J. Neurovirol. (2012) 18:231–243
DOI 10.1007/s13365-012-0105-2

Direct evidence that HSV DNA damaged by ultraviolet (UV) irradiation can be repaired in a cell type-dependent manner

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Received: 27 January 2012 / Revised: 6 April 2012 / Accepted: 13 April 2012 / Published online: 12 May 2012

Abstract Infection of permissive cells, in tissue culture, with herpes simplex virus (HSV) has been reported to induce host DNA damage repair responses that are necessary for efficient viral replication. However, direct repair of the damaged viral DNA has not, to our knowledge, been shown. In this report, we detect and determine the amount of damaged HSV-1 DNA, following introduction of experimentally damaged HSV genomes into tissue cultures of permissive Vero, NGF differentiated PC12 cells and primary rat neurons, using a method of detection introduced here. The results show that HSV-1 strain 17 DNA containing UV-induced DNA damage is efficiently repaired, in Vero, but not NGF differentiated PC12 cells. The primary rat neuronal cultures were capable of repairing the damaged viral DNA, but with much less efficiency than did the permissive Vero cells. Moreover, by conducting the experiments with either an inhibitor of the HSV polymerase (phosphonoacetic acid [PAA]) or with a replication defective DNA polymerase mutant virus, HP66, the results suggest that repair can occur in the absence of a functional viral polymerase, although polymerase function seems to enhance the efficiency of the repair, in a replication independent manner. The possible significance of varying cell type mediated repair of viral DNA to viral pathogenesis is discussed.

Keywords HSV-1 DNA damage · Ultraviolet · PCR · DNA repair · Latent infection

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Introduction

Herpes simplex virus (HSV) has been shown to influence cellular (host) DNA repair systems, during productive infection in permissive cells (Lilley et al. 2005, 2007, 2010; Mohni et al. 2010, 2011; Wilkinson and Weller 2004, 2006). Less is known about if and how HSV DNA is repaired in non-permissive, neuronal like cells during productive or latent infection. Viral DNA, as isolated from the virion appears to contain multiple single stranded nicks, and thus some degree of repair would seem to be necessary (Frenkel et al. 1972; Kieff et al. 1971; Wilkie 1973). We have observed that HSV DNA derived from quiescently infected NGF differentiated PC12 cells, which had harbored the virus for weeks, appeared to contain significantly greater numbers of nicks and other possible damage, rendering it alkali sensitive as compared to viral DNA derived from productively infected cells (Millhouse et al. 2010). It therefore seemed possible that the host DNA repair

systems were differentially used or available to the virus during productive infection in Vero and quiescent infection in neuronal type cells.

We thus set out to determine, firstly if the host cell can repair HSV DNA following infection with virions, and, if so, if the repair efficiency differed in different cell types. For this, we infected permissive Vero, Nerve Growth Factor (NGF) differentiated PC12 and primary explants of rat CNS tissue. Vero cells, derived from monkey kidneys, are highly permissive for HSV-1 replication and are routinely used for the production of high titer viral stocks. PC12 cells are derived from a rat pheochromocytoma (Tischler and Greene 1975). Following differentiation with NGF, PC12 cells extend neuritic processes, become post mitotic. They have been used as a model of HSV-1 "latent" or quiescent" infection, since the viral genomes can persist in them for weeks in the absence of productive infection (Block et al. 1994; Danaher et al. 1999; Moxley et al. 2002; Su et al. 1999, 2000, 2002). CNS cultures can be generated from rat tissue reasonably easily, and be used as models of primary, non-transformed type, neuronal cells (Brewer 1995). Each were infected with HSV-1 virions that had been irradiated with amounts of ultraviolet (UV) light sufficient to induce polymerase chain reaction (PCR)-detectable cyclobutane pyrimidine dimers (CPDs) and other lesions in the viral genome.

Since UV-induced DNA damage blocks the processivity of the *Taq* polymerase during the PCR (Murray et al. 1992; Ponti et al. 1991), we adapted a PCR method that had been described to detect and quantify the amount of UV damage on DNA, and monitor its repair, following infection of the UV irradiated DNA. Briefly, by using an amount of UV damage that introduced approximately 1–2 lesions per thousand nts, we could use the differential PCR efficiencies of amplification of a short, 70 nt stretch ("short amplicon") of the viral DNA, versus longer, 2-kb stretches ("long amplicon") of the genome, as an indication of the amount of damage a virion genome contained. Virions containing the UV-induced damage can be used to infect cells and the amount of damage remaining can be followed by analyzing DNA recovered from the infected cell. In doing this, we determined that virion DNA containing UV-induced damage can be repaired following infection of Vero cells. The repair occurs much less efficiently in CNS derived primary neurons and, not to any detectable level, in the NGF differentiated PC12 cells. Both host and viral functions were shown to be involved. Although DNA repair functions have been shown to be important in HSV replication, this, to our knowledge the first example showing directly that HSV DNA can be a substrate for repair, itself. The cell type differences in repair efficiencies may have biological significance to viral pathogenesis, and this is discussed.

Materials and methods

Cells, viruses, and media

PC12 cells were originally obtained from the American Type Culture Collection. The PC12 cells were maintained in an RPMI medium supplemented with 10 % horse serum, 5 % fetal bovine serum, and 1 % antibiotic–antimycotic agent. HSV-1, strains 17 and F, were prepared and titered in Vero cells as described previously (Moxley et al. 2002). HSV-1 strain KOS mutant HP66 (Marcy et al. 1990), provided by Donald Coen of Harvard Medical School, was grown and titered in the HSV-1 pol expressing Vero cell line PolB3 (Hwang et al. 1997).

