Journal of NeuroVirology, 12: 375–386, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280600975358

informa
healthcare

The stability of herpes simplex virus type I genomes in infected Vero cells undergoing viral induced apoptosis

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Maintaining the viral genome intact following infection and prior to replication is critical to the virus life cycle. Here we report an analysis of the stability of herpes simplex virus type 1 (HSV-1) genomes, relative to host chromosomal DNA, in infected cells as a function of viral induced apoptosis. The results show that, in the absence of DNA replication, the input genomes of wild-type (KOS), and replication compromised ICP27 deleted (d27-1) virus are remarkably stable. Intracellular half-lives of their genomes exceeded 24 hours. In contrast, the half-life of replication incompetent ICP4 deleted (d120) viral genomes were significantly less (approximately 8 hours). Interestingly, it was also noted that in cells infected under conditions permissible for replication, viral DNA replication occurs, even in cells undergoing apoptosis. The possibility that the genome structure and replication compartment formation provide protection to the HSV-1 genome from degradation is discussed. *Journal of NeuroVirology* (2006) **12,** 375–386.

Keywords: HSV-1 DNA stability; viral induced apoptosis

Introduction

For HSV-1 to complete its life cycle, it is essential that its genome is protected from cellular nucleases. Infection with various pathogens, in some circumstances, may lead to apoptosis. Apoptosis is one mechanism whereby innate host defenses can limit infection. Numerous publications have shown that HSV-1 blocks or delays apoptosis induced by heat-shock (Leopardi and Roizman, 1996), tumor necrosis factor alpha (Halford and Schaffer, 2001; Yu *et al*, 1999), Fas ligand (Galvan and Roizman, 1998; Jerome *et al*, 2001; Sieg *et al*, 1996), osmotic shock (Galvan *et al*, 1999; Koyama and Miwa, 1997), and virtually every pro-apoptotic substance tested to date.

In order to be able to block or delay apoptosis induced by various substances, HSV-1 encodes not only several anti-apoptotic genes such as Us3, Us5, ICP27, ICP4 and gamma 34.5 expressed during the lytic infection (Jerome *et al*, 2001; Aubert and Blaho, 1999; Leopardi *et al*, 1997; Galvan and Roizman, 1998), but also LAT microRNA (Gupta *et al*, 2006; Cui *et al*, 2006) that down regulates of pro-apoptotic pathways. Thus, the mechanism that HSV-1 protects infected cells from apoptosis, appear to be pathway specific depending on the host cells and pro-apoptotic agents studied (Galvan and Roizman, 1998; Galvan *et al*, 1999; Galvan *et al*, 2000; Munger and Roizman, 2001; Taddeo *et al*, 2003; Jerome *et al*, 2001; Jerome *et al*, 1999; Aubert and Blaho, 1999; Aubert *et al*, 1999; Koyama and Miwa, 1997). The infection of HSV-1 mutants defective in genes involved in blocking the

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The authors thank Mr Mengjun Wang for statistical analysis. This work was supported by National Institute of Health Grant NS 33768, The Institute for Hepatitis and Virus Research and an appropriation from the Commonwealth of Pennsylvania.

Received 18 May 2006; revised 21 July 2006; accepted 15 August 2006.

cellular apoptosis pathway has been shown to cause an apoptotic cytopathology that does not occur after infection of the parental HSV-1 (Leopardi and Roizman, 1996; Galvan *et al*, 1999; Aubert *et al*, 2001; Leopardi *et al*, 1997).

Once apoptosis is triggered, a series of programmed events leads to the death of the cell, which is manifested by morphological changes and the degradation of cellular DNA. It is, however, unclear if viral genomes, in cells undergoing apoptosis as a result of HSV-1 infection, are selectively sensitive or resistant to degradation, relative to the cellular chromosomes. It is possible that the association of viral genomes with DNA replication compartments provides protection to DNA from nuclease degradation.

A hallmark for the formation of replication complexes in HSV-1 infected cells is the genomes association with ICP8, a specified single-stranded, DNA-binding protein (Lukonis and Weller, 1996; Liptak *et al*, 1996; Quinlan *et al*, 1984), and replication compartments (Quinlan *et al*, 1984; Liptak *et al*, 1996). The detection of ICP 8 has been used to define and to identify these structures (Kops and Knipe, 1988; Lukonis and Weller, 1996; Uprichard and Knipe, 2003; Liptak *et al*, 1996).

In this study, two known apoptosis inducing HSV-1 mutant viruses, ICP27 deleted strain d27-1, and ICP4 deleted, Us3 defective strain d120, were used to induce apoptosis in infected Vero cells. The stability of viral DNA in infected cells, following these infections, was determined relative to host chromosomal DNA. Interestingly, our data suggest that the stability of viral DNA, in cells undergoing apoptosis, is related to its competency to form a viral DNA replication complex.

Materials and methods

Virus and cells

HSV-1 wild-type strain KOS was prepared in Vero cells. HSV-1 mutant d120, containing a 4.1 kb deletion in both copies of the ICP4 gene (DeLuca *et al*, 1985), and the complementary cell line E5 (DeLuca and Schaffer, 1987) were kindly provided by Dr. Priscilla Schaffer (Harvard Medical School, Boston, MA). Another mutant d27-1 (alpha 27-deleted, replication-compromised) and its complementary cell line V27 (Rice and Knipe, 1990; Aubert *et al*, 1999; Aubert and Blaho, 1999) were kindly provided by Dr. David Knipe (Harvard Medical School, Boston, MI). The stock of mutant d120 virus was prepared in E5 cells. The stock of mutant d27-1 virus was prepared in V27 cells.

Note that the parental strain of d120 is strain KOS and the parental strain of d27-1 is strain KOS1.1. Since strain KOS1.1 is a plaque-purified laboratory isolate from strain KOS, which was originally derived from a clinical isolate, there has been no published evidence to show that KOS and KOS 1.1 are different since they are derived from the same isolate. Thus, in this study we used KOS as the wild-type strain for both d120 and d27-1 mutants.

Induction of apoptosis by HSV-1 mutant virus infections on Vero cells

Vero cells were infected with HSV-1 parental strain KOS, mutant strains d27-1 and d120, at a moi of 5 to ensure that almost every cell was infected. Cultures were photographed in phase contrast microscopy 24 h post infection (PI) for morphological study prior to harvesting for the DNA degradation study.

To determined cellular DNA degradation, twenty-four hours PI, total cellular DNA of each culture was harvested, and 3 μg of total DNA from each sample was resolved on a 2% agarose gel with a 100 bp molecular weight marker (M.W.). After gel electrophoresis, and staining with ethidium bromide (EtBr), gels were photographed under an UV illuminator.

