Journal of NeuroVirology, 9: 477–488, 2003

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280390218788

Taylor & Francis
Taylor & Francis Group

The herpes simplex virus (HSV) protein ICP34.5 is a virion component that forms a DNA-binding complex with proliferating cell nuclear antigen and HSV replication proteins

June Harland,[1] Paul Dunn,[1] Euan Cameron,[1] Joe Conner,[2] and S Moira Brown[1]

[1]*Glasgow University, Neurovirology Research Laboratories, Institute of Neurological Sciences, Southern General Hospital, Glasgow, United Kingdom;* [2]*Crusade Laboratories Ltd., Southern General Hospital, Glasgow, United Kingdom*

The replicative ability of ICP34.5-null herpes simplex virus (HSV) is cell type and state dependent. In certain cells, ICP34.5 interacts with protein phosphatase 1 to preclude host cell protein synthesis shutoff by dephosphorylation of the eukaryotic initiation factor eIF-2α. However, host cell shutoff is not induced by ICP34.5-null HSV in most cells, irrespective of type and state. In general, dividing cells support replication of ICP34.5-null HSV; nondividing cells cannot. Previously the authors showed that ICP34.5 binds to proliferating cell nuclear antigen (PCNA), a protein necessary for cellular DNA replication and repair. Here the authors demonstrate that (1) the interaction between ICP34.5 and PCNA involves two regions of the virus protein; (2) ICP34.5 forms a complex with HSV replication proteins that is DNA binding; (3) at early times in infection, ICP34.5 colocalizes with PCNA and HSV replication proteins in cell nuclei, before accumulating in the cytoplasm; and (4) ICP34.5 is a virion protein. In light of ongoing clinical trials assessing the safety and efficacy of ICP34.5-null HSV, it is vital that the roles of ICP34.5 in HSV replication are understood. The authors propose that in nondividing cells, ICP34.5 is required to switch PCNA from repair to replication mode, a prerequisite for the initiation of HSV replication. *Journal of NeuroVirology* (2003) **9,** 477–488.

Keywords: HSV replication proteins; ICP34.5; PCNA

Introduction

The herpes simplex virus (HSV) protein ICP34.5 is a specific determinant of virulence (Chou *et al*, 1990). *In vivo*, the ICP34.5-null mutant HSV1716 fails to replicate in both the central nervous system (MacLean *et al*, 1991; McKie *et al*, 1998a) and the peripheral nervous system (Robertson *et al*, 1992), although in many tissue culture lines, its replication is indistinguishable from that of the parental HSV-1 strain 17. The proposal that this selective pattern of growth might allow ICP34.5-null HSV to replicate in tumor cells, while sparing surrounding normal tissue, has been tested in a number of animal systems (Markert *et al*, 1993; Chambers *et al*, 1995; Randazzo *et al*, 1995, 1996, 1997; Lasner *et al*, 1996; Kucharczuk *et al*, 1997). Tumor regression and improved survival times were demonstrated. Subsequently we have completed three phase 1 toxicity trials in patients with primary and recurrent glioma, in which HSV1716 was injected into tumors (Rampling *et al*, 2000; Papanastassiou *et al*, 2002; Harrow *et al*, manuscript in preparation).

Address correspondence to S Moira Brown, Glasgow University, Neurovirology Research Laboratories, Institute of Neurological Sciences, Southern General Hospital, Glasgow, G51 4TF, United Kingdom. E-mail: smbrown@clinmed.gla.ac.uk

The authors are indebted to colleagues who generously provided reagents used in this study. Dr. Derrick Dargan supplied the HSV virions, light particles, and PREPs; Dr. Bruce Stillman the pT7-hPCNA plasmid; Dr. Liz McKie the 34.5pGEM plasmid; and Drs. Howard Marsden and Susan Graham supplied antibodies.

Received 6 August 2002; revised 11 November 2002; accepted 3 January 2003.

Additionally, a trial using HSV1716 in patients with metastatic melanoma has been completed (MacKie *et al*, 2001). Elsewhere, a trial using G207, a mutant of HSV-1 strain F, lacking both the ribonucleotide reductase and ICP34.5 genes, has been performed in glioma patients (Markert *et al*, 2000). The safety results are good and, whilst the trials were not designed to assess efficacy, the demonstration of viral replication in tumor tissue and indications of prolonged survival times of glioma patients are encouraging. The potential of these viruses in cancer therapy makes it important to determine the mechanism whereby they infect and selectively lyse tumor cells.

One function of ICP34.5 demonstrated in certain cells is the preclusion of host cell protein synthesis shutoff controlled through the RNA-dependent protein kinase (PKR) pathway (Chou and Roizman, 1992). Interaction of ICP34.5 with protein phosphatase 1 to dephosphorylate the eukaryotic initiation factor eIF-2α allows protein synthesis to proceed (Chou *et al*, 1995; He *et al*, 1997). This phenotype is, however, separable from the virulence phenotype conferred by expression of ICP34.5 (Markovitz *et al*, 1997). Deregulation of U_S11 expression, which can overcome host cell protein synthesis shutoff (Mohr and Gluzman, 1996), has no bearing on virulence (Mohr *et al*, 2001).

A 63–amino acid region of ICP34.5, which is highly conserved between both sero types of HSV, shares significant homology with two cell-cycle regulation proteins, MyD116 and GADD34 (McGeoch and Barnett, 1991). The functions of these proteins remain unclear, but they are expressed following myeloid differentiation (Lord *et al*, 1990) and growth arrest plus DNA damage (Fornace *et al*, 1989), respectively, and may promote cell survival (Lieberman and Hoffman, 1998). The homology between ICP34.5, MyD116 and GADD34 suggests a common function for at least the conserved parts of the proteins, and it has been shown that the carboxyl terminus of MyD116 can substitute for the equivalent domain in ICP34.5 to preclude the premature shutoff of protein synthesis (He *et al*, 1996).

We have shown that bacterially expressed ICP34.5 complexes with proliferating cell nuclear antigen (PCNA) (Brown *et al*, 1997), a cellular protein involved in DNA replication (Prelich *et al*, 1987) and repair (Shivji *et al*, 1992). We were able to confirm the relevance of the *in vitro* data by demonstrating that PCNA complexes with ICP34.5 *in vivo*, by immunoprecipitation of ICP34.5 with a specific PCNA antibody (Brown *et al*, 1997). We have also shown complexing between PCNA and the region of MyD116, which shares homology with ICP34.5. The function of PCNA is regulated by other MyD and GADD proteins (reviewed in Kelman, 1997), and it is believed that this regulation may act as a switch between the processes of DNA replication and repair (reviewed in Cox, 1997). It is possible, therefore, that ICP34.5 has a similar role in regulating the function of PCNA.

ICP34.5 renders HSV virulent. Its expression is an absolute requirement for HSV replication in terminally differentiated cells (Bronn *et al*, 1994). Proof of this has been obtained when ICP34.5-null HSV1716 has been injected into human brain and failed to cause disease or elicit a toxic response (Rampling *et al*, 2000; Papanastassiou *et al*, 2002; Harrow *et al*, manuscript in preparation). Our study has been designed to determine how the interaction of ICP34.5 with PCNA enables HSV to replicate in nondividing cells. We demonstrate that, *in vitro*, PCNA interacts with at least two separate regions of ICP34.5, that ICP34.5 complexes with HSV replication proteins, and that (possibly through this complex) ICP34.5 binds to DNA. We also demonstrate that ICP34.5 is a virion protein, which colocalizes *in vivo* in the cell nucleus with PCNA at early times in infection and subsequently accumulates in the cytoplasm. We show that, in response to damage induced in the cellular DNA by HSV infection, PCNA is recruited to sites of DNA repair within cell nuclei. Based on these observations, we propose that in nondividing cells, ICP34.5 switches PCNA from the repair to replication mode required for the initiation of HSV DNA replication.

