung, T. D., Wymer, J. P., Smith, C. C., Kulka, M. & Aurelian, L. (1989).** Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *J Virol* **63**, 3389–3398.
- Conner, J. (1999).** The unique N terminus of herpes simplex virus type 1 ribonucleotide reductase large subunit is phosphorylated by casein kinase 2, which may have a homologue in *Escherichia coli*. *J Gen Virol* **80**, 1471–1476.
- Cooper, J., Conner, J. & Clements, J. B. (1995).** Characterization of the novel protein kinase activity present in the R1 subunit of herpes simplex virus ribonucleotide reductase. *J Virol* **69**, 4979–4985.
- Davis, R., Thelander, M., Mann, G. J., Behravan, G., Soucy, F., Beaulieu, P., Lavalley, P., Graslund, A. & Thelander, L. (1994).** Purification, characterization, and localization of subunit interaction area of recombinant mouse ribonucleotide reductase R1 subunit. *J Biol Chem* **269**, 23171–23176.

- Depto, A. S. & Stenberg, R. M. (1992).** Functional analysis of the true late human cytomegalovirus pp28 upstream promoter: *cis*-acting elements and viral *trans*-acting proteins necessary for promoter activation. *J Virol* **66**, 3241–3246.
- Engstrom, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L. & Tribukait, B. (1985).** Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J Biol Chem* **260**, 9114–9116.
- Eriksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnstrom, K., Sjoberg, B. M. & Eklund, H. (1997).** Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* **5**, 1077–1092.
- Flemington, E. K. (2001).** Herpesvirus lytic replication and the cell cycle: arresting new developments. *J Virol* **75**, 4475–4481.
- Fortunato, E. A., McElroy, A. K., Sanchez, I. & Spector, D. H. (2000).** Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. *Trends Microbiol* **8**, 111–119.
- Gallina, A., Simoncini, L., Garbelli, S. & 7 other authors (1999).** Polo-like kinase 1 as a target for human cytomegalovirus pp65 lower matrix protein. *J Virol* **73**, 1468–1478.
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992).** Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**, 493–501.
- Gerna, G., Percivalle, E., Baldanti, F., Sozzani, S., Lanzarini, P., Genini, E., Lilleri, D. & Revello, M. G. (2000).** Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. *J Virol* **74**, 5629–5638.
- Goldmacher, V. S., Bartle, L. M., Skaletskaya, A. & 10 other authors (1999).** A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc Natl Acad Sci U S A* **96**, 12536–12541.
- Guttet, O., Hakansson, P., Voevodskaya, N., Fridt, S., Graslund, A., Arakawa, H., Nakamura, Y. & Thelander, L. (2001).** Mammalian p53R2 protein forms an active ribonucleotide reductase *in vitro* with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J Biol Chem* **276**, 40647–40651.
- Hahn, G., Jores, R. & Mocarski, E. S. (1998).** Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc Natl Acad Sci U S A* **95**, 3937–3942.
- 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.
- Heineman, T. C. & Cohen, J. I. (1994).** Deletion of the varicella-zoster virus large subunit of ribonucleotide reductase impairs growth of virus *in vitro*. *J Virol* **68**, 3317–3323.
- Hobom, U., Brune, W., Messerle, M., Hahn, G. & Koszinowski, U. H. (2000).** Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. *J Virol* **74**, 7720–7729.
- Idowu, A. D., Fraser-Smith, E. B., Poffenberger, K. L. & Herman, R. C. (1992).** Deletion of the herpes simplex virus type 1 ribonucleotide reductase gene alters virulence and latency *in vivo*. *Antiviral Res* **17**, 145–156.
- Jacobson, J. G., Leib, D. A., Goldstein, D. J., Bogard, C. L., Schaffer, P. A., Weller, S. K. & Coen, D. M. (1989).** A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. *Virology* **173**, 276–283.
- Johnson, R. A., Huong, S. M. & Huang, E. S. (2000).** Activation of the mitogen-activated protein kinase p38 by human cytomegalovirus infection through two distinct pathways: a novel mechanism for activation of p38. *J Virol* **74**, 1158–1167.