To measure the caspase 3 activity of each infected culture, Vero cells were plated at the density of 1×10^6cells in 25 cm^2culture flasks, followed by infection with HSV-1 wild-type strain KOS and mutant strains d27-1 (with or without 400 μg/ml of PAA), and d120 at a moi of 5. The infected cells were harvested at 8 and 24 h PI. As the control, Vero cells were either treated with 2% dimethyl sulfoxide (DMSO) for 24 h or left untreated (Mock). The cell lysates were prepared and assayed for caspase 3 activity using the BD ApoAlert Caspase Colorimetric Assay Kit (BD Bioscience, USA) according to the manufacture's specification. The caspase 3 inhibitor, DEVD-fmk (Asp-Glu-Val-Asp-fluoromethylketone), was added in the d120 infected cell lysate 24 h PI to show the specificity of assay. The background level for the assay was the reading of the untreated Vero cell lysate assayed without substrate. The caspase activity of each sample was calculated by subtracting the background reading.

Nuclear DNA isolation

To isolate nuclear DNA, cells were scraped into medium and collected by centrifugation at 1,000 rpm for 10 min at 4°C. The cell pellet was resuspended into nuclei lysis buffer (1 mM $CaCl_2$, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris, pH 7.5, 5% sucrose) containing 0.5% NP-40, homogenized with a Dounce homogenizer, and washed in nuclei lysis buffer containing 0.1% of sodium deoxycholate. Then, the nuclei were collected by centrifugation at 2,000 rpm for 10 min at 4^0C. The nuclei pellet was subjected to DNA isolation by SDS/proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation.

Virion DNA preparation

The method to isolate HSV virion DNA was described previously (Su *et al*, 2002). Briefly, HSV-1 infected CV-1 cells were scraped into medium and collected by centrifugation at 1,500 rpm for 10 min at 4°C. The

cells were lysed by sonication at 40% power (Heat System Ultrasonicator, Farmington, NY) for 1 min. Cell debris was separated from virions by centrifugation at 2,000 rpm for 15 min at 4°C. HSV-1 virions were pelleted by centrifugation at 28,000 rpm using a SW41 rotor for 1 hour at 10°C. Isolated virions were then lysed in virion lysis buffer (0.25% Triton X-100, 10 mM EDTA, 10 mM Tris, pH 8.0), and viral capsids were isolated by centrifugation through a 20% sucrose cushion at 28,000 rpm using a SW55 rotor for 50 min at 4°C, and DNA was isolated by SDS/proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation.

Quantification of viral DNA and cellular DNA in infected cells: (A) Southern blot hybridization and (B) real-time PCR

Southern blot hybridization: Vero cells were infected with HSV-1 wild-type strain KOS and two mutant strains d27-1 and d120 at a moi of 5 in the presence (+) or absence (−) of DNA replication inhibitor PAA, or were mock infected (M). Total DNA was isolated at 3, 8 and 24 h PI and quantified by spectrophotometry. 2 μg of infected cell DNA, mock infected Vero DNA, or Vero DNA with 10 ng of virion DNA (V) was digested with Restriction Endonuclease BamHI. Digested DNA was resolved by 1% agarose gel, stained with EtBr, and photographed under an UV illuminator. The gel was transferred to a nylon membrane as described previously (Su *et al*, 2002), and hybridized with the ^{32}P labeled 3.6 kb tk probe, prepared by labeling the tk fragment (isolated from the BamHI digests of pHSV106 (McKnigh and Gavis, 1980) with alpha ^{32}P dCTP using random primer labeling kit (Radprime DNA labeling system, Invitrogen, Carlsbad, CA) per manufacture's specification. Finally the image was generated and quantified by a Bio-Rad PhosphoImager (Bio-Rad Laboratories, Inc., Hercules, CA).

To quantify the total cellular DNA, the intensity of EtBr stained, BamHI digested DNA migrating in the range of 3.5–4 kb in the agarose gel, was determined by the AlpaImager gel documentation instruments (Alpha Innotech Corporation, Leandro, CA). The intensity of EtBr stained DNA compared to the quantity of HSV-1 DNA (determined by the tk probe) at 3 h PI in the presence of PAA was set as a 1:1 ratio. The relative amount of HSV-1 DNA to cellular DNA was calculated by normalizing to 3 h PI sample.

Real-time PCR: To quantify the viral DNA and cellular DNA, isolated DNA was quantified by the LightCycler (Roche Diagnostics, Germany) real-time PCR using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Germany) with the primers specific for the cellular gene albumin (Alb) (Wang *et al*, 2004) and the viral capsid gene Vp5 (Devi-Rao *et al*, 1994) according to manufacture's specifications. The sizes of both PCR products are 99 bp for Alb and 149 bp for Vp5. As the standards for quantification, a serially diluted genomic DNA and virion DNA were used.

Western blot analysis

Vero cells were infected with HSV-1 strain KOS, mutant strains d27-1, and d120 at a moi of 5. After 3 h or 10 h PI, infected cells were harvested and pelleted at 1,500 rpm at 4°C for 10 min. The cell pellet was lysed by addition of the lysis buffer (0.25% Triton-X100, 10 mM EDTA and 10 mM Tris, pH 8.0) as infected cell lysate.

Cell lysates were electrophoretically separated in a 9% of SDS-polyacrimide gel under denaturing and reducing conditions. The gel was transferred to a PVDF membrane manufactured by Amersham Biosciences (Piscataway, NJ) at 0.03A at 4°C overnight. The HSV-1 ICP4, ICP8, and cell beta-actin, were detected respectively with specific antibodies: mouse anti-ICP4 (H1101) and anti-ICP8 (H1115) monoclonal antibodies purchased from The Rumbaugh-Goodwin Institute for Cancer Research Inc. (Plantation, FL); mouse monoclonal anti-actin antibody (MAB1501) purchased from Chemicon Corp. (Temecula, CA). The desired protein-antibody complexes were visualized by using the appropriate species-specific secondary antibodies, followed by the SuperSignal Western Chemiluminescent Detection according to the manufacturer's instructions (Pierce, Rockford, IL).

Immunocytochemistry and fluorescent in situ hybridization (FISH)

Immunostaining was performed on cells grown on coverslips after fixation with 1% paraformaldehyde (10 min at room temperature) and permeabilization in 0.2% Triton (20 min on ice) by sequential incubation with primary and Texas Red- or FITC-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 min each (all solutions in PBS). For simultaneous detection of HSV-1 ICP8 and HSV-1 DNA, cells were first immunostained for ICP8 protein using monoclonal antibody against ICP8 (m-395, a gift from R. Everett, MRC Virology Unit, Glasgow, UK) and then treated for 1 h at 37°C with RNase, 100 μg/ml in PBS). After re-fixation in 4% paraformaldehyde (10 min at room temperature), samples were equilibrated in 2× SSC, dehydrated in ethanol (70%, 80% and 100% ethanol for 3 min each at –20°C), air-dried, and incubated overnight at 37°C with the hybridization mixture. For DNA detection, the FITC-avidin labeled probes, prepared by made by nick translation using HSV-1 cosmid DNAs as template, and cells were simultaneously heated at 94°C for 4 min to denature DNA. After hybridization, samples were washed at 37°C with 55% formamide in 2× SSC (twice for 15 min each), 2× SSC (10 min) and 0.25× SSC (twice for 5 min each). The hybridized signal was amplified using biotinylated anti-avidin (Vector Laboratories, 1:250), followed by another round of FITC-avidin staining. Finally, cells were equilibrated in PBS, stained for

DNA with Hoechst 33258 (0.5 μg/ml) and mounted in Fluoromount G (Fisher Scientific, Newark, DE).