Results

We demonstrated previously (Brown *et al*, 1997) that ICP34.5 interacts *in vitro* with PCNA. As actively dividing cells are permissive for ICP34.5-null HSV replication and have high levels of nuclear PCNA (Celis *et al*, 1984, 1987), it is reasonable to propose that the interaction between ICP34.5-null and PCNA is significant in the replication-competent phenotype of ICP34.5-null HSV in rapidly dividing cells. The interaction between PCNA and a MyD116/GST fusion protein (containing only the region of MyD116 homologous to ICP34.5) demonstrated that the interaction between ICP34.5 and PCNA was at least through this region (Brown *et al*, 1997). To further define the sites of interaction among ICP34.5, PCNA, and other proteins, a series of truncated ICP34.5/GST (glutathione *S*-transferase) fusion proteins has been expressed (shown in Figure 1).

Identification of ICP34.5/GST fusion constructs that interact with PCNA

Bacterially expressed GST fusion proteins of full-length ICP34.5 and the nine constructs illustrated in Figure 1 plus control GST protein were immobilized on glutathione agarose beads. Extracts from BHK cells, either mock infected (MI) or infected with $HSV17^+$ or HSV1716, or from bacteria transformed with the plasmid pT7-hPCNA to express human PCNA, were mixed with the beads and unbound proteins washed off. Proteins remaining bound to the beads were Western blotted with antibody to PCNA to identify which ICP34.5 constructs interact

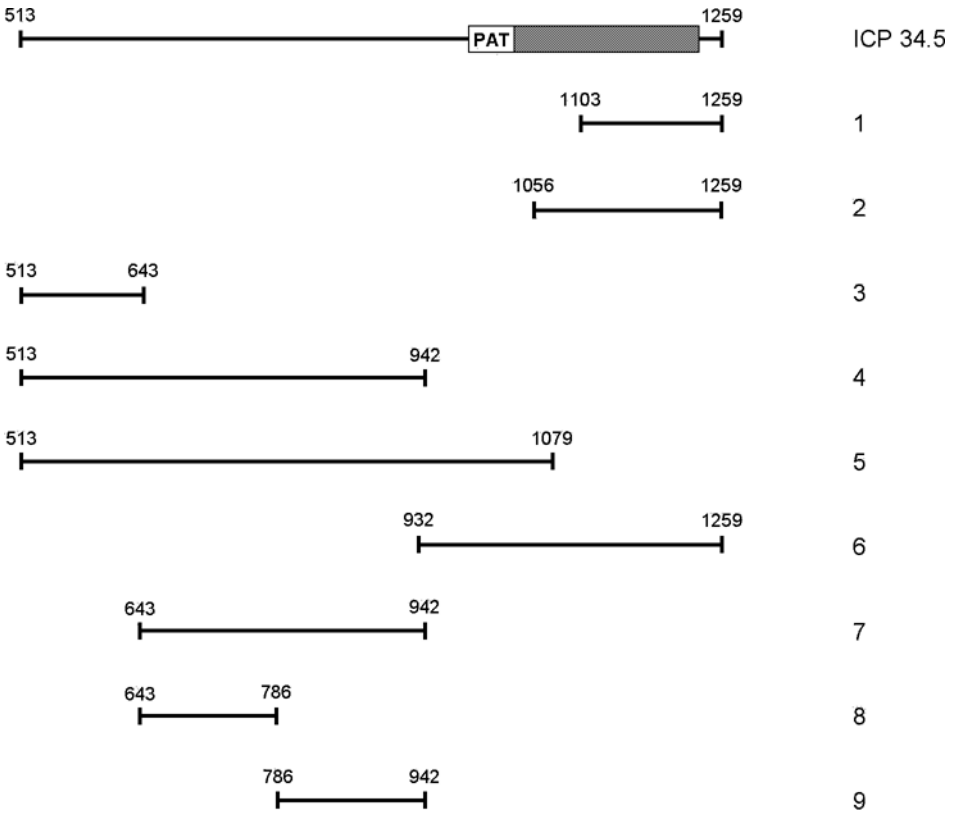

Figure 1 Schematic diagram showing the portions of ICP34.5 expressed as GST fusion proteins. The top line represents full length ICP34.5, showing the proline/alanine/threonine (PAT) region and the 63–amino acid conserved region as a dark box. The truncated portions, numbered 1 to 9, are shown above. The numbers denote nucleotide positions based on the TR_L copy of the ICP34.5-encoding gene.

with PCNA. Duplicate samples of the extracts (mock-infected, $HSV17^+$- or HSV1716-infected BHK cells, or bacterially expressed PCNA), used in each of the pull-down experiments were blotted with the pull-down samples, showing that the amount of PCNA present in each of the extracts was comparable. When using extracts from mock-infected BHK cells, PCNA bound to the full length ICP34.5 and to constructs 2, 6, 9, and possibly 8 (Figure 2**a**). The faint bands, representing PCNA bound to constructs 4 and 5, are at the same level as GST alone and can be regarded as background. In both $HSV17^+$- and HSV1716-infected cell extracts (Figure 2**b**, **c**, respectively), PCNA bound to constructs 2, 6, and 9, although in this experiment the binding to the full-length ICP34.5 was barely detectable. Despite a certain degree of variability inherent in this protocol, we observed binding with each of the extracts to full-length ICP34.5, and constructs 2, 6, and 9 over several experiments. These results with eukaryotic cell extracts are in agreement with those using bacterially expressed human PCNA (Figure 2**d**). Constructs 2 and 6 overlap (see Figure 1); construct 2 consists of most of the conserved 63–amino acid region and construct 6 contains the whole conserved region, the proline/alanine/threonine (PAT) repeat, and a limited portion of the nonconserved region. The binding of PCNA to construct 2, but not to construct 1, indicates that interaction within the conserved region adjacent to the PAT repeat. Constructs 8 and 9 are in the nonconserved region and do not overlap. Construct 9, and to a lesser extent construct 8, contain stretches (16 and 18 amino acids, respectively) that share significant homology with ICP34.5 of HSV-2, strain HG52. The anomaly that construct 7, which comprises constructs 8 plus 9, fails to pull down PCNA is difficult to understand. It is possible that the larger fusion protein is folded in such a way that it impedes interactions, which can occur with the smaller constructs.

Interaction of ICP34.5 with HSV DNA polymerase (U_L30) and the 65-kDa DNA-binding protein (U_L42)

By screening a HSV phage expression library against bacterially expressed GST/ICP34.5, we identified interaction of ICP34.5 with the U_L30 HSV polymerase and the U_L18 capsid protein VP23 (data not shown). As ICP34.5 complexes with PCNA, a protein involved in cellular DNA replication, the demonstration that ICP34.5 also associates with HSV DNA polymerase was unsurprising. To confirm the interaction

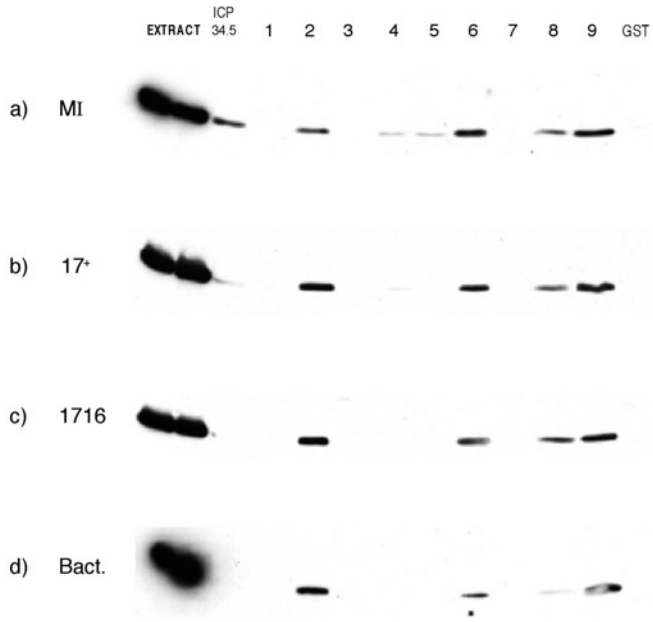

Figure 2 Western blots showing PCNA pulled down by GST fusion proteins of full-length ICP34.5 and truncations 1 to 9, compared to GST alone. The extracts used in the pulldown experiment were blotted in duplicate on the left of the gel. The extracts used were (**a**) mock-infected BHK; (**b**) HSV17$^+$-infected BHK; (**c**) HSV1716-infected BHK; and (**d**) bacterially expressed human PCNA.