Differentiation of PC12 cells and establishment of quiescent infection

NGF-differentiated PC12 cultures were established as described previously (Su et al. 1999). PC12 cells were dispersed from clusters by syringe, and 6×10^5 cells were seeded onto T-75 flasks coated with poly-L-ornithine hydrobromide (Sigma, St. Louis, MO, USA) in the same media for PC12 cell culture with an additional supplement of 100 ng/ml of 2.5S NGF (BD Bioscience, Franklin Lakes, NJ, USA).

Differentiated PC12 cultures were infected with HSV-1 strain 17 or pol mutant HP66 at an MOI (multiplicity of infection) of 20 based on Vero cell titers. Following 1 h incubation at 37 °C, the cultures were treated with 3 ml sodium citrate buffer (pH=3) for 30 s to 1 min to inactivate residual virus modified as described (Su et al. 1999). After low pH treatment, cultures were incubated at 37 °C with fresh medium containing NGF.

Primary rat neuron cultures

Hippocampal tissue was obtained commercially through Brain Bits (Springfield, IL, USA) and cultures prepared with slight modifications to methods previously described (Brewer 1995). The hippocampal neurons were platted at low density (10,000 cell/well) on pre-coated poly-L-lysine coated plates in a 96-well format and maintained in serum-free medium consisting of Neurobasal Medium supplemented with B25 and GlutaMAX (Gibco). Neuronal cultures were infected with HSV-1 14 days post-plating at an MOI of 20. The culture medium was tested for HSV-1 pfu (plaque forming units) by Vero cell-based plaque assays. DNA from infected neurons was prepared by pooling cells from eight wells followed by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. HSV-1 DNA was measured by SyBr green real-time PCR using short (70 nt) amplicon thymidine kinase (TK)-specific primers: TK1F 5′- GCAGCGACCCGCTTAACA −3′ and

TK1R 5′- GAAGAGGTGCGGGAGTTTCAC-3′. For additional PCR details, see the Discussion below.

Ultraviolet light irradiation

HSV DNA was damaged by exposure to UVC (254 nm wavelength) irradiation generated by a handheld wand (Model UVG-54 mineral light lamp; UVP, Inc.) suspended 5 cm directly above the sample for the indicated exposure times. During virion irradiation, approximately 400 μl of PBS-diluted virus was distributed in the bottom of a single well of a chilled 12-well plate.

Detection of cyclobutane pyrimidine dimers by a "dot blot" method

CPDs were measured using a dot blot of infected cell DNA. Briefly, DNA was prepared by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. Total DNA was measured by spectrophotometry at A260 by NanoDrop (Thermo Scientific). Two micrograms of total DNA (7 μl) was spotted on a membrane (Amersham Hybond-N+purchased from GE Healthcare), dried overnight at room temperature and baked for 2 h under a heat lamp. Membranes were blocked with 5 % nonfat milk in PBS for 1 h, then incubated for 2 h with mouse anti-CPD monoclonal antibody clone KTM53 (Kamiya Biomedical Company, Seattle, WA, USA) diluted 1:2,000 in 5 % milk/PBS. Anti-mouse HRP conjugated secondary antibody was diluted 1:5,000 and incubated with membrane for 45 min. HRP was measured using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Blots were photographed and quantified with a Gel Logic 1500 Molecular Imaging System (Care Stream Health Inc.).

The DNA damage assay — quantitative real-time and endpoint polymerase chain reaction

DNA to be analyzed for damage was prepared from cells by proteinase K digestion, phenol/chloroform digestion and ethanol precipitation. DNA was resuspended in a minimal volume of TE and allowed to solubilize overnight at 4 °C. For samples in which HSV DNA levels would be very high due to viral replication, the DNA was further diluted by as much as 2,000-fold. Each DNA sample was tested by quantitative real-time PCR in duplicate using TK-specific primers and SYBR green and in some cases SYBR green and Taqman hydrolysis probe detection systems. Primers used for real-time PCR are TK1.70F 5′-GAAG AGGTGCGGGAGTTTCAC-3′ and TK1.70R 5′-GCAGCGACCGCTTAACA-3′. The Taqman probe when used was [6FAM]-CAGCGTGCCGCAGA TCTTG-[BHQ1]. In some experiments cellular GAPDH specific primers were used to normalize viral DNA quantities. The GAPDH primers used were rGAP.2F 5′-CCTGTTC TAGAGACAGCCGC-3′ and rGAP.3R 5′-CACCTGGC ACTGCACAAG-3′. The real-time PCR quantification reactions were performed using the LightCycler 480 (Roche Biochemical, Germany), at 95 °C for 10 min, then 45 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 10 s. SYBR green Mastermix reagents (Roche Biochemical, New Jersey, USA) were used to test for double-stranded DNA products resulting from the PCRs, and melting curve analysis was used to verify the specificities of these PCR products. Serially diluted DNA prepared from productively infected Vero cells was used to compute a standard curve and to assign relative concentrations. All samples typically measured HSV-1 DNA at 1,000-fold or more than the maximum sensitivity of the assay. All samples, now diluted to equal HSV-1 DNA concentration, were tested in two standard endpoint PCR reactions, in which one utilized a short (~70 bp) amplicon and the other utilized a ~2-kb amplicon. The PCR cycle number was optimized for each so that differences in PCR product reflected amplification efficiency as assessed by the amplification of serially diluted virion DNA standards (typically 30 cycles were used). The two HSV-1 primer sets used for endpoint PCR were: TK2.70F 5′-GCAGCGAC CCGCTTAACA-3′ and TK2.70R 5′-GAAGAGGTGC GGGAGTTTCAC-3′ for a 76-bp amplicon and TK1.2000F 5′-GCAGCGACCCGCTTAACA-3′ and TK1.2000R 5′-ATGGCTTCGTACCCCTGCCAT-3′ for a 1,917-bp amplicon. The endpoint PCR utilized Qiagen Hotstart Taq polymerase and the standard suggested reagents. PCR products were viewed on 2 % agarose gels.