- Johnson, R. A., Ma, X. L., Yurochko, A. D. & Huang, E. S. (2001).** The role of MKK1/2 kinase activity in human cytomegalovirus infection. *J Gen Virol* **82**, 493–497.
- Jordan, A. & Reichard, P. (1998).** Ribonucleotide reductases. *Annu Rev Biochem* **67**, 71–98.
- Kalejta, R. F. & Shenk, T. (2002).** Manipulation of the cell cycle by human cytomegalovirus. *Front Biosci* **7**, D295–306.
- Kauppi, B., Nielsen, B. B., Ramaswamy, S., Larsen, I. K., Thelander, M., Thelander, L. & Eklund, H. (1996).** The three-dimensional structure of mammalian ribonucleotide reductase protein R2 reveals a more-accessible iron-radical site than *Escherichia coli* R2. *J Mol Biol* **262**, 706–720.
- Langelier, Y., Champoux, L., Hamel, M., Guilbault, C., Lamarche, N., Gaudreau, P. & Massie, B. (1998).** The R1 subunit of herpes simplex virus ribonucleotide reductase is a good substrate for host cell protein kinases but is not itself a protein kinase. *J Biol Chem* **273**, 1435–1443.
- Langelier, Y., Bergeron, S., Chabaud, S., Lippens, J., Guilbault, C., Sasseville, A. M., Denis, S., Mosser, D. D. & Massie, B. (2002).** The R1 subunit of herpes simplex virus ribonucleotide reductase protects cells against apoptosis at, or upstream of, caspase-8 activation. *J Gen Virol* **83**, 2779–2789.
- Lembo, D., Griboudo, G., Hofer, A. & 7 other authors (2000).** Expression of an altered ribonucleotide reductase activity associated with the replication of murine cytomegalovirus in quiescent fibroblasts. *J Virol* **74**, 11557–11565.
- Lindberg, U. & Skoog, L. (1970).** A method for the determination of dATP and dTTP in picomole amounts. *Anal Biochem* **34**, 152–160.
- Lycksell, P. O., Ingemarson, R., Davis, R., Graslund, A. & Thelander, L. (1994).** 1H NMR studies of mouse ribonucleotide reductase: the R2 protein carboxyl-terminal tail, essential for subunit interaction, is highly flexible but becomes rigid in the presence of protein R1. *Biochemistry* **33**, 2838–2842.
- Maciejewski, J. P., Bruening, E. E., Donahue, R. E., Mocarski, E. S., Young, N. S. & St Jeor, S. C. (1992).** Infection of hematopoietic progenitor cells by human cytomegalovirus. *Blood* **80**, 170–178.
- Mocarski, E. S., Jr (1996).** Cytomegaloviruses and their replication. In *Fields Virology*, 3rd edn, vol. 2, pp. 2447–2492. Edited by B. N. Fields, D. M. Knipe, P. M. Howley. Philadelphia: Lippincott–Raven.
- Nordlund, P. & Eklund, H. (1993).** Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2. *J Mol Biol* **232**, 123–164.
- Paradis, H., Gaudreau, P., Massie, B., Lamarche, N., Guilbault, C., Gravel, S. & Langelier, Y. (1991).** Affinity purification of active subunit 1 of herpes simplex virus type 1 ribonucleotide reductase exhibiting a protein kinase activity. *J Biol Chem* **266**, 9647–9651.
- Pari, G. S. & Anders, D. G. (1993).** Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus ori-Lyt-dependent DNA replication. *J Virol* **67**, 6979–6988.
- Pari, G. S., Kacica, M. A. & Anders, D. G. (1993).** Open reading frames UL44, IRS1/TRS1, and UL36–38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. *J Virol* **67**, 2575–2582.

- Pavloff, N., Rivard, D., Masson, S., Shen, S. H. & Mes-Masson, A. M. (1992).** Sequence analysis of the large and small subunits of human ribonucleotide reductase. *DNA Seq* **2**, 227–234.