of ICP34.5 with HSV replication proteins, we carried out pulldown experiments using HSV17$^+$- and HSV1716-infected BHK cell extracts. Western blots of these extracts and of the proteins pulled down from these extracts by the full-length and by the nine truncated ICP34.5/GST fusion products compared to a GST control, carried out using antibodies against the 65K$_{DBP}$ (U$_L$42) polymerase accessory protein (Gallo *et al*, 1988) and HSV DNA polymerase (U$_L$30), are shown in Figures 3 and 4. When the extract is from HSV17$^+$-infected cells, there is binding of the 65K$_{DBP}$ to full length ICP34.5 and to every one of the truncated forms but not to GST (Figure 3**a**). The amount of protein bound to constructs 2 and 9 was consistently less. Using HSV1716-infected BHK extracts, there is essentially no binding of the 65K$_{DBP}$ (Figure 3b). Similarly, the HSV polymerase from HSV17$^+$-infected extracts bound to all of the constructs, except construct 2 and 9 (Figure 4**a**), with no detectable binding from HSV1716-infected cell extracts (Figure 4**b**). The blots show that the HSV17$^+$- and HSV1716-infected cell extracts used in the pulldown experiments contain comparable levels of 65K$_{DBP}$ and HSV DNA polymerase (Figures 3 and 4). Confirmation that there was no significant difference in the amount of 65K$_{DBP}$ present in the two extracts was obtained by performing a Western blot with doubling dilutions of both extracts, which showed that the difference in amount was less than twofold (data not shown). As the BHK cells in which the infected cell extracts were produced are fully permissive for ICP34.5-null HSV, the only known difference between HSV17$^+$- and HSV1716-infected BHK extracts is the ICP34.5 in the HSV17$^+$ extract. It, therefore, appears that ICP34.5 must have mediated the pulldown of the HSV replication proteins by the ICP34.5/GST constructs.

Binding of ICP34.5 to DNA

The pulldown data demonstrating complexing *in vitro* of ICP34.5, PCNA, and two HSV replication proteins indicated that a common factor could be DNA. To determine whether ICP34.5 binds to DNA, protein extracts from mock-infected, HSV17$^+$- and HSV1716-infected BHK cells were mixed with double- or single-stranded DNA cellulose. Increasing salt concentrations were used to elute bound protein. Figure 5 shows a Western blot, using the 137 polyclonal antiserum against ICP34.5, of proteins remaining bound to double-stranded DNA following washes of increasing NaCl concentrations, from 50 mM to 2 M. In HSV17$^+$-infected cell extracts, ICP34.5 binds to double-stranded DNA and is not eluted by up to 2 M NaCl; in mock-infected or HSV1716-infected extracts, no ICP34.5 is present. Exactly the same

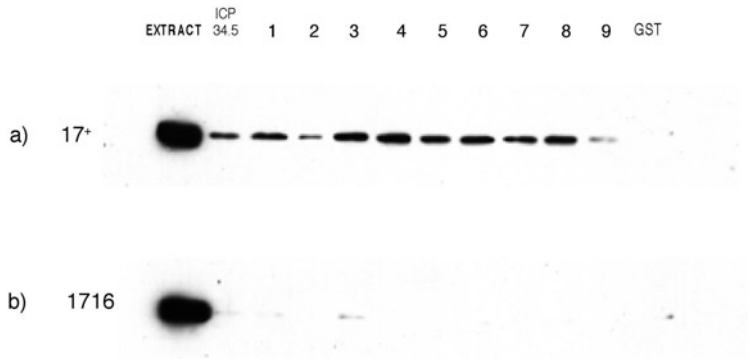

Figure 3 Western blots showing 65K$_{DBP}$ (U$_L$42) pulled down by GST fusion proteins of the full-length ICP34.5 and truncations 1 to 9, compared to GST alone. The extracts used in the pulldown experiment were blotted on the left of the gel. The extracts used were (**a**) HSV17$^+$-infected BHK and (**b**) HSV1716-infected BHK.

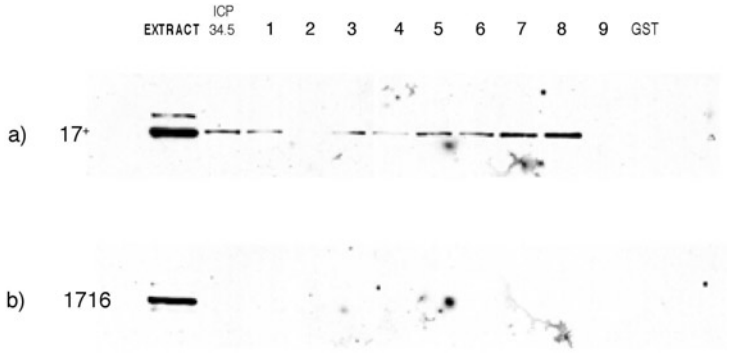

Figure 4 Western blots showing HSV polymerase (UL30) pulled down by GST fusion proteins of full-length ICP34.5 and truncations 1 to 9, compared to GST alone. The extracts used in the pulldown experiment were blotted on the left of the gel. The extracts used were (**a**) HSV17$^+$-infected BHK and (**b**) HSV1716-infected BHK.

pattern pertained when single-stranded DNA was used in place of double-stranded DNA (data not shown). We have shown previously that infection of BHK cells by both HSV17$^+$ and HSV1716 induces a 70-kDa protein, which is detected by antibodies to ICP34.5. This 70-kDa protein is apparent in Figure 5 and is not eluted with 2 M NaCl. The 21-kDa band at the bottom of the gel is thought to be the 21-kDa product of U_S11, which is known to be both DNA and RNA binding (MacLean *et al*, 1987; Roller and Roizman, 1990; Roller *et al*, 1996) and is consistently detected by our antibodies to ICP34.5.

Cellular localization of PCNA, ICP34.5, and HSV 65-kDa DNA-binding protein

The changes in cellular localization of PCNA as cells pass through the cell cycle are well documented (Bravo and Celis, 1985; Bravo and Macdonald-Bravo, 1985). In the G1 phase, PCNA is detected in small punctate, prereplicative sites within the nucleus. In S phase, the distribution changes to large clumps, representing DNA replication factories. Figure 6**a** shows the PCNA distribution in resting 3T6 cells, either mock infected or infected with HSV17$^+$ or HSV1716. Mock-infected, resting 3T6 cells fixed with methanol show a fairly uniform pattern of nuclear PCNA staining. Lysis of cells prior to staining releases unbound proteins and only PCNA bound to DNA is detected. In resting cells, there is virtually no bound PCNA in the nuclei of mock-infected cells. Within 2 h of infection with either HSV17$^+$ or HSV1716, bound PCNA starts to appear as punctate nuclear staining, which subsequently coalesces into large brightly staining foci.