In the PCR reaction, lesion frequency, f, within an amplicon is calculated by the zero class Poisson function $f=-\ln(A_D/A_O)$, where A_D is the amplification of damaged template and A_O is the amplification of non-damaged template (Yakes et al. 1996). This is true at least for lesions that block PCR polymerase progression. For example, an average of three lesions per strand in a given amplicon would be expected to decrease the amplification to 5.0 % of normal. In theory, if the amount of DNA damage is appropriately controlled, a set of conditions can be established that yield an 18-fold or greater difference in PCR product formation when the 2-kb amplicon PCR is compared to a 70-bp amplicon PCR.

Results

Detection of UV-damaged HSV DNA using a PCR-based system

Since our goal was to detect the damage and repair of HSV-1 DNA in infected cells, systems that allow for detection of very small (nanogram) amounts of viral DNA were necessary. We therefore adapted an assay that depends upon the inability of the Taq and other PCR polymerases, in a standard PCR, to

proceed past damages on the template strand, such as CPDs or single-stranded nicks (Yakes et al. 1996). There exist polymerases that can proceed through a CPD on the template strand but these typically do not have sufficient processivity for use in PCR and their utility appears restricted to preventing the stalling of a DNA replication fork at a damaged base in vivo. For our purposes, the ability of the polymerase to elongate long stretches of a DNA template is reduced as a function of damage frequency and interruptions on the amplicon template.

As illustrated in (Fig. 1), DNA containing lesions such as nicks, AP sites and CPDs, block or impair DNA polymerase progression during PCR, thus PCR product formation of damaged DNA becomes a function of the average lesion frequency in the amplicon (Ponti et al. 1991; Yakes et al. 1996). The PCR product amount then becomes proportional to the frequency of lesions within the template. For a given frequency of DNA damage (lesions per kb), the longer the template, the more likely there is to be an interruption in the PCR, and the more sensitive is the assay in detecting damage. As the illustration shows, if an amount of UV light is delivered such that damage is at a frequency of less than 1 per lesion per ~70 bp (see Materials and methods), amplicons that are 70 bp can be much more efficiently amplified than those that that are longer. Therefore, we use two sizes of amplicons derived from the same region of the HSV genome (see Fig. 1), an ~70 bp amplicon and an ~2 kb amplicon, which, following introduction of UV damage to HSV genomes, we refer to as short and long amplicon PCRs.

Figure 2 shows a validation of this approach by amplifying the 70-bp and 2-kb amplicons from the HSV 1 thymidine kinase (tk) gene, using either control or UV irradiated viral DNA. UV exposures as long as 100 s duration resulted in undetectable changes to the small (70 bp) amplicon PCR product. On the other hand, 2-kb stretches of the tk gene (which include the 70 kb amplicon region) cannot be efficiently amplified following either 10- or 100-s UV exposures, and, although the 70 bp short amplicon product is similar in the 0- and 100-s UV irradiation samples, the 2-kb amplicon product is beneath the level of detection, following 100 s of

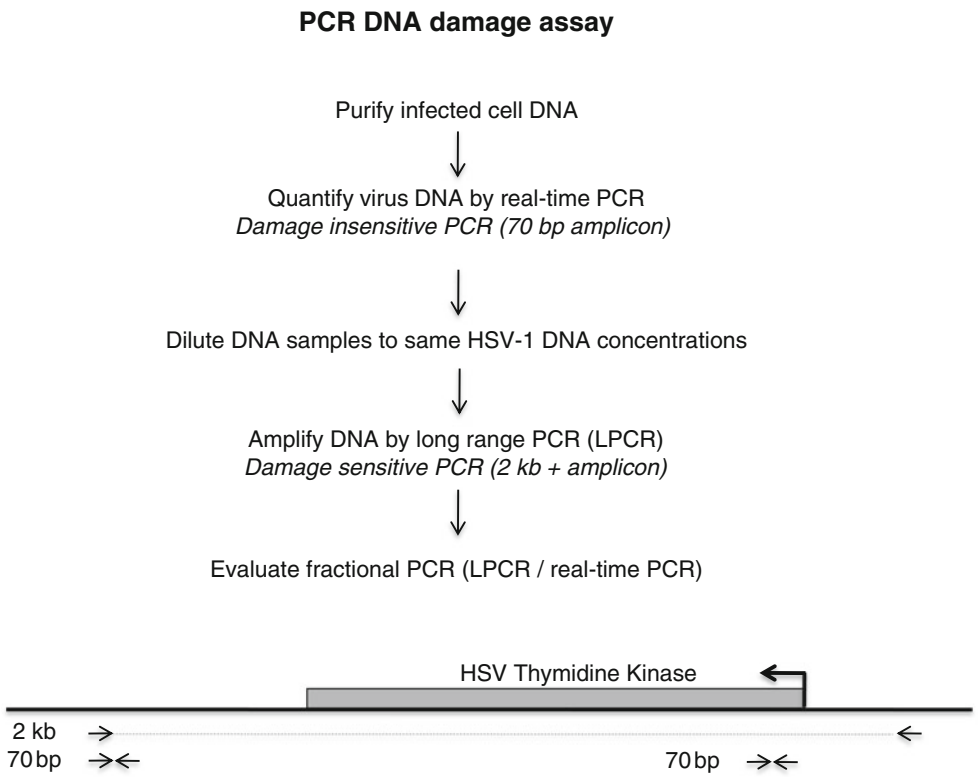

Fig. 1 PCR-based DNA damage assay was performed by determining the sensitivity of a DNA preparation to long amplicon PCR. DNA to be tested was quantified by quantitative real-time PCR using an HSV-1 TK standard sized small amplicon primer pair whose product is approximately 70 bp in length. The amount of DNA damage in these studies was not so extensive as to impair the PCR reaction with these small amplicons. Based on the qPCR, samples were diluted to equal HSV DNA concentration and used in subsequent endpoint PCR reactions in which a second small (~70 bp) and a long amplicon (2 kb) PCR were used. The small amplicon endpoint PCR was used as a verification of the qPCR and should remain equal in all samples. The 2-kb amplicon spanned the two small amplicon sites. PCR reactions containing serial dilutions of an HSV-1 DNA standard were run with the experimental samples to ensure that differences in PCR product reflected changes in the amount of template. Endpoint PCR reactions were run on 2 % agarose gels to monitor changes in the fractional PCR efficiencies of long and short amplicons