Results

Induction of apoptosis in Vero cells following infection with mutant viruses, d120 or d27-1

To study the relative stability of HSV-1 DNA in infected cells undergoing viral induced apoptosis, the wild-type virus (strain KOS) and two KOS derived mutants that lack HSV-1 anti-apoptosis gene functions and reportedly induce apoptosis following infection, were used. The two KOS mutants used were d120 (ICP4-deleted, Us3-defective, replication-incompetent) (Minagawa *et al*, 1994; Munger *et al*, 2001; Leopardi and Roizman, 1996; Benetti *et al*, 2003; DeLuca *et al*, 1985) and d27-1 (ICP27-deleted, replication-compromised) (Rice and Knipe, 1990; Aubert *et al*, 1999; Aubert and Blaho, 1999).

To confirm that the known apoptosis inducing mutant viruses d120 and d27-1 induced apoptosis in infected cells, we assessed DNA fragmentation, induction of caspase 3 activity, and altered cell morphology in Vero cells. The effect of each virus on the morphology of Vero cells after 24 h infection was observed by phase-contrast microscopy (Figure 1A). After infection with a moi of 5, the wild-type KOS strain

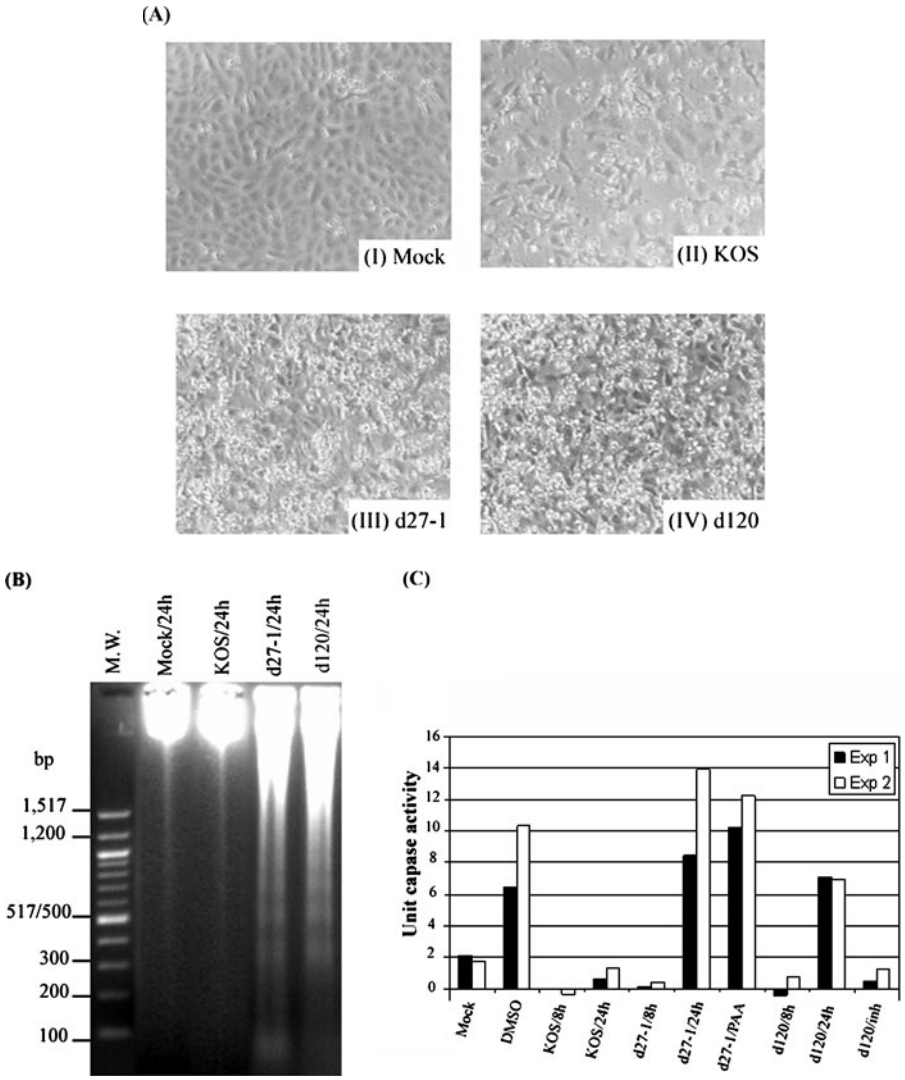

Figure 1 The induction of apoptosis by HSV-1 mutant virus infections on Vero cells. (**A**) The morphologies of HSV-1 infected Vero cells after 24 h of infection. Vero cells were infected with HSV-1 viruses strain KOS (II), d27-1 (III), or d120 (IV), at a moi of 5, or were left infected (I). (**B**) Cellular DNA degradation induced by HSV-1 infection. Vero cells were infected as described above or mock infected (Mock). Twenty-four h PI, total cellular DNA was harvested and analyzed for DNA degradation with a 100 bp molecular weight marker (M.W.), as described in Methods. (**C**) The caspase 3 activity induced by HSV-1 infection. Vero cells were infected with HSV-1 wild-type strain KOS and mutant strains d27-1 (with or without 400 μg/ml of PAA), and d120 at a moi of 5. The infected cells were harvested at 8 and 24 h PI. As the control, Vero cells were either treated with 2% DMSO for 24 h or left untreated (Mock). The cell lysates were prepared and assayed for the caspase 3 activity as described in Methods. The data from two independent experiments are shown.

caused a typical cytopathic effect (CPE) due to a productive infection: cells lost obvious cell-cell contacts and were ballooning and detaching from the culture flask, when compared with mock infected cells. This severe CPE resulted in the lyses and death of infected cells. As expected, although cytophatlogy resulting from infection by either of the two apoptosis inducing mutants, d120 or d27-1 was evident (compare Figure 1A(III) and Fig. 1A(IV), were smaller and irregularly shaped, compared to KOS-infected cells (Figure 1A(II).

After morphological evaluation, cellular DNA was isolated and analyzed for cellular DNA degradation in 2% agarose gels. As shown in Figure 1B, DNA isolated from mock and KOS infected cells consisted almost entirely of high molecular weight molecules, remaining close to the wells of the gel, and thus showing little evidence of degradation. On the other hand, DNA isolated from cells infected with either d27-1 or d120 migrated with a smear from the high to very low molecular weight. These results show that both d120 and d27-1 mutant viruses induced cellular DNA degradation in infected Vero cells, whereas, no evidence of DNA degradation was observed in KOS-infected Vero cells 24 hours post infection (PI).