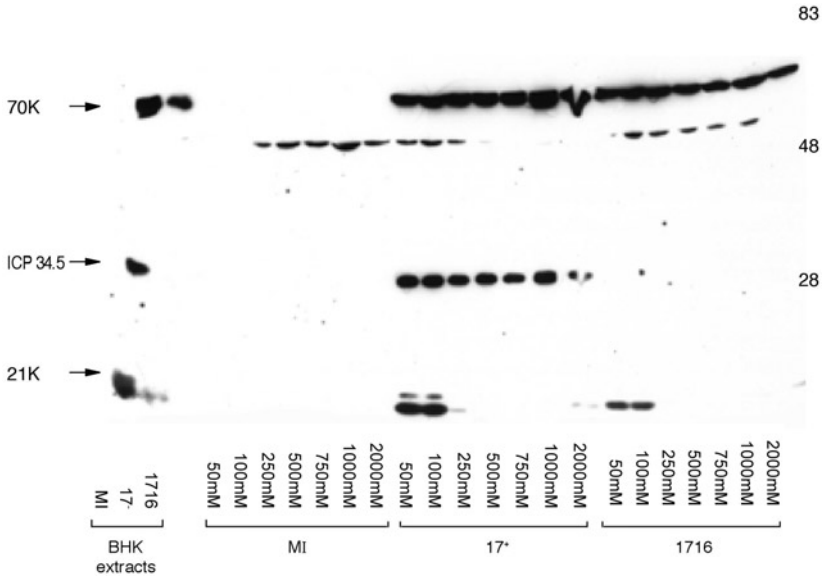

Figure 5 Western blot using antibody 137, raised against ICP34.5/GST fusion protein. The blot shows proteins from mock infected (MI), HSV17$^+$-infected and HSV1716-infected BHK extracts bound to double-stranded DNA/cellulose after washes with increasing salt concentrations (50 mM to 2 M NaCl). Blots of the extracts used in the experiment are shown on the left. The positions of the molecular weight markers are shown on the right (in kDa) and the positions of the 70-kDa protein, ICP34.5, and the 21-kDa proteins are shown on the left.

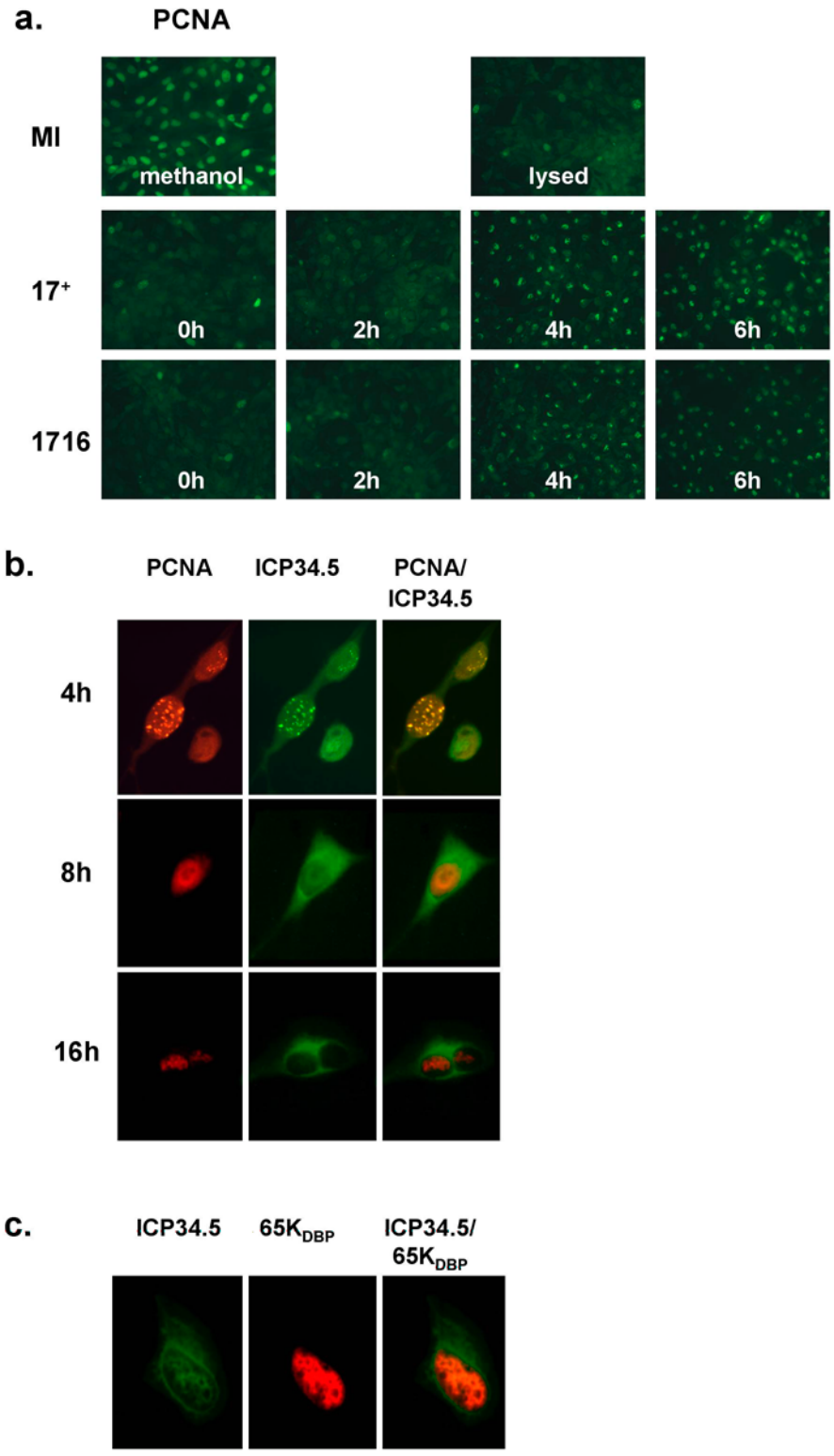

Figure 6 (**a**) Distribution of PCNA (*green*) in the nuclei of mock-infected, resting 3T6 cells, methanol fixed following lysis with a hypotonic buffer, compared to cells fixed directly with methanol. The distribution of PCNA in resting 3T6 cells infected with HSV17^{+} or HSV1716 for 0, 2, 4, or 6 h and lysed prior to fixation is shown below. (**b**) Distribution of PCNA (*red*) and ICP34.5/EGFP (*green*) 4, 8, and 16 h after infection with 1781. The *right hand panel* shows the *red* and *green* superimposed. (**c**) Distribution of ICP34.5/EGFP (*green*) and 65K$_{DBP}$ (UL42) (*red*) in a 3T6 cell 8 h after infection with 1781. The *right hand panel* shows the *red* and *green* superimposed.

Our antibodies against ICP34.5 have failed to yield useful immunofluorescence data. Therefore, to detect ICP34.5, we constructed two viruses expressing enhanced green fluorescent protein (EGFP). The first (1781) is HSV1 strain 17 in which EGFP is expressed as a fusion protein with ICP34.5 from the ICP34.5 promoter. The second (1782) is HSV1716 in which EGFP is inserted into the nonessential U_L43 locus and is expressed from the cytomegalovirus (CMV) IE promoter. Insertion of EGFP has not altered the *in vitro* phenotype of either virus (data not shown). 3T6 cells were infected with 1781 and, at various times post infection, fluorescence studies carried out to localize EGFP and hence ICP34.5. The cells were also stained for PCNA. Figure 6**b** shows 3T6 cells after infection with 1781. In some cells at 4 h post infection, ICP34.5/EGFP localized to the same structures in the nucleus as PCNA, in others, EGFP was detected in the cytoplasm as well as in the nucleus (data not shown). At 8 h post infection, the majority of infected cells showed strong EGFP staining in the cytoplasm, although some continued to colocalize with PCNA in the nucleus; by 16 h, ICP34.5 is almost exclusively in the cytoplasm. Infection with 1782 showed uniform nuclear distribution throughout infection, confirming that the punctate nuclear localization of ICP34.5/EGFP fusion protein seen in 1781-infected cells was not due to the EGFP portion of the protein (data not shown).

The distribution of $65K_{DBP}$ was examined with respect to ICP34.5/EGFP in 3T6 cells. At 4 h post infection, $65K_{DBP}$ staining was faint but in some cells there was colocalization with EGFP (data not shown). By 8 h post infection, although there was ICP34.5 in the cytoplasm, there is also colocalization with $65K_{DBP}$ in the nucleus (Figure 6**c**). At 16 h post infection (data not shown), the $65K_{DBP}$ retains an exclusively nuclear distribution whereas most ICP34.5 is in the cytoplasm. The immunofluorescence experiments confirm the previous *in vitro* evidence of the interaction of ICP34.5 with both PCNA and HSV DNA replication proteins.