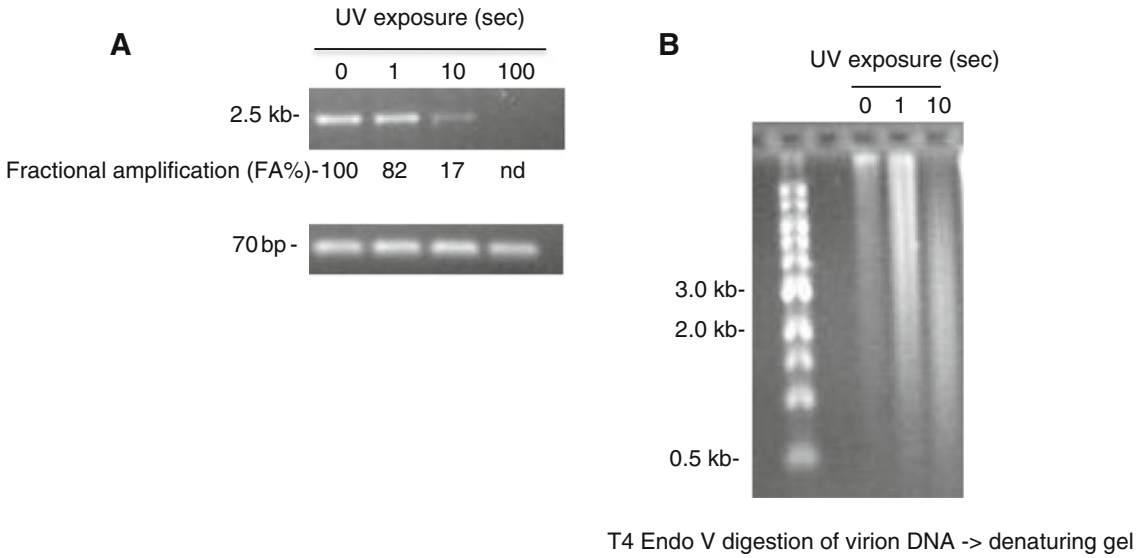

Fig. 2 Analysis of ultraviolet irradiation induced DNA damage using the T4EndoV digests and a PCR processivity assay. **a** Purifed HSV-1 DNA was irradiated as described in Materials and methods for 0, 1, 10 and 100 s. In the experiment, 10 pg of each virion DNA sample was subjected to PCR using TK primer pair TK1.70 (70 bp amplicon) and TK1.2500 (2.5 kb amplicon). "nd" represents "not determined." **b** Irradiated DNA samples (100 ng) were digested with T4EndoV nuclease and subsequently run on a denaturing alkaline agarose gel

UV irradiation. We note that "endpoint" PCR read-outs, rather than real-time PCR, are used in this case, since amplification of 2-kb templates by real-time PCR is problematic. Endpoint PCR was always carried within the linear range of amplification, as assured by carrying out the reactions at multiple dilutions of templates (not shown here).

To determine if the PCR inhibition could be explained by the introduction of UV-induced CPDs, the same DNA samples used in Fig. 2a were examined by T4 Endonuclease V (EndoV) digestion, which cleaves DNA backbones at CPDs, followed by denaturing alkaline gel electrophoresis (Fig. 2b). This resulted in the fragmentation of single stranded DNA into sizes approximately 2–3 kb for the 10-s exposure (Fig. 2b). This suggests that the samples irradiated for 10 s contain an average one CPD lesion per 2.5 kb amplicon, and that a 2.5-kb amplicon PCR should be reduced to 34 % according to the Poisson calculations, which is similar to that observed in experimental analysis (Fig. 2a). This result is consistent with the notion that CPD frequency in the amplicon template is causing the amplicon length-dependent PCR reduction (Fig. 2a). Small differences between the T4 EndoV data and the PCR data would be explained by the presence of additional types of UV-induced lesions such as the (6–4) photoproduct which would be expected to inhibit PCR polymerase progression but would not be detected by T4 EndoV digestion.

These data show that the relative amounts of UV-induced damage of HSV genomes can be detected and quantified using this PCR based system, and that much of the damage resulting from UV irradiation is, as expected, CPDs.

Repair and amplification of UV-damaged HSV genomes in Vero cells

With a system to detect and quantify UV-damaged viral genomes, it was now possible to determine the extent to, if any, HSV-1 damaged genomes could be detected and repaired within the infected cell, in culture. Therefore, HSV-1 strain 17 viral stocks were diluted and irradiated at various exposures of UV light as previously performed with viral DNA. Vero cells were infected with each virus at an MOI of 5, and infected cells were harvested for DNA purification at selected time points. Figure 3a shows changes in relative intracellular viral genome copies at each time point based on analysis of the thymidine kinase (tk) gene. As shown in Fig. 3a, the infected cultures reproducibly show nearly identical amounts of tk gene amplicons at time 0 h post-infection, but this is followed by a period of genome copy decline by as much as 1 log during the first 2 h after infection. The decline in viral DNA amounts is similar for DNA from irradiated and non-irradiated viruses, when all of the experiments are taken into consideration (e.g., see Fig. 4), although the reductions seem more extreme in the cells receiving irradiated virions in the experiments in Fig. 3. By 24 h post-infection, viruses receiving lower UV doses (1–10 s) replicated similarly to unirradiated virus (approx. 3–4 log expansion), whereas the highly irradiated virus (100-s UV exposure) replicated little or not at all compared to time 0 copy numbers (Fig. 3a).