Next, induction of caspase 3 activity, another marker of apoptosis, was determined as a function of time following infection with wild-type or mutant viruses. The levels of caspase 3 activity were measured from cell lysates prepared from cells either mock infected, or 8 and 24 hours post infection, with virus, or from Vero cells treated with 2% DMSO. The concentration of DMSO that was used has been shown to induce apoptosis in Vero cells, and thus, was intended to serve as a positive control for apoptosis (Liu *et al*, 2001).

The results, shown in Figure 1C, indicate that the level of caspase 3 activity in cells infected with parental strain KOS is similar to those in mock-infected cells, even late in infection. This is consistent with the results of the DNA degradation analysis in which it was shown that cellular DNA degradation did not detectably occur in parental stain KOS infected cells (Figure 1B).

However, a substantial level of caspase 3 activation (at least 5–10 fold) relative to the level of mock-infected cells was seen in both d27-1 and d120 infected cells. As expected, Vero cells treated with 2% DMSO exhibited elevated caspase 3 activity. The results of evaluation of morphology, cellular DNA degradation, and the elevation of caspase 3 activity, are all consistent with the conclusion that the mutant viruses d27-1 and d120, induced apoptosis in infected Vero cells.

Stability of HSV-1 DNA in infected cells undergoing viral induced apoptosis

To compare the stability of viral DNA and host DNA in cells undergoing apoptosis the relative half-life of the input viral DNA as compared to cellular DNA were determined. Phosphonoacetic acid (PAA) (400 μg/ml), was included to prevent viral DNA replication to ensure that the amount of viral DNA measured was input DNA.

Therefore, Vero cells were infected with parental strain KOS, and one of the two apoptosis inducing mutants, d27-1 and d120, at a moi of 5, in the presence or absence of DNA replication inhibitor PAA. Infected cellular DNA was harvested at 3, 8, and 24 h PI. The viral DNA in the sample taken from cells treated with PAA at 3 hours PI is defined as the amount of *input viral DNA*. The amount of input viral DNA and cellular DNA, at this time point, is set as 1:1.

The amount of viral DNA and cellular DNA in each sample was measured by both Southern blot hybridization (as illustrated in Figure 2 and summarized in Table 1) and real-time PCR (Table 1). In all cases, the results from Southern blot analysis and PCR quantification, were comparable.

As illustrated in Figure 2, by 24 hours PI, the HSV-1 parental virus strain KOS replicated, and the ratio of viral DNA to host DNA increased to almost one thousand-fold. To permit analysis on the same membrane as the other samples, dilutions of KOS 24h (without PAA) samples were used. By PhosphoImager analysis, an approximately 10-fold difference in reading between 10-fold diluted and 100-fold diluted samples was observed demonstrating that quantification was in the linear range.

In the presence of PAA, the relative amount of KOS to cellular DNA remains unchanged for 8 h PI. After 24 h, a ratio of 0.7 (viral DNA to cellular DNA) was observed in this particular experiment. In the absence of PAA, by 8 h PI, substantial viral replication has occurred and was reflected in the increase of relative amounts of viral to cellular genomes. The experiment was repeated and the results summarized in Table 1.

The viral to cellular DNA ratio was also quantified by real-time PCR, using primers specific for the cellular gene albumin (Alb) (Wang *et al*, 2004) and the viral capsid gene Vp5 (Devi-Rao *et al*, 1994). The sizes of both PCR products are 99 bp for Alb and 149 bp for Vp5. The results from real-time PCR are summarized in Table 1 and are comparable to those from Southern blot hybridization.

Under conditions where viral DNA replication was blocked with PAA, the ratio of infecting genomic KOS to cellular DNA remained similar between 3 and 8 h PI (Table 1). By 24 h, the ratio of viral to cellular DNA was approximately 0.6, suggesting that the relative half-life of the input viral genome is more than 24 h.

The data from d120, a replication incompetent virus that induces apoptosis, was significantly different from parental strain KOS as summarized in Table 1. By 8 h PI, approximately 40% [by both Southern hybridization analysis and real-time PCR (Table 1) of viral DNA remained, whereas, there was no viral DNA degradation detected in KOS-infected cultures in the presence of PAA [$P < 0.001$ (Southern) or $P = 0.039$ (real-time PCR)]. By 24 hours, the

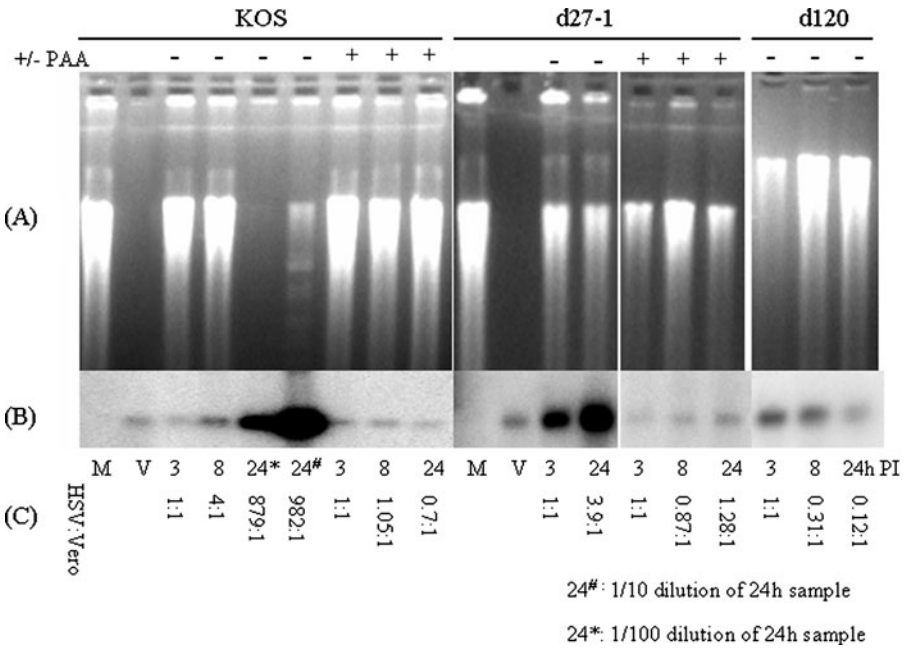

Figure 2 Stability of viral DNA following infection in Vero cells as analyzed by Southern blot hybridization. Vero cells were infected with HSV-1 wild-type strain KOS and two mutant strains d27-1 and d120 at a moi of 5 in the presence (+) or absence (−) of DNA replication inhibitor PAA, or were mock infected (M). Total DNA was isolated at 3, 8, and 24 h PI and quantified by spectrophotometry. 2 μg of infected cell DNA, mock infected Vero DNA, or Vero DNA with 10 ng of virion DNA (V) was digested with Restriction Endonuclease BamHI. Digested DNA was resolved by 1% agarose gel, stained with ethidium bromide (EtBr), and photographed under an UV illuminator as shown in Panel **A**. The gel was transferred to a nylon membrane as described previously (Su *et al*, 2002), and hybridized with the ^{32}P labeled 3.6 kb tk probe (generated from BamHI digestion of pHSV106 (McKnight and Gavis, 1980)). Then the image was generated and quantified by Bio-Rad PhosphoImager as shown in panel **B**. To quantify the total cellular DNA, the intensity of EtBr stained, and digested DNA migrating in the range of 3.5–4 kb in the agarose gel, was determined by the AlpaImager gel documentation instruments (Alpha Innotech Corporation, Leandro, CA). The intensity of EtBr stained DNA compared to the quantity of HSV-1 DNA (determined by the tk probe) at 3 h PI in the presence of PAA was set as a 1:1 ratio. The relative amount of HSV-1 DNA as to cellular DNA was calculated and shown in panel **C**.