ICP34.5 in virions, light particles, and PREPs

The observation, from the screening of a HSV expression library, that ICP34.5 interacts with the U_L18 capsid protein VP23 led us to investigate whether ICP34.5 could be a component of the virus particle. Samples of HSV-1 strain 17 virions, light particles (composed of envelope and tegument), and previral DNA replication enveloped particles (PREPs) (Dargan *et al*, 1995) were compared with $HSV17^+$- and HSV1716-infected BHK and 3T6 cell extracts. Figure 7 shows a Western blot of proteins recognized by the 137 antibody against ICP34.5/GST. As expected, ICP34.5 is detected in the $HSV17^+$-infected cell extracts but not in the HSV1716 extracts. Although the quantity is low, ICP34.5 is present in the $HSV17^+$ particle preparations irrespective of the presence of DNA. These results, in combination with the association of ICP34.5 with VP23, strongly indicate that ICP34.5 is a virion component.

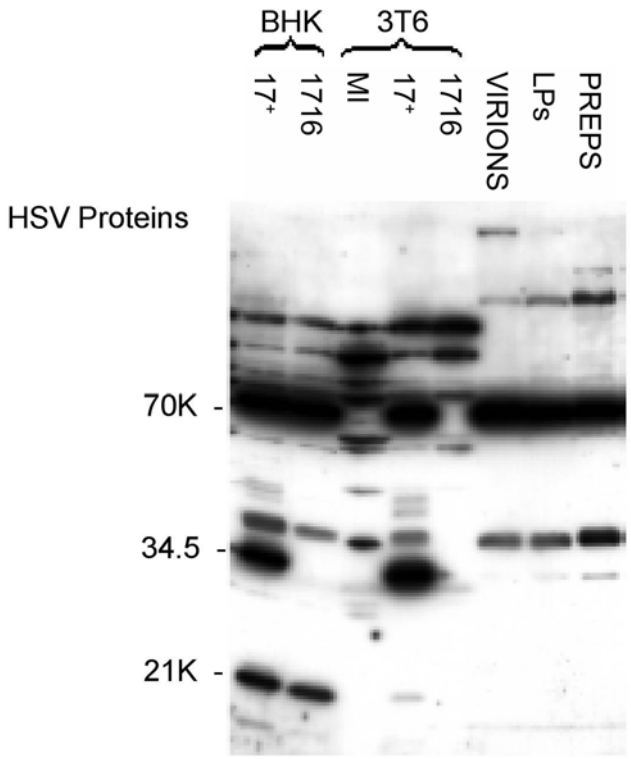

Figure 7 Western blot using antibody 137 (against ICP34.5), hybridized to proteins run on a 10% acrylamide gel. Extracts of BHK cells infected with $HSV17^+$ and HSV1716 and 3T6 cells mock infected and infected with $HSV17^+$ and HSV1716 were compared to extracts of 2×10^9 particles of HSV-1 strain 17 virions, light particles, and PREPs. The positions of the 70-kDa protein, ICP34.5, and the 21-kDa protein are shown on the left.

Discussion

It is established that one function of ICP34.5 is to preclude host cell protein synthesis shutoff through interaction with the PKR pathway (Chou and Roizman, 1992). In a recent paper, Leib *et al* (2000) show that full virulence is restored to the ICP34.5 mutant 17TermA when PKR knockout mice are infected. They conclude that the PKR pathway is the specific target for ICP34.5 *in vivo*. However, 17TermA, which has a termination signal inserted 30 amino acids from the amino terminus, is 10,000 fold less attenuated than HSV1716 (Bolovan *et al*, 1994), and cannot therefore be classified as avirulent. It is clear that ICP34.5 is a multifunctional protein, which can overcome a block in protein synthesis induced as an antiviral defence mechanism in certain cells. We propose that its primary function relevant to the phenotype of ICP34.5-null HSV *in vivo* is its interaction with PCNA to overcome the block in DNA replication in nondividing cells.

A 63–amino acid domain at the carboxyl end of ICP34.5 of HSV-1 is highly conserved with the equivalent HSV-2 protein (McGeoch and Barnett, 1991), despite divergence at the amino termini. This conserved region shares significant homology with part of an African swine fever virus protein (Sussman *et al*, 1992), also a determinant of virulence, and with regions of two cellular proteins MyD116 and GADD34 (McGeoch and Barnett, 1991). The functions of the cellular homologues are not fully understood, but they are expressed following myeloid differentiation and DNA damage, respectively. The expression of MyD116 is cyclic; it is rapidly induced following stimulation of myeloid cells with interleukin (IL)-6, it declines during the intermediate stages of differentiation and increases prior to apoptosis. The use of antisense oligonucleotides to block late expression of MyD116 promotes cell death, indicating that MyD116 can play a role in cell survival (Lieberman and Hoffman 1998). In GST pulldown experiments, we have shown that ICP34.5 and the conserved domain of MyD116 both interact with PCNA (Brown *et al*, 1997) and concluded that the interaction must be through at least the conserved domain. To define the sites of interaction, we constructed pGEX plasmids, which express truncated forms of ICP34.5 fused to GST and used these in pulldown experiments. PCNA interacts with at least two separate regions of ICP34.5, one in the conserved and another in the nonconserved part of the protein. In each of the two constructs from the nonconserved region that interacted with PCNA, there are amino acid stretches that share homology with the amino acid sequence in the equivalent region of ICP34.5 of HSV-2. The functional significance of the two sites of interaction is at present unknown.

Screening a HSV phage expression library against bacterially expressed GST/ICP34.5 revealed an interaction between ICP34.5 and the HSV U_L30-encoded polymerase, one of seven genes (U_L5, U_L8, U_L9, U_L29, U_L30, U_L42, and U_L52), necessary and sufficient for origin-dependent DNA replication (Challberg, 1986; Wu *et al*, 1988; McGeoch *et al*, 1988). In Western blots using specific antibodies, we have demonstrated that in extracts of BHK cells infected with HSV1716, there was negligible binding of the HSV replication proteins to the full length or truncated forms of ICP34.5. In $HSV17^+$-infected cell extracts, there was pull down of the HSV DNA replication proteins by the full length ICP34.5 and by most of the truncated forms. As the extracts were made in fully permissive BHK cells, the only difference in the extracts is the presence of ICP34.5. During $HSV17^+$ infection, a complex of ICP34.5 and HSV DNA replication proteins must be produced, which is required for interaction with ICP34.5/GST fusion proteins. The ICP34.5/GST fusions that bound PCNA most strongly, were those that bound the HSV replication proteins poorly or not at all. The interaction between the fusion protein and the complex probably occurs through ICP34.5, indicating that the protein forms dimers or multimers. This is in agreement with data showing ICP34.5 in a high-molecular-weight complex containing PP1α (He *et al*, 1998).

The interactions of ICP34.5 with PCNA and HSV DNA replication proteins could be mediated by DNA. Results using both double-stranded and single-stranded DNA cellulose showed that ICP34.5 is strongly DNA binding. It cannot, however, be discounted that the DNA-binding results from the interaction of ICP34.5 with another protein in the complex. The high concentration of salt would be expected to disrupt DNA binding of $65K_{DBP}$. Binding of ICP34.5 to DNA, either directly or indirectly, is consistent with its role in determining whether HSV replicates.

Using the 1781 virus, expressing ICP34.5 tagged with GFP, we demonstrated colocalization of ICP34.5 and PCNA in discrete foci within the nuclei of 3T6 cells shortly after infection, with subsequent accumulation in the cytoplasm. ICP34.5 and HSV (U_L42) $65K_{DBP}$ also colocalize, a finding in agreement with previous studies showing that PCNA colocalizes with HSV DNA replication proteins at replication sites within the nucleus (Wilcock and Lane, 1991). The colocalization of PCNA, ICP34.5, and HSV replication proteins in cells *in vivo* highlights the functional significance of these interactions.