- Perkins, D., Pereira, E. F., Gober, M., Yarowsky, P. J. & Aurelian, L. (2002).** The herpes simplex virus type 2 R1 protein kinase (ICP10 PK) blocks apoptosis in hippocampal neurons, involving activation of the MEK/MAPK survival pathway. *J Virol* **76**, 1435–1449.
- Ripalti, A., Bocconi, M. C., Campanini, F. & Landini, M. P. (1995).** Cytomegalovirus-mediated induction of antisense mRNA expression to UL44 inhibits virus replication in an astrocytoma cell line: identification of an essential gene. *J Virol* **69**, 2047–2057.
- Rodems, S. M. & Spector, D. H. (1998).** Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. *J Virol* **72**, 9173–9180.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanchez, V., Sztul, E. & Britt, W. J. (2000a).** Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. *J Virol* **74**, 3842–3851.
- Sanchez, V., Greis, K. D., Sztul, E. & Britt, W. J. (2000b).** Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. *J Virol* **74**, 975–986.
- Schmolke, S., Kern, H. F., Drescher, P., Jahn, G. & Plachter, B. (1995).** The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture. *J Virol* **69**, 5959–5968.
- Sinzger, C., Grefte, A., Plachter, B., Gouw, A. S. H., 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.
- Skaletskaya, A., Bartle, L. M., Chittenden, T., McCormick, A. L., MocarSKI, E. S. & Goldmacher, V. S. (2001).** A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. *Proc Natl Acad Sci U S A* **98**, 7829–7834.
- Skoog, L. (1970).** An enzymatic method for the determination of dCTP and dGTP in picomole amounts. *Eur J Biochem* **17**, 202–208.
- Smith, C. C. & Aurelian, L. (1997).** The large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is associated with the virion tegument and has PK activity. *Virology* **234**, 235–242.
- Smith, C. C., Peng, T., Kulka, M. & Aurelian, L. (1998).** The PK domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is required for immediate-early gene expression and virus growth. *J Virol* **72**, 9131–9141.
- Smith, C. C., Nelson, J., Aurelian, L., Gober, M. & Goswami, B. B. (2000).** Ras-GAP binding and phosphorylation by herpes simplex virus type 2 RR1 PK (ICP10) and activation of the Ras/MEK/MAPK mitogenic pathway are required for timely onset of virus growth. *J Virol* **74**, 10417–10429.
- Soderberg-Naucler, C., Fish, K. N. & Nelson, J. A. (1997).** Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **91**, 119–126.
- Somia, N. V., Miyoshi, M. J., Schmitt, H. & Verma, I. M. (2000).** Retroviral vector targeting to human immunodeficiency virus type 1-infected cells by receptor pseudotyping. *J Virol* **74**, 4420–4424.
- Song, Y. J. & Stinski, M. F. (2002).** Effect of the human cytomegalovirus IE86 protein on expression of E2F-responsive genes: a DNA microarray analysis. *Proc Natl Acad Sci U S A* **99**, 2836–2841.
- Stubbe, J., Ge, J. & Yee, C. S. (2001).** The evolution of ribonucleotide reduction revisited. *Trends Biochem Sci* **26**, 93–99.
- Sun, Y. & Conner, J. (1999).** The U28 ORF of human herpesvirus-7 does not encode a functional ribonucleotide reductase R1 subunit. *J Gen Virol* **80**, 2713–2718.
- Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. & Nakamura, Y. (2000).** A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* **404**, 42–49.
- Uhlin, U. & Eklund, H. (1994).** Structure of ribonucleotide reductase protein R1. *Nature* **370**, 533–539.
- Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M. A., Nakamura, Y. & Arakawa, H. (2001).** p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. *Cancer Res* **61**, 8256–8262.
- Yu, Y. & Alwine, J. C. (2002).** Human cytomegalovirus major immediate-early proteins and simian virus 40 large T antigen can inhibit apoptosis through activation of the phosphatidylinositolide 3'-OH kinase pathway and the cellular kinase Akt. *J Virol* **76**, 3731–3738.
- Zhu, H., Shen, Y. & Shenk, T. (1995).** Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J Virol* **69**, 7960–7970.