Total DNA prepared from the infected cells was analyzed for damage using the PCR assay described in Figs. 1 and 2. Irradiated and non-irradiated HSV genomes were taken up and internalized to similar amounts, usually ~1–3 genomes per cell

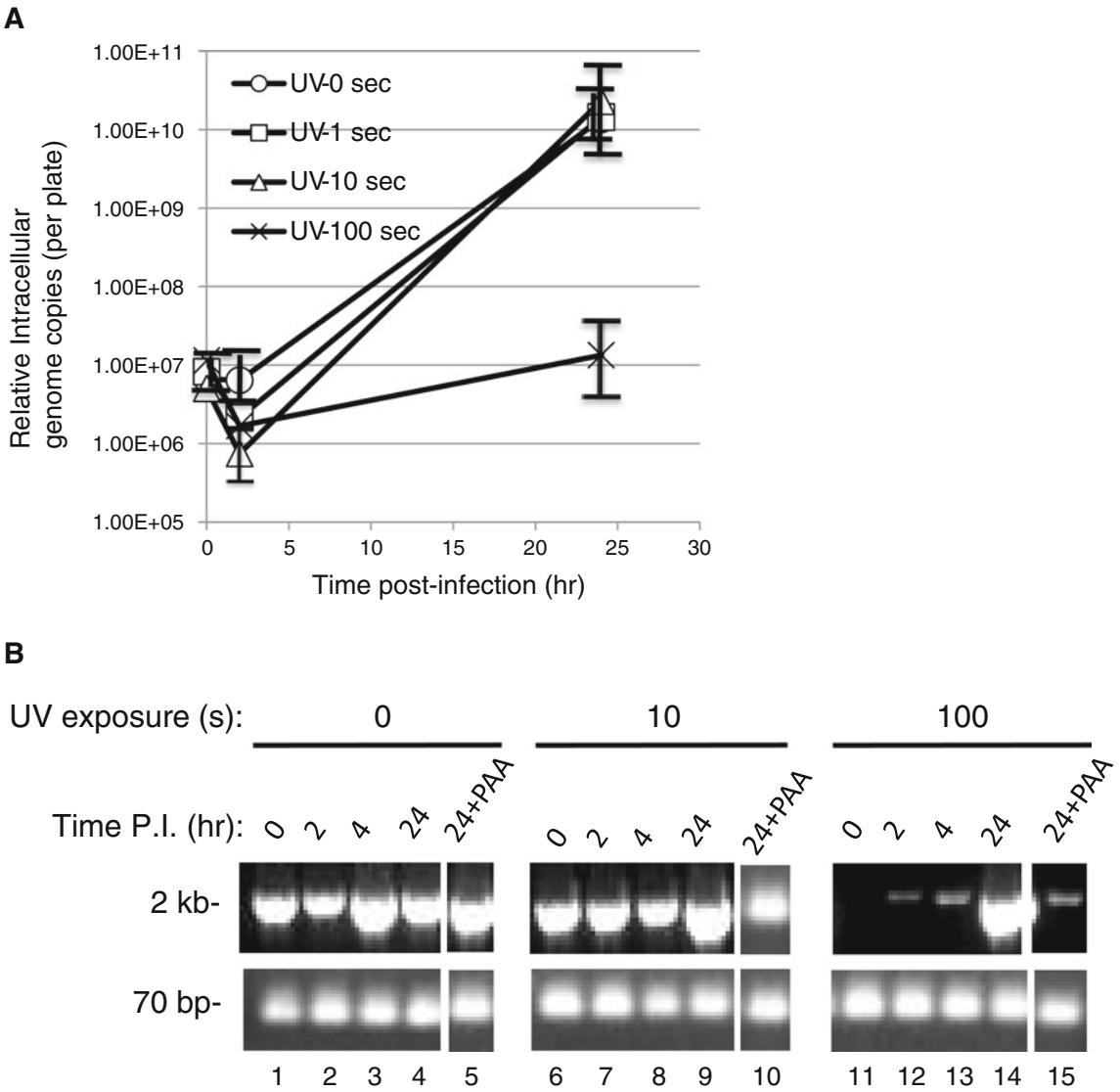

Fig. 3 UV-damaged HSV-1 infections in Vero cells show conversion of damaged DNA to undamaged DNA by 24 h post-infection. **a** Vero cells were infected with unirradiated or irradiated HSV-1 strain 17+ (MOI=5) for the indicated time points after which DNA was prepared and quantified for HSV-1 DNA content by SYBR green real-time PCR using standard primers (TK1.70). Data from three experiments were averaged to show standard deviation at the 0, 2 and 24 h time points. **b** DNA was diluted as necessary to equalize HSV-1 amounts and a long-range (2 kb amplicon) and short-range (70 bp amplicon) endpoint PCR was performed using TK1.2000 primer and TK2.70 primers, respectively. PCR products were separated on 2 % agarose gels and stained with ethidium bromide. PCR results for 24-h infections containing phosphono acetic acid (*PAA*) are labeled 24 + PAA

(data not shown). Only virus exposed to the longest UV exposure (100 s) showed loss of long amplicon PCR at time 0, while maintaining short amplicon PCR (Fig. 3b, compare lane 11 to lanes 1 and 6). For simplicity, this virus will subsequently be referred to as HSV-UV100. This suggests that within the context of the live virus, a 10-fold higher UV dose was required to create lesions at a frequency that can be detected by this assay compared to naked DNA (Fig. 2b). Interestingly, by 24 h post-infection, HSV-UV100 DNA yielded similarly amplifiable long and short amplicons to low dose irradiated virus (Fig. 3b, compare lane 14 to lanes 4 and 9). This result could be explained by the near complete repair of the CPDs by the host nucleotide excision repair machinery. Alternatively, it could have resulted from the replication of what would have had to have been a small (undetectable) number of viral genomes that would have had to have experienced no damage or damage that did not prevent replication. This is considered unlikely, but is addressed below.