relative amount of viral DNA compared to cellular DNA, had decreased by more than 5-fold. This suggests that the viral DNA, in d120-infected cells, was being degraded at a rate much faster ($P < 0.001$) than the KOS DNA in KOS-infected culture. Results were similar from PAA treated cells (not shown).

To determine if the induction of apoptosis is necessarily associated with the accelerated viral DNA degradation found in d120 infection, the stability of viral DNA in d27-1 infected cells was examined. Both d27-1 and d120 mutant viruses induce apoptosis to a comparable level as determined by cellular DNA degradation and activation of caspase 3 activity. However, when PAA was included, to prevent viral DNA replication, there was no selective degradation in the input d27-1 viral DNA as compared to cellular DNA (Table 1). In fact, the ratio of d27-1 DNA to cellular DNA was almost unchanged (1:1) throughout the entire 24 h of infection, suggesting that the d27-1 DNA is not more susceptible to nuclease degradation when compared to cellular DNA. Thus, the induction of apoptosis is, in itself, not sufficient to cause an accelerated degradation of viral DNA as seen in d120 infected cells.

Previous studies have suggested that ICP27 protein is required to achieve wild-type levels of viral DNA synthesis, although not an absolute requirement for viral DNA replication. The onset of d27-1 DNA replication is slower than that found in parental strain infected cells (Margolis *et al*, 1992; Rice and Knipe, 1990). This is consistent with the results shown in Table 1. Briefly, there is an approximately 10-fold increase in the amount of viral DNA after 24h of infection with d27-1 in the absence of PAA.

d27-1 is replication defective, however, as compared to its parental strain KOS. The efficiency of d27-1 DNA replication in Vero cells is about 50- to 100-fold less than KOS. Interestingly, a limited d27-1 viral DNA replication occurs even in cells undergoing apoptosis, thus viral replication might occur in a compartment that is protected from nuclease degradation.

Taken together, the two apoptosis inducing mutant viruses, d120 and d27-1, were significantly different in their relative susceptibility as compared to host DNA ($P = 0.05$ as determined by the Mann-Whitney test) in apoptosis induced DNA degradation at both 8 and 24 h PI.

Table 1 The ratio of HSV-1 DNA and host DNA as a function of time after infection as determined by Southern blot hybridization and Real-time PCR

	KOS/Vero				*d27-l/Vero*					
	−PAA		*+PAA*		*−PAA*		*+PAA*		*d120/Vero*	
Exp. (PI)	*Southern*	*PCR*	*Southern*	*PCR*	*Southern*	*PCR*	*Southern*	*PCR*	*Southern*	*PCR*
8h										
Mean ± SE	8.80 ± 3.7	.63 ± 3.58	0.96 ± 0.09	0.75 ± 0.07	0.933 ± 0.06	1.67 ± 0.17	0.98 ± 0.07	0.88 ± 0.05	0.42 ± 0.06	0.45 ± 0.05
P-value*	ND	ND	<0.001	0.039	ND	ND	0.001	0.0051	–	–
24h										
Mean ± SE	1,398.31 ± 735.68	580.63 ± 309.02	0.60 ± 0.10	0.55 ± 0.05	9.40 ± 3.72	11.97 ± 6.13	1.15 ± 0.14	0.92 ± 0.08	0.19 ± 0.04	0.09 ± 0.03
P-value*	ND	ND	<0.001	<0.001	ND	ND	<0.001	< 0.001	–	–

The quantity and integrity of cellular DNA and viral DNA were determined by Southern blot hybridization (column As) and real-time PCR (column Bs). The ratio of the amount of HSV-1 DNA (wt KOS and d27-1) to cellular DNA obtained for 3 h samples in the presence of PAA are set as 1:1, and served as the reference to normalize the ratio of 8 h and 24 h samples (as illustrated in Figure 2). For d120 infected samples, PAA is not included; the 3 h samples were set as 1:1 and used as the reference for the 8 h and 24 h samples. The data presented is from three independent experiments. SE: standard error of the mean.

*Statistical analysis was performed and *P*-values between KOS/Vero/+PAA and d120/Vero and *P*-values between d27-1/Vero/+PAA and d120/Vero were calculated by Yates Corrected Chi-square test.

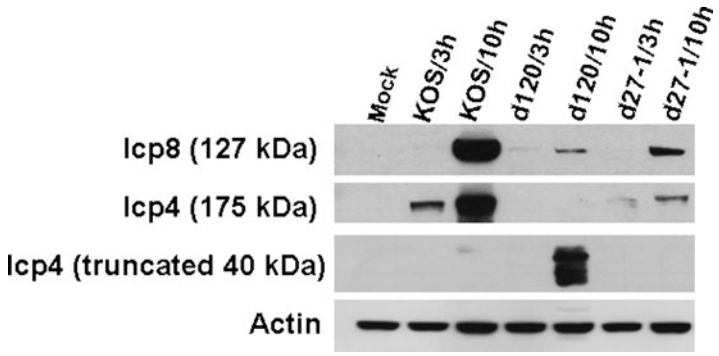

Figure 3 Expression of ICP8 and ICP4 proteins in Vero cells infected with wild-type strain KOS or mutant strains d27-1 or d120. Total cell extract (50 μg) prepared at 3 and 10 h PI from infected Vero cells were used for immunoblot with the monoclonal anti-ICP8 (1115, Rumbaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL), anti-ICP4 (1101, Rumbaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL), and anti-actin (MAB1501, Chemicon, Temecula, CA) antibodies, as described in Methods.

Localization of the d27-1 genomes to pre-replicative domains in the absence of viral DNA replication by immunocytochemistry and FISH analyses

The possibility that viral genome localization and/or association of replication compartments were involved in the protection of viral genomes from nuclease degradation was explored. Western blots analysis of the ICP8 protein (for abundance), immunohistochemistry (for localization), and fluorescent in situ hybridization (FISH) to detect the location of viral genome, were used.