We demonstrate that infection with HSV-1 (either with or without ICP34.5) causes a rapid influx of PCNA into the cell nucleus. It has been shown (Aranda-Anzaldo, 1992; Aranda-Anzaldo and Dent, 1997) that HSV infection induces single-stranded breaks in host cell DNA, comparable to those produced by moderate doses of ultraviolet radiation (2 to 5 J/m^2). These breaks are mostly repaired within 8 h post infection and are not related to the major breakdown of host cell DNA observed after 12 h post infection. We postulate that PCNA recruited into the nuclei following HSV infection is regulated to allow DNA repair, but not replication, and that ICP34.5 is required to de-regulate the PCNA into replication mode. This mechanism could also explain why irradiated cells allow increased HSV replication from the attenuated HSV-1 mutant R7020, which contains one copy of the ICP34.5 gene (Chung *et al*, 2002).

In conclusion, we believe that ICP34.5 acts as a regulatory protein for PCNA. It is required to switch on cellular DNA replication in nondividing cells so that HSV can then subvert the cells' machinery to replicate its own DNA. To function in this way, ICP34.5 would be required very early in the virus replication cycle, explaining its presence in virus particles and its interaction with the capsid protein VP23 (U_L18). In ICP34.5, there are nuclear localization signals (RRRRHRGPRRPRPP [amino acids 3 to 16] and RRGSWARERADRARFRR [amino acids 200 to 217]), which would facilitate its rapid transfer into the cell nucleus. In dividing cells, PCNA is actively involved in DNA replication and hence ICP34.5 is

not required for the initiation of viral replication. It is possible that the homologous regions of MyD116 and GADD34 also regulate the DNA replication mode of PCNA.

Work is ongoing to determine a three-dimensional structure for ICP34.5. This should allow identification of functional domains of ICP34.5, particularly those involved in complexing with DNA and PCNA.

Materials and methods

Cells
BHK cells were grown in Glasgow modified Eagle medium with tryptose phosphate broth and 10% newborn calf serum, and 3T6 cells in Dulbecco's modified Eagle medium with 10% fetal calf serum.

Viruses
The parental HSV-1 strain 17 (Brown *et al*, 1973) and the ICP34.5-null mutant HSV1716 (MacLean *et al*, 1991) were used throughout the study. The generation of 1781 (strain 17 expressing ICP34.5/EGFP) and 1782 (1716 expressing EGFP in the U_L43 locus under the HCMV IE promoter) is described below.

Screening of a HSV phage expression library
To identify proteins binding to ICP34.5, a phage lambda (Lambda UniZAP) HSV expression library (kindly provided by Prof. J. B. Clements) was screened. Purified GST/ICP34.5 fusion protein was incubated with blotted filters and positive colonies identified using an anti-GST antibody. Positive colonies were purified, their DNA extracted and sequenced.

Expression of regions of HSV-1 strain 17 ICP34.5 as GST fusion proteins
As described in (Brown *et al*, 1997), the whole of ICP34.5 was expressed as a GST fusion protein using the pGEX GST system (Smith and Johnson, 1998). We generated nine additional clones expressing GST fused to the portions of ICP34.5 encoded between the nucleotide positions shown in Figure 1. Constructs 1 and 2 were generated from PCR-amplified fragments, whereas the remainder were cloned from restriction enzyme fragments.

Analysis of cellular and HSV proteins bound to GST fusion proteins (pulldown)
The method (Brown *et al*, 1997) used to analyze proteins pulled down by the ICP34.5/GST fusion proteins is described briefly here. Proteins were expressed in protease-deficient *Escherichia coli* BL21 (Stratagene), from the pGEX plasmid constructs containing the sequences for the full-length ICP34.5, and the nine truncated constructs fused to GST plus the control pGEX plasmid encoding GST alone. The proteins were bound to freshly prepared glutathione agarose beads. Infected cell extracts were prepared by infecting confluent BHK monolayers with HSV wild-type strain 17 or HSV1716 at a multiplicity of infection (MOI) of 5 plaque forming units (pfu)/cell for 16 h at 37°C. Mock-infected cells were treated identically with the omission of the infecting virus. Cells were resuspended in buffer (50 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitors [Boehringer Mannheim]), at approximately 1 ml per 3×10^7 cells. Fifty microlitres of the glutathione agarose bead slurry with bound GST fusion protein were mixed with 300 μl of infected cell extract and incubated at 4°C for 1 h. Beads were harvested by brief centrifugation and washed three times in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitors. The beads were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel loading buffer, run on a 10% polyacrylamide gel, and Western blotted using the appropriate antibody. The plasmid, pT7-hPCNA (Fien and Stillman, 1992), a kind gift from Dr. Bruce Stillman, was used to prepare bacterially expressed human PCNA.

Western blotting
Western blotting was carried out as described (McKay *et al*, 1993). The following antisera were used: anti-ICP34.5/GST rabbit polyclonal serum 137 (Brown *et al*, 1997); anti-PCNA mouse monoclonal IgG2a PC10 (Novocastra); anti-HSV (U_L42) 65kDa DNA-binding protein ($65K_{DBP}$) (Marsden *et al*, 1987); mouse monoclonal serum Z1F11 (Schenck *et al*, 1988; Murphy *et al*, 1989); and anti-HSV (U_L30) polymerase mouse monoclonal serum 13185 (Marsden *et al*, 1996). The Z1F11 and 13185 antibodies were kindly supplied by Dr. Howard Marsden and Dr. Susan Graham.

DNA binding
BHK cells were either mock infected or infected with HSV-1 strain 17 or HSV1716 at a MOI of 5 pfu/cell and incubated at 37°C for 16 h. The cells (1×10^7) were washed once with phosphate-buffered saline (PBS) and scraped into 1 ml of B2 buffer (50 mM NaCl, 20 mM Tris, pH 8.2, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol). After brief sonication, the debris was spun out and the supernatant used as the cellular extract. The extract was mixed with 1/10 volume of 50% (either single- or double-stranded) DNA cellulose slurry (Sigma) rehydrated in B2 buffer. The slurry and extract were mixed for 1 h at room temperature and washed three times with B2 buffer. The DNA cellulose pellets were washed three times with B2 buffer containing concentrations of NaCl from 50 mM to 2 M. A sample of the first wash was mixed with polyacrylamide gel loading buffer, run on a gel, and Western blotted with the rabbit

antibody (137) against ICP34.5/GST to check for the presence of eluted protein. The pellets were resuspended in gel loading buffer, run on a gel, and Western blotted with the 137 antibody to detect bound protein.

Construction of HSV-1 strain 17 expressing ICP34.5/EGFP fusion protein (1781)

The plasmid pGEM34.5 containing the entire gene, its promoter, and flanking sequences between the *Alu*I and *Rsa*I sites (McKie *et al*, 1994) was cut with *Nco*I. The EGFP gene was excised in a 750-bp fragment from the plasmid pEGFP-C3 (Clontech) using *Nco*I and *Bgl*II. To facilitate cloning of the 750-bp EGFP fragment, a linker was designed to introduce a *Bgl*II site into pGEM 34.5:

Linker: 5′-CATGGAGATCT-3′

3′-CTCTAGAGTAC-5′

Clones of pGEM34.5 were screened for the presence of the *Bgl*II site, the orientation of the linker insert verified by restriction enzyme and sequence analyses, and the 750-bp EGFP fragment inserted at the *Nco*I-*Bgl*II sites. The new plasmid, pGEM34.5/EGFP, was cotransfected with-wild type HSV-1, strain 17, DNA into BHK cells and recombinant virus identified by the presence of fluorescent plaques when illuminated with blue (488 nm) light. A recombinant virus, HSV-1 34.5/EGFP (designated 1781), was plaque purified and a stock grown.