Along these lines, even at the earliest times after infection tested (2 h), partial reconstitution of long amplicon PCR is reproducibly observed (Fig. 3b, lanes 12 and 13), consistent with repair having occurred prior to DNA replication, which under these conditions occurs after 4 h of infection (Fig. 3 and other data not shown).

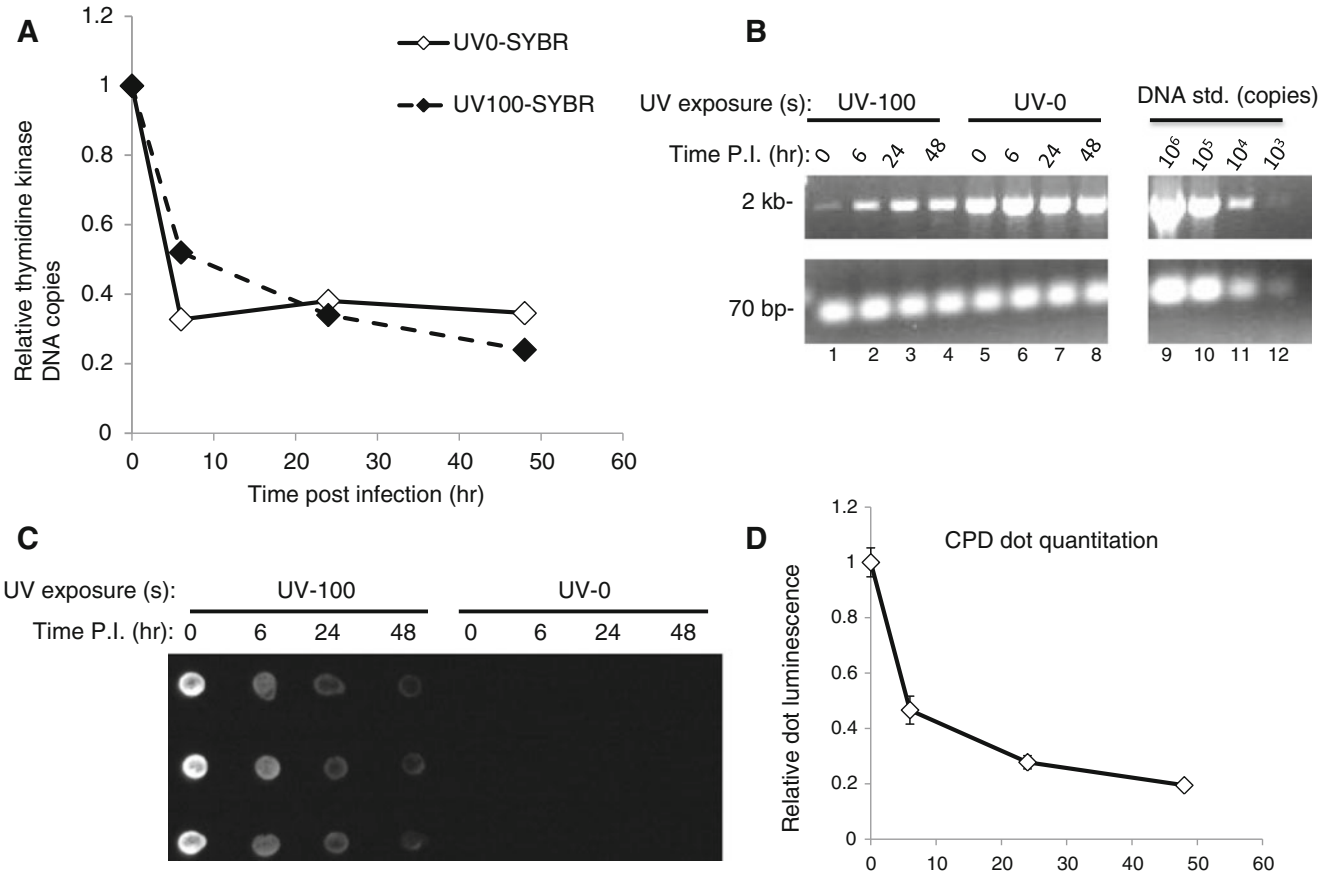

Fig. 4 Repair of UV-damaged virus DNA can occur in the absence of viral polymerase. **a** Vero cells were infected with unirradiated or irradiated HSV-1 strain HP66 (MOI=5) for the indicated time points after which total DNA was prepared and quantified for HSV-1 DNA content by SYBR green real-time PCR using standard primers (TK1.70). Error bars represent the standard deviation of four data points derived from two independent experiments. **b** DNA was diluted as necessary to equalize HSV-1 DNA amounts and a long range (2 kb amplicon) and short range (70 bp amplicon) endpoint PCR was performed using TK1.2000 primer and TK2.70 primers, respectively. PCR products were separated on 2 % agarose gels and stained with ethidium bromide. **c** Total DNA preparations at each time point were quantified by A260 spectrophotometry and equal amounts (2 μg) were spotted onto nitrocellulose membranes in triplicate. Membranes were probed for cyclobutane pyrimidine dimers (*CPDs*) by antibodies directed against CPDs. **d** CPD dot blots were quantified. Data points represent the average of three spots with standard deviation

To address the role of viral DNA replication, we included the viral polymerase inhibitor, phosphonoacetic acid (PAA), into the 24-h infections (Fig. 3b), which has been shown — at these concentrations — to block HSV-1 DNA replication in a non-cytotoxic manner (Furman et al. 1979). Virus genome copy number at 24 h in the presence of PAA in this experiment ranged from 0.5 to 1.0 copies per cell, which approximately matches the lower levels seen at earlier time points in the absence of PAA (Fig. 3a). This suggests that PAA was effective in blocking viral DNA replication, as expected.