Briefly, Vero cells were infected with the parental strain KOS and the two apoptosis inducing mutant viruses, d27-1 and d120, in the presence of PAA. Total cell lysate was harvested at 3 h and 10 h PI and subjected to Western blot analysis as shown in Figure 3.

As expected, an abundant amount of ICP8 was detected at 10 h PI in the KOS-infected Vero lysate. The amount of ICP8 protein in the d27-1 infected cell lysate was less compared to that in KOS at 10 h PI.

ICP8 was clearly detectable in the lysate of d120-infected Vero cells 10 h PI, although the amount was significantly less than that of KOS lysate. ICP8 is an early protein, thus requiring simulation of its gene by protein ICP4 for expression in the infected cell. d120 contains a deletion within the ICP4 gene which results in production of a truncated ICP 4 polypeptide that is 40 kDa, rather than the full length 175 kDa species (DeLuca *et al*, 1985). This 40 kDa truncated protein was also detected in the d120 infected Vero lysate by the anti-ICP4 antibody. No functional full-length (175 kDa) protein was seen in d120-infected Vero lysate. As expected, the full length ICP4 protein, not the truncated ICP4 protein, was readily detected in both parental strain KOS and mutant d27-1 infected cell lysate.

Immunocytochemistry and FISH was performed to localize ICP8 in the infected cells, as shown in Figure 4. As expected, in the absence of PAA at 3 h, KOS DNA replication (viral DNA, green) occurred and was associated with ICP8 immunostaining (red) replication compartments (as indicated by the red-green merge) at 3 h PI (Figure 4A). By 6 h PI, cellular chromosomes appear to have been displaced to the side of infected nuclei, presumably secondary to the development of viral replication compartments.

In contrast, in the presence of PAA, only pre-replicative sites that are rich in ICP8 (Kops and Knipe, 1988; Quinlan *et al*, 1984) were found in Vero cells infected with KOS (Figure 4J) due to the successful blockage of viral replication.

Although DNA replication by d27-1 was much less efficient than replication by parental strain KOS virus (Table 1), and less ICP8 was made (Figure 3) by d27-1, the immunocytochemistry and FISH staining patterns shown in Figure 4 (C, F, I, & L) suggest that replication compartments were still formed. Briefly, by 6 h PI (Figure 4F), punctuate spots of green (DNA) and red (ICP8) representing pre-replicative sites and replication compartments can be seen. More surprisingly, in the presence of PAA (Figure 4L), the d27-1 genomes inside the nucleus were found juxtaposed or embedded in minor aggregates of ICP8. In nuclei with large amounts of ICP8, such juxtaposition might be considered random. Careful analysis of the image shows that nearly every green spot is associated with a red spot (Figure 4L; both nuclei on the right).

On the other hand, in Vero cells infected with the ICP4 deletion mutant d120, pre-replicative sites and replication compartments were not detected (Figure 4B). Cytoplasmic and nuclear diffraction sized spots of viral DNA (green), a typical indication of viral entry, can be seen with careful examination (Figure 4B and E). At 24 h PI, viral genome staining was only seen in the cytoplasm or on the surface of the nuclear membrane (Figure 4H). This may be the result of input viral genomes remaining outside of nucleus, probably still inside of the capsid.

d120 viral DNA was not found to be in nuclear punctuated spots, and there was no evidence of the