Construction of HSV1716 expressing EGFP in U_L43 under the HCMV IE promoter (1782)

The plasmid p35(*Pac*I), derived from pGEM-2 containing the HSV1 U_L43 gene and its flanking sequences into which a multicloning site has been inserted resulting in disruption of the gene (McKie *et al*, 1998b), was used. The p35(*Pac*I) was linearized with *Xho*I and blunt ended using Klenow polymerase. The pEGFP-C3 plasmid was cut with *Nsi*I and *Mlu*I and the 1.6-kb fragment containing the EGFP gene, the CMV IE promoter, and the simian virus 40 (SV40) polyA sequences, was isolated and blunt ended with Klenow polymerase. The 1.6-kb EGFP fragment was ligated into the linearised p35(*Pac*I) plasmid. Recombinant plasmids were isolated and screened by restriction enzyme (RE) digestion. Plasmids with the EGFP insert in either orientation can be used to generate the recombinant HSV1716. Recombinant virus was obtained following cotransfection of HSV1716 DNA and the constructed plasmid as described above. The virus HSV1716/EGFP is designated 1782.

Immunofluorescence

Three coverslips in a 35-mm dish were seeded with 3T6 cells to give a final concentration of $\sim 1 \times 10^5$ cells/plate following incubation at 37°C overnight. To synchronise cells, incubation was continued at 37°C for 24 h in serum-free medium. Cells were infected and incubation continued for various times up to 16 h post infection. Cells were fixed in methanol for 10 min at −20°C and washed with PBS. To visualize bound PCNA, the samples were treated with a hypotonic solution (10 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40) for 10 min at 4°C prior to methanol fixation (Savio *et al*, 1998). The following antibodies were used: Rabbit polyclonal antibody (Ab) 137 (against ICP34.5/GST), monoclonal Ab PC10 (against PCNA), and monoclonal Ab Z1F11 (against $65K_{DBP}U_L42$). 137 was used at a 1:400 dilution, PC10 1:1000, and Z1F11 1:2000. Incubation with the specific antibody was carried out at room temperature for 1 h and, following thorough washes, the conjugated second antibody incubated on the cells for 1 h at room temperature. The coverslips and the attached cells were washed thoroughly with PBS and mounted using Citifluor.

References

Aranda-Anzaldo A (1992). Early induction of DNA single-stranded breaks in cells infected by herpes simplex virus type 1. *Arch Virol* **122:** 317–330.

Aranda-Anzaldo A, Dent MAR (1997). Loss of DNA loop supercoiling and organization in cells infected by herpes simplex virus type 1. *Res Virol* **148:** 397–408.

Bolovan CA, Sawtell NM, Thompson RL (1994). ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J Virol* **68:** 48–55.

Bravo R, Celis JE (1985). Changes in the nuclear distribution of cyclin (PCNA) during S-phase are not triggered by post-translational modifications that are expected to moderately affect its charge. *FEBS* **182:** 435–440.

Bravo R, Macdonald-Bravo H (1985). Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *EMBO J* **4:** 655–661.

Brown SM, Harland J, MacLean AR, Podlech J, Clements JB (1994). Cell type and cell state determine differential *in vitro* growth of non-neurovirulent ICP34.5-negative herpes simplex virus. *J Gen Virol* **75:** 2367–2377.

Brown SM, MacLean AR, McKie EA, Harland J (1997). The herpes simplex virus virulence factor ICP34.5 and the cellular protein MyD116 complex with proliferating cell nuclear antigen through the 63-amino-acid domain conserved in ICP34.5, MyD116 and GADD34. *J Virol* **71:** 9442–9449.

Brown SM, Ritchie DA, Subak-Sharpe JH (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. *J Gen Virol* **18:** 329–346.

Celis JE, Bravo R, Larsen PM, Fey SJ (1984). Cyclin: a nuclear protein whose level correlates directly with the

proliferative state of normal as well as transformed cells. *Leukoc Res* **8:** 143–157.

Celis JE, Madsen P, Celis A, Nielsen HV, Gesser B (1987). Cyclin (PCNA, auxiliary protein of DNA polymerase delta) is a central component of the pathway(s) leading to DNA replication and cell division. *FEBS Lett* **220:** 1–7.

Challberg MD (1986). A method for identifying the viral genes required for herpesvirus DNA-replication. *Proc Natl Acad Sci U S A* **83:** 9094–9098.

Chambers R, Gillespie GY, Soroceanu L, Andreansky S, Chatterjee S, Chou J, Roizman B, Whitley RJ (1995). Comparison of genetically engineered herpes simplex viruses for the treatment of brain tumours in a scid mouse model of human malignant glioma. *Proc Natl Acad Sci U S A* **92:** 1411–1415.

Chou J, Chen J-J, Gross M, Roizman B (1995). Association of a M_r90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2α and premature shutoff of protein synthesis after infection with $\gamma_1$34.5$^-$ mutants of herpes simplex virus 1. *Proc Natl Acad Sci U S A* **92:** 10516–10520.

Chou J, Kern ER, Whitley RJ, Roizman B (1990). Mapping of herpes simplex virus-1 neurovirulence to gamma 1 34.5, a gene nonessential for growth in culture. *Science* **250:** 1262–1266.

Chou J, Roizman B (1992). The γ34.5 gene of herpes simplex virus type 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc Natl Acad Sci U S A* **89:** 3266–3270.

Chung S-M, Advani S, Bradley J, Kataoka Y, Vashistha K, Yan S, Market J, Gillespie G, Whitley RJ, Roizman B, Weichselbaum RR (2002). The use of a genetically engineered herpes simplex virus (R7020) with ionising radiation for experimental hepatoma. *Gene Therapy* **9:** 75–80.

Cox LS (1997). Review article–who binds wins: competion for PCNA rings out cell-cycle changes. *Trends Cell Biol* **7:** 493–498.

Dargan DJ, Patel AH, Subak-Sharpe JH (1995). PREPs: herpes simplex virus type 1-specific particles produced by infected cells when viral DNA replication is blocked. *J Virol* **69:** 4924–4932.

Fien K, Stillman B (1992). Identification of replication factor-C from *Saccharomyces cerevisiae*—a component of the leading-strand DNA replication complex. *Mol Cell Biol* **12:** 155–163.

Fornace AJ, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fragnoli J, Holbrook NJ (1989). Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol Cell Biol* **9:** 4196–4203.

Gallo ML, Jackwood DH, Murphy M, Marsden HS, Parris DS (1988). Purification of the herpes simlex virus type1 65kilodalton DNA-binding protein-properties of the protein and evidence of its association with the virus encoded DNA polymerase. *J Virol* **62:** 2874–2883.

He B, Chou J, Liebermann DA, Hoffman B, Roizman B (1996). The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma 34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *J Virol* **70:** 84–90.

He B, Gross M, Roizman B (1997). The γ_1 34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1α to dephosphorylate the α subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* **94:** 843–848.

He B, Gross M, Roizman B (1998). The gamma 143.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. *J Biol Chem* **273:** 20737–20743.

Kelman Z (1997). Review article—PCNA: structure, functions and interactions. *Oncogene* **14:** 629–640.

Kucharczuk JC, Randazzo BP, Chang MY, Amin KM, Elshami AA, Sterman DH, Rizk NP, Molnar-Kimber KL, Brown SM, MacLean AR, Litzky LA, Fraser NW, Abelda SM, Kaiser LR (1997). Use of replication restricted herpes virus to treat malignant mesothelioma. *Cancer Res* **57:** 466–471.

Lasner TM, Kesari S, Brown SM, Lee VM-Y, Fraser NW, Trojanowski JQ (1996). Therapy of a murine model of paediatric brain tumours using a herpes simplex virus type 1 ICP34.5 mutant and demonstration of viral replication within the CNS. *J Neuropathol Exp Neurol* **55:** 1259–1269.

Leib DA, Machalek MA, Williams BRG, Silverman RH, Virgin HW (2000). Specific phenotypic restoration of attenuated virus by knockout of a host resistance gene. *Proc Natl Acad Sci U S A* **97:** 6097–6101.