Based on the large increase in the amount of long amplicon (2 kb) HSV DNA detected at 24 h relative to 0 h (Fig. 3b, comparing lane 14 with lane 11), following infection with UV-damaged HSV virions, it can be seen that inhibition of the viral polymerase, or the agent PAA itself, significantly reduces, but does eliminate, the ability of the damaged viral DNA to be repaired (compare Fig. 3b, lanes 15 and 14). This suggests that for repair of damaged viral DNA, either an active viral DNA polymerase (or the viral replication complex) is necessary, or that PAA inhibits cellular repair functions. The possibility that viral DNA repair can occur in a viral polymerase free fashion was addressed in the next set of experiments, using polymerase knock out mutants.

Partial repair of UV damage in the absence of viral polymerase

To further examine the role of the HSV-1 polymerase, UL30, we used a mutant of HSV-1 strain KOS, HP66, that does not express the UL30 protein (Marcy et al. 1990). The HP66 virus used here did not replicate DNA in Vero cells, although it did replicate in a U_L30 complementing Vero cell line, indicating that the absence of genome replication is due to the absence of U_L30 protein and the mutant behaves as reported (data not shown).

The HP66 viral stock was irradiated for 100 s (HP66-UV100) as performed with wild-type HSV-1 used in Fig. 3 and in the Materials and methods section. The resultant HP66-UV100 virus and unirradiated virus were used to infect Vero cells. We note that there was no detectable level of infectious HSV-1 DNA produced Vero cells infected with HP66, suggesting that there was no inadvertent passing of wild type virus or wild type pol in the HP66 stocks (data not shown).

When "total" intracellular genome copy number was assessed at 0, 6, 24 and 48 h after infection (Fig. 4a), there was, as expected, no increase in viral genome copy observed over the period, since there is no viral replication in the absence of a functional DNA polymerase. Indeed, there was a 70 % drop in total genome copies for the HP66-UV100 and a similar drop was also observed for unirradiated HP66 virus by 48 h after infection. Thus, it appears that, in the absence a functional polymerase, some degradation of viral DNA occurs in Vero cells.

For the DNA damage assay, as expected, HP66-UV100 virus DNA at time 0 showed a loss of long amplicon PCR (Fig. 4b, lane 5 compared to lane 1). Partial reconstitution of long amplicon PCR could be seen by 6–24 h after infection and was stable between 24 and 48 h after infection, indicating that repair of the damaged long 2-kb amplicon can occur in the absence of a functional HSV-1 polymerase. Indeed, by 6 h, at least 35 % of the damaged DNA was restored to non-damaged DNA values, as measured by the amount of long amplicon DNA produced, compared to the amount detected in unirradiated samples (compare Fig. 4b, 2 kb 6 h from the UV100 lane with the 6-h UV 0 lane).

Therefore, the viral polymerase U_L30 is not strictly required for the re-establishment of long amplicon PCR efficiency, and we believe that this data, along with the results with PAA studies (Fig. 3c), suggests that UV-damaged HSV-1 DNA can be repaired by cellular mechanisms, in the absence of the viral polymerase, within the first several hours after infection. We note, however, that in both cases, only partial repair was observed. It is not clear if this result is due to partial repair of all the DNA or if full repair was occurring in a fraction of infected cells, or if only certain types of DNA damage are able to be repaired in a viral pol independent fashion.

To directly detect CPDs with HP66 DNA and its repair analyzed UV-irradiated HP66 DNA recovered from infected cells and immobilized on dot blots using a CPD-specific antibody, as a function of time after infection. As shown in Fig. 4c, the cells with UV-irradiated HSV contained the greatest amount of detectable CPD viral DNA at the earliest times after infection, followed by a time-dependent loss of CPD detectable DNA, as quantified in Fig. 4d. In contrast, there is no CPD containing DNA detected in cells infected with HP66 that was not irradiated (Fig. 4c, "UV-0"). These data are consistent with the HP66 DNA derived from the virions that were UV-irradiated being damaged, with CPD containing residues, detected upon infection, followed by their repair presumably by nucleotide excision repair mechanisms. This is further appreciated when considering the fact that the absolute amounts of HSV DNA delivered into the infected was similar from the unirraduated virions and irradiated virions as shown in Fig. 4b (compare the 70 nt bands in the "UV-0," lanes 1–4 and "UV-100," lanes 5–8 of Fig. 4b).

UV-damaged viral DNA is not repaired in NGF-differentiated PC12 cells

NGF differentiated PC12 cells have been used as a model of the peripheral nervous system, since they share many properties in common with sensory nerves (Greene and Tischler 1976). They have also been used to study HSV latency, since the virus establishes quiescent infections in which the viral genome circularizes and is transcriptionally repressed for many weeks after infection (Block et al. 1994).