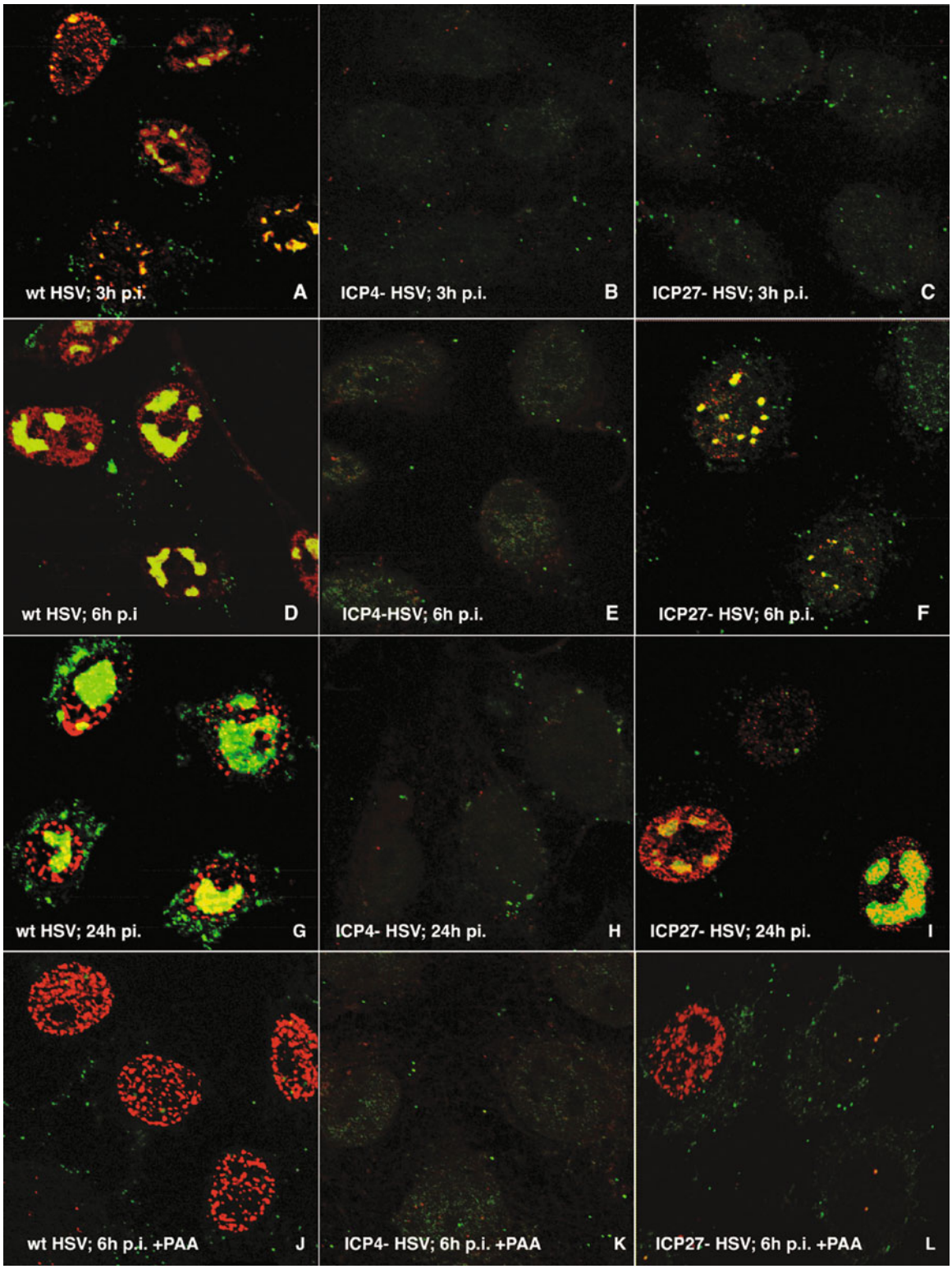

Figure 4 Localization of infected viral genomes and ICP8 proteins by Immunocytochemistry and fluorescent in situ hybridization (FISH). Vero cells were grown on coverslips and infected with wild-type strain KOS (wt HSV) or mutant strains d27-1 (ICP27-HSV) or d120 (ICP4-HSV) in the absence or presence of a viral DNA replication inhibitor (PAA). Immunostaining was performed at various times PI using a previously described method (Tang *et al*, 2003). For simultaneous detection of HSV-1 ICP8, and HSV-1 DNA, cells were first immunostained against ICP8 proteins using monoclonal antibody (m-395, a gift from R. Everett). The cells were then subjected to in situ hybridization with FITC-avidin (Vector Laboratories) labeled HSV-1 probe (made by nick translation using HSV-1 cosmid DNAs (Cunningham and Davison, 1993) as the template) and followed by biotinylated anti-avidin (Vector Laboratories, 1:250), FITC-avidin staining, and then mounted in Fluoromount G (Fisher Scientific, Newark, DE). Confocal images of cells were obtained by using a Leica TCS SPII confocal laser scanning system as previously described (Tang *et al*, 2003).

formation or association of the genomes of d120 with pre-replicative sites. While this is not entirely unexpected, since d120 is defective in viral DNA replication and lack the functional ICP4 protein, the inability of ICP4 genomes to form replication complexes could not be assumed.

It is possible that d120 DNA, which is uncoated following entry (Su *et al*, 2006), is left relatively unprotected, and therefore, more susceptible to nucleases, during apoptosis.

Discussion

To study the susceptibility of the HSV-1 genome to degradation in cells undergoing apoptosis, two apoptosis-inducing HSV-1 mutants, d27-1 and d120, were used to establish viral-induced apoptosis in infected Vero cells. Apoptosis was determined by DNA degradation and the elevation of caspase 3 activity. Three main findings are presented in this study. Firstly, in the KOS- infected cell culture in which apoptosis was not detectable, the half-life of HSV-1 DNA was approximately 24 h PI. Secondly, in cells in which virus induced apoptosis occurred, mutant d120 genomes were more susceptible to degradation (half-life of approximately 8 hours) than were d27-1 genomes (half-life of more than 24 hours, $P < 0{,}001$). The differential susceptibility to DNA degradation between these two apoptosis-inducing mutant virus genomes was surprising. The mechanism by which this selectivity is achieved is not clear but may relate to the degree to which each genome interacts with replication complexes. By immunocytochemistry and FISH analyses, almost every d27-1 genome found inside of the nucleus was associated with the ICP8 protein, indicating that the viral genome is in a pre-replicative domain. This is in contrast to the d120 genome found in infected nucleus, where no association of the d120 genome with the ICP8 protein was detected. Thus, we hypothesize that the association of viral genomes with pre-replicative domains might play a role in protecting DNA from nuclease degradation.

Among HSV-1 mutants that induce apoptosis in various host cells, the mutant strain d120 is known to be highly cytotoxic to the cells it infects. Infection of strain d120 resulted in apoptosis in almost all cell lines tested (Vero, SK-N-SH, HEp-2, and HEL cells) (Galvan and Roizman, 1998; Galvan *et al*, 1999; Leopardi and Roizman, 1996; Zhou and Roizman, 2000). Interestingly, we found that the mutant d120 failed to induced apoptosis in neuronal-like nerve growth factor differentiated PC12 (NGF-PC12) cells. In contrast to its genome's short half-life in infected Vero cells (less than 8h / Table 1), the half-life of the d120 genome inside of infected neuronal-like NGF-PC12 cells was shown to be up to 30 days (Su *et al*, 2006).

The unusually long half-life of linear d120 genomes in NGF-PC12 cells might be due to the following reasons: 1, infected NGF-PC12 cells do not undergo apoptosis; 2, relative less DNA degradative activity in NGF-PC12 cells, as evidence by reduced amounts of DNA repair as discussed in (Moxley *et al*, 2002).

Compared with KOS, d27-1, and d120 infected cells, the amount of ICP8 accumulating in d120 infected cells was the least, but detectable. Perhaps a critical amount of ICP8 is needed for formation of pre-replicative domains, and this amount is not achieved in d120 infected cells. It is also possible that lack of ICP4 expression in d120-infected Vero cells contributes to the failure of formation of DNA pre-replicative domains, since ICP4 protein is known to be essential for the formation of viral DNA pre-replicative domains.

Parenthetically, since ICP8 synthesis is thought to require ICP4, it is not clear how ICP8 was made in d120-infected Vero cells, since d120 does not induce de novo synthesis of ICP4 in these cells. We have found that ICP4 protein contained within the d120 virion was predominately the truncated form, with a small fraction of full-length ICP4 protein, made from the complementary E5 cells (Su *et al*, 2006). It is possible that this small amount of functional ICP4, derived from d120 virion, was sufficient to activate the expression of ICP8 gene, although the level of expression is limited.

Our work and that of others has shown that infection with ICP27 and ICP4 mutant viruses results in apoptosis (Munger *et al*, 2001; Benetti *et al*, 2003; Leopardi and Roizman, 1996; Aubert and Blaho, 1999). However, it is worth noting that the mechanisms involved in induction of ICP27 and ICP4 mutant mediated apoptosis may differ. For instance, in the case of ICP27 mutants, de novo protein synthesis appears to be necessary in Vero cells, but not in HEp-2 cells. It was suggested (Nguyen *et al*, 2005) that the induction of apoptosis in Vero cells infected with another ICP27 deleted mutant strain (vBSd27) is dependent upon de novo protein synthesis. In contrast, the apoptosis occurred in HEp-2 cells infected with vBSd27 in the presence or absence of de novo protein synthesis.

In addition to blocking or delaying the onset of apoptosis in infected cells by encoding several anti-apoptosis genes, this study has revealed additional possible mechanisms by which viral genomes protect themselves from degradation challenges. This could be achieved by the association with a protein complex or the formation of an endless genome structure. More work is needed to determine if this is a mechanism whereby d27-1, and more importantly, wild type virus genomes, protect themselves from degradation during productive infection and perhaps, even, latency.

References

Aubert M, Blaho JA (1999). The herpes simplex virus type 1 regulatory protein ICP27 Is required for the prevention of apoptosis in infected human cells. *J Virol* **73:** 2803–2813.

Aubert M, O'Toole J, Blaho JA (1999). Induction and prevention of apoptosis in human HEp-2 cells by herpes simplex virus type 1. *J Virol* **73:** 10359–10370.

Aubert M, Rice SA, Blaho JA (2001). Accumulation of herpes simplex virus type 1 early and leaky-late proteins correlates with apoptosis prevention in infected human HEp-2 cells. *J Virol* **75:** 1013–1030.