Lieberman DA, Hoffman B (1998). MyD genes in negative growth control. *Oncogene* **17:** 3319–3329.

Lord KA, Hoffman-Liebermann B, Liebermann DA (1990). Complexity of the immediate early response of myeloid cells to terminal differentiation and growth arrest includes ICAM-1, Jun-B and histone variants. *Oncogene* **5:** 387–396.

McGeoch DJ, Barnett BC (1991). Neurovirulence factor. *Nature* **353:** 609.

McGeoch DJ, Dalrymple MA, Dolan A, McNab D, Perry LS, Taylor P, Challberg MD (1988). Structures of herpes simplex virus type 1 genes required for replication of virus DNA. *J Virol* **62:** 444–453.

McKay EM, McVey G, Marsden HS, Brown SM, MacLean AR (1993). The herpes type 1 strain 17$^+$ open reading frame RL1 encodes a polypeptide of apparent M_r 37k, equivalent to ICP34.5 of herpes simplex virus type 1 strain F. *J Gen Virol* **74:** 2493–2497.

McKie EA, Brown SM, MacLean AR, Graham DI (1998a). Histopathological responses in the CNS following inoculation with a non-neurovirulent mutant (1716) of herpes simplex virus type 1 (HSV1): relevance for gene and cancer therapy. *Neuropathol Appl Neurobiol* **24:** 367–372.

McKie EA, Graham DI, Brown SM (1998b). Selective astrocytic transgene expression in vitro and in vivo from the GFAP promoter in a HSV RL1 null mutant vector—potential glioblastoma targeting. *Gene Therapy* **5:** 440–450.

McKie EA, Hope RG, Brown SM, MacLean AR (1994). Characterization of the herpes simplex virus type 1 strain 17$^+$ neurovirulence gene RL1 and its expression in a bacterial system. *J Gen Virol* **75:** 733–741.

MacKie RM, Stewart B, Brown SM (2001). Intralesional injection of herpes simplex virus 1716 in patients with metastatic melonoma. *Lancet* **357:** 525–526.

MacLean AR, Fareed MU, Robertson L, Harland J, Brown SM (1991). Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence related sequences in Glasgow strain 17^+ between immediate early gene 1 and the 'a' sequence. *J Gen Virol* **72:** 631–639.

MacLean CA, Rixon FJ, Marsden HS (1987). The products of gene U_S11 of herpes simplex virus type 1 are DNA binding and localize to the nucleoli of infected cells. *J Gen Virol* **68:** 1929–1937.

Markert JM, Malick A, Coen DM, Martuza RL (1993). Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. *Neurosurgery* **32:** 597–603.

Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, Palmer CA, Feigenbaum F, Tornatore C, Tufaro F, Martuza RL (2000). Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase 1 trial. *Gene Therapy* **7:** 867–874.

Markovitz NS, Baunoch D, Roizman B (1997). The range and distribution of murine central nervous system cells infected with the $\gamma_1 34.5^-$ mutant of herpes simplex virus 1. *J Virol* **71:** 5560–5569.

Marsden HS, Campbell MEM, Haarr L, Frame MC, Parris DS, Murphy M, Hope RG, Muller MT, Preston CM (1987). The 65,000-M_r DNA-binding virion and *trans*-inducing proteins of herpes simplex virus type 1. *J Virol* **61:** 2428–2437.

Marsden HS, Cross AM, Francis GJ, Patel AH, MacEachran K, Murphy M, McVey G, Haydon D, Abbotts A, Stow ND (1996). The herpes simplex virus type 1 UL8 protein influences the intracellular localization of the UL52 but not the ICP8 or POL replication proteins in virus-infected cells. *J Gen Virol* **77:** 2241–2249.

Mohr I, Gluzman Y (1996). A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. *EMBO J* **15:** 4759–4766.

Mohr I, Sternberg D, Ward S, Leib D, Mulvey M, Gluzman Y (2001). A herpes simplex virus type 1 $\gamma 34.5$ second-site suppressor mutant that exhibits enhanced growth in cultured glioblastoma cells is severely attenuated in animals. *J Virol* **75:** 5189–5196.

Murphy M, Schenk P, Lankinen HM, Cross AM, Taylor P, Owsianka A, Hope RG, Ludwig H, Marsden HS (1989). Mapping epitopes on the 65K DNA-binding protein of herpes simplex virus type 1. *J Gen Virol* **70:** 2357–2364.

Papanastassiou V, Rampling R, Fraser M, Petty R, Hadley D, Nicoll J, Harland J, Mabbs R, Brown M (2002). The potential for efficacy of the modified (ICP34.5$^-$) herpes simplex virus HSV1716 following intratumoural injection into human malignant glioma: a proof of principal study. *Gene Therapy* **9:** 398–406.

Prelich G, Tan C-K, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature* **326:** 517–520.

Rampling R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Brennan D, Petty R, MacLean A, Harland J, McKie E, Mabbs R, Brown M (2000). Toxicity evaluation of replication-competent herpes simplex virus (ICP34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Therapy* **7:** 859–866.

Randazzo BP, Bhat MG, Kesari S, Fraser NW, Brown SM (1997). Treatment of experimental subcutaneous human melanoma with replication restricted herpes simplex virus mutant. *J Invest Dermatol* **108:** 933–937.

Randazzo BP, Kesari S, Gesser RM, Alsop D, Ford JC, Brown SM, MacLean AR, Fraser NW (1995). Treatment of experimental intracranial murine melanoma with neuroattenuated herpes simplex virus type 1 mutant. *Virology* **211:** 94–101.

Randazzo BP, Kucharczuk JC, Litzky LA, Kaiser LR, Brown SM, MacLean AR, Abelda SM, Frasern NW (1996). Herpes simplex 1716- an ICP34.5 mutant is severely replication restricted in human xenografts *in vivo*. *Virology* **223:** 392–396.

Robertson LM, Maclean AR, Brown SM (1992). Peripheral replication and latency reactivation kinetics of the non-neurovirulent herpes simplex virus type 1 variant 1716. *J Gen Virol* **73:** 967–970.

Roller RJ, Roizman B (1990). The herpes simplex virus US11 open reading frame encodes a sequence-specific RNA-binding protein. *J Virol* **64:** 3463–3470.

Roller RJ, Monk LL, Stuart D, Roizman B (1996). Structure and function in the herpes simplex virus 1 RNA-binding protein U_S11: mapping of the domain required for ribosomal and nucleolar association and RNA binding *in vitro*. *J Virol* **70:** 2842–2851.

Savio M, Stivala LA, Bianchi L, Vannini V, Prosperi E (1998). Involvement of the proliferating cell nuclear antigen (PCNA) in DNA repair induced by alkylating agents and oxidative damage in human fibroblasts. *Carcinogenesis* **19:** 591–596.

Schenck P, Pietschmann S, Gelderblom H, Pauli G, Ludwig H (1988). Monoclonal antibodies against herpes simplex virus type 1-infected nuclei defining and localizing the ICP8 protein, 65K DNA-binding protein and polypeptides of the ICP35 family. *J Gen Virol* **69:** 99–111.

Shivji MKK, Kenny MK, Wood RD (1992). Proliferating cell nuclear antigen is required for DNA excision repair. *Cell* **69:** 367–374.

Smith DS, Johnson KS (1998). Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S transferase. *Gene* **67:** 31–40.

Sussman MD, Lu Z, Kutish G, Afonso CL, Roberts P, Rock L (1992). Identification of an African swine fever virus gene with similarity to a myeloid differentiation primary response gene and a neuruvirulence-associated gene of herpes simplex virus. *J Virol* **66:** 5586–5589.

Wilcock D, Lane DP (1991). Localization of P53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. *Nature* **349:** 429–431.

Wu CA, Nelson NJ, McGeoch DJ, Challberg MD (1988). Identification of herpes simplex virus type-1 genes required for origin dependent DNA synthesis. *J Virol* **62:** 435–443.