Benetti L, Munger J, Roizman B (2003). The herpes simplex virus 1 Us3 protein kinase blocks caspase-dependent double cleavage and activation of the proapoptotic protein BAD. *J Virol* **77:** 6567–6573.

Cui C, Griffiths A, Li G, Silva LM, Kramer MF, Gasterland T, Wang XJ, Coen DM (2006). Prediction and identification of herpes simplex virus 1-encoded microRNAs. *J Virol* **80:** 5499–5508.

Cunningham C, Davison AJ (1993). A cosmid-based system for constructing mutants of herpes simplex virus type 1. *Virology* **197:** 116–124.

DeLuca NA, Schaffer PA (1987). Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. *Nucl Acids Res* **15:** 4491–4509.

DeLuca NA, McCarthy A, Schaffer PA (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J Virol* **56:** 558–570.

Devi-Rao GB, Bloom DC, Stevens JG, Wagner EK (1994). Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latently infected murine sensory ganglia. *J Virol* **68:** 1271–1282.

Galvan V, Brandimarti R, Munger J, Roizman B (2000). Bcl-2 blocks a caspase-dependent pathway of apoptosis activated by herpes simplex virus 1 infection in HEp-2 cells. *J Virol* **74:** 1931–1938.

Galvan V, Brandimarti R, Roizman B (1999). Herpes simplex virus 1 blocks caspase-3-independent and caspase-dependent pathways to cell death. *J Virol* **73:** 3219–3226.

Galvan V, Roizman B (1998). Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. *Proc Natl Acad Sci U S A* **95:** 3931–3936.

Gupta A, Gartner JJ, Sethupathy P, Hatzigeorgiou AG, Fraser NW (2006). Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature* **442:** 82–85.

Halford WP, Schaffer PA (2001). ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. *J Virol* **75:** 3240–3249.

Jerome KR, Chen Z, Lang R, Torres MR, Hofmeister J, Smith S, Fox R, Froelich CJ, Corey L (2001). HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas. *J Immunol* **167:** 3928–3935.

Jerome KR, Fox R, Chen Z, Sears AE, Lee Hy, Corey L (1999). Herpes simplex virus inhibits apoptosis through the action of two genes, Us5 and Us3. *J Virol* **73:** 8950–8957.

Kops ADB, Knipe DM (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell* **55:** 857-868.

Koyama AH, Miwa Y (1997). Suppression of apoptotic DNA fragmentation in herpes simplex virus type 1-infected cells. *J Virol* **71:** 2567–2571.

Leopardi R, Roizman B (1996). The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. *Proc Natl Acad Sci U S A* **93:** 9583–9587.

Leopardi R, Van Sant C, Roizman B (1997). The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. *Proc Natl Acad Sci U S A* **94:** 7891–7896.

Liptak LM, Uprichard SL, Knipe DM (1996). Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. *J Virol* **70:** 1759–1767.

Liu C, Xu HY, Liu DX (2001). Induction of caspase-dependent apoptosis in cultured cells by the avian coronavirus infectious bronchitis virus. *J Virol* **75:** 6402–6409.

Lukonis CJ, Weller SK (1996). Characterization of nuclear structures in cells infected with herpes simplex virus type 1 in the absence of viral DNA replication. *J Virol* **70:** 1751–1758.

Margolis T, Sedarati F, Dobson A, Feldman LT, Stevens J (1992). Pathways of viral gene expression during acute neuronal infection with HSV-1. *Virology* **189:** 150–160.

McKnight S, Gavis E (1980). Expression of the herpes thymidine kinase gene in Xenopus laevis oocytes: an assay for the study of deletion mutants constructed in vitro. *Nucl Acids Res* **8:** 5940.

Minagawa H, Tanaka S, Toh Y, Mori R (1994). Detection of herpes simplex virus type 1-encoded RNA by polymerase chain reaction: different pattern of viral RNA detection in latently infected murine trigeminal ganglia following *in vitro* or *in vivo* reactivation. *J Gen Virol* **75:** 647–650.

Moxley M, Block T, Liu H-C, Fraser N, Perng G-C, Wechsler S, Su Y-H (2002). Herpes simplex virus type 1 infection prevents detachment of nerve growth factor-differentiated PC12 cells in culture. *J Gen Virol* **83:** 1591–1600.

Munger J, Chee A, Roizman B (2001). The U_S3 protein kinase blocks apoptosis induced by the *d*120 mutant of herpes simplex virus 1 at a premitochondrial stage. *J Virol* **75:** 5491–5497.

Munger J, Roizman B (2001). The U_s3 protein kinase of herpes simplex virus 1 mediated the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins. *Proc Natl Acad Sci U S A* **98:** 10410–10415.

Nguyen ML, Kraft RM, Blaho JA (2005). African green monkey kidney Vero cells require de novo protein synthesis for efficient herpes simplex virus 1-dependent apoptosis. *Virology* **336:** 274–290.

Quinlan MP, Chen LB, Knipe DM (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36:** 857–868.

Rice SA, Knipe DM (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. *J Virol* **64:** 1704–1715.

Sieg S, Yildirim Z, Smith D, Kayagaki N, Yagita H, Huang Y, Kaplan D (1996). Herpes simplex virus type 2 inhibition of Fas ligand expression. *J Virol* **70:** 8747–8751.

Su Y-H, Moxley M, Ng AK, Lin J, Jordan R, Fraser NW, Block TM (2002). Stability and circularization of herpes simplex virus type 1 genomes in quiescently infected PC12 cultures. *J Gen Virol* **83:** 2943–2950.

Su Y-H, Zhang X, Wang X, Fraser NW, Block TM (2006). Evidence that the immediate early gene product, ICP4, is necessary for HSV-1 ICP4 deletion mutant strain d120 genome circularization in infected cells. *J Virol* (in press).

Taddeo B, Luo TR, Zhang W, Roizman B (2003). Activation of NF-κB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. *Proc Natl Acad Sci U S A* **100:** 12408–12413.

Tang Q, Li L, Ishov AM, Revol V, Epstein AL, Maul GG (2003). Determination of minimum herpes simplex virus type 1 components necessary to localize transcriptionally active DNA to ND10. *J Virol* **77:** 5821–5828.

Uprichard SL, Knipe DM (2003). Conformational changes in the herpes simplex virus ICP8 DNA-binding protein coincident with assembly in viral replication structures. *J Virol* **77:** 7467–7476.

Wang M, Block TM, Steel L, Brenner DE, Su YH (2004). Preferential isolation of fragmented DNA enhances the detection of circulating mutated k-ras DNA. *Clin Chem* **50:** 211–213.

Yu KY, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS (1999). A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J Biol Chem* **274:** 13733–13736.

Zhou G, Roizman B (2000). Wild-type herpes simplex virus 1 blocks programmed cell death and release of cytochrome *c* but not the translocation of mitochondrial apoptosis-inducing factor to the nuclei of human embryonic lung fibroblasts. *J Virol* **74:** 9048–9053.