Previous studies suggested that quiescent HSV-1 DNA in NGF-differentiated PC12 cells accumulate damage as a function of time in culture (Millhouse et al. 2010), implying that viral DNA may be unable to access repair functions in neuronal like cells. It seemed possible that the accumulation of damaged viral DNA in NGF-PC12 cells might be due to poor DNA repair functions in these cells. To explore the relative ability of damaged HSV DNA to be repaired in NGF differentiated cells, UV-irradiated HSV-1 was used to infect NGF-differentiated PC12 cells and the integrity of the DNA was examined and monitored by the PCR processivity assay. As shown in Fig. 5, in complete contrast to infections in Vero cells, intracellular HSV-1 DNA copy number remained nearly stable for unirradiated virus for the first 3 days after infection. The copy numbers of irradiated virus DNA were generally reduced 2- to 5-fold within 3 days of infection (Fig. 5a), which is similar to the reduction seen in Vero cells for irradiated and unirradiated DNA from replication defective virus (HP66).

In the NGF-differentiated PC12 cells infected with UV-damaged virus, detection of UV damage was recognized as a near complete loss of long amplicon PCR at time 0 (Fig. 5b, lane 7 compared to lane 1). In contrast to Vero cell infections, the UV-induced loss of long amplicon PCR was never restored, even when DNA was examined from cells harvested 1 or 3 days post-infection. These data suggests that NGF-differentiated PC12 cells are impaired in the UV damage response, at least within the context of viral infection and when compared to Vero cell infections. Similar experiments with undifferentiated PC12 cells also showed the same evidence of DNA repair deficiency (data not shown). This suggests that PC12 cells are inherently DNA repair deficient and that this phenotype is not caused by NGF signaling pathways.

We reported previously that a limited productive infection occurs in a culture of NGF-differentiated PC12 cells

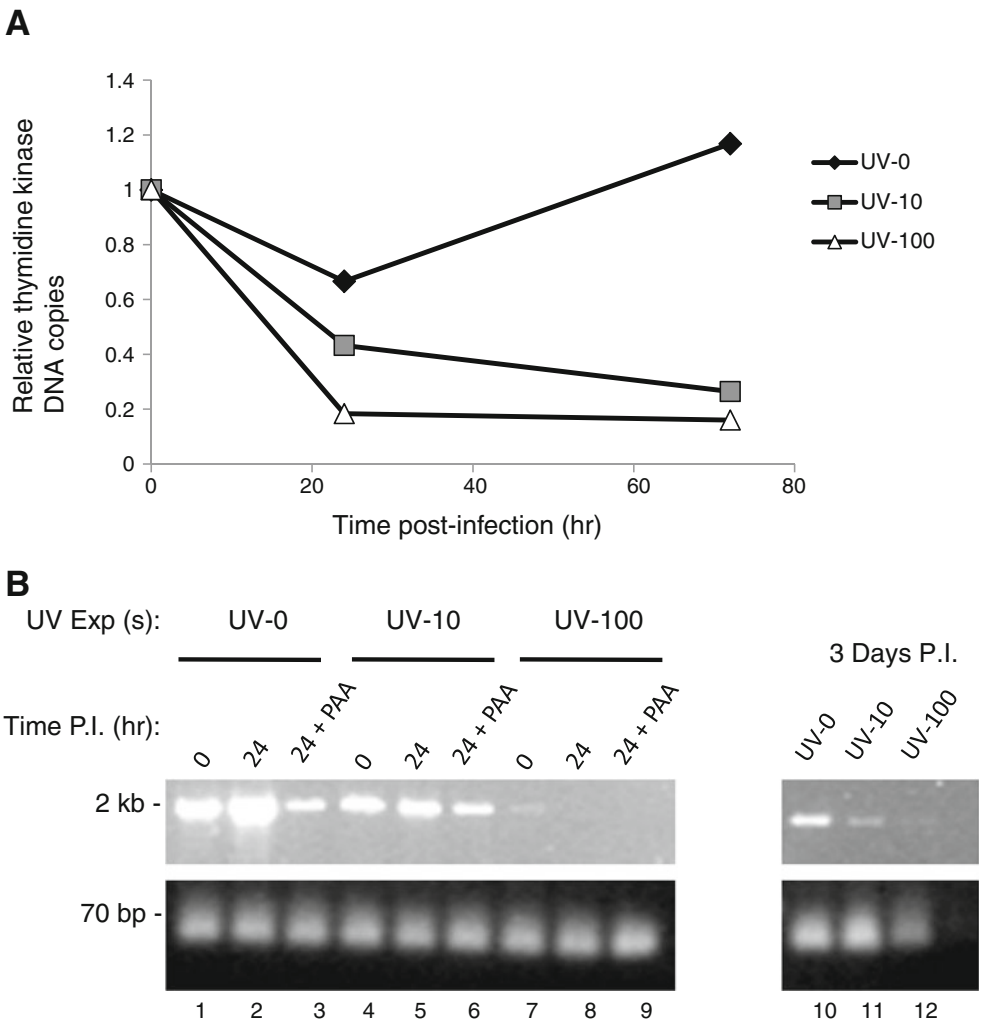

Fig. 5 HSV-1 infection of NGF differentiated PC12 cells shows no evidence of repair of UV-damaged HSV. **a** NGF differentiated PC12 cells were infected with unirradiated (UV-0), 10 s (UV-10) irradiated, or 100 s (UV-100) irradiated HSV-1 strain 17+ (MOI=20) for the indicated time points after which DNA was prepared and quantified for HSV-1 DNA content by SYBR green real-time PCR using standard primers (TK1.70) Error bars represent the standard deviation of three independent experiments. **b** DNA was diluted as necessary to equalize HSV-1 amounts and a long range (2 kb amplicon) and short range (70-bp amplicon) endpoint PCR was performed using TK1.2000 primer and TK2.70 primers, respectively. PCR products were separated on 2 % agarose gels and stained with ethidium bromide

during the first several days of infection (Block et al. 1994; Su et al. 1999, 2000, 2002). As such, the absence of genome copy increase by 1 and 3 days post-infection was somewhat surprising. To examine this issue, we tested the 3-day culture supernatants for infectious virus and we observed an average titer from two independent experiments of 1.5×10^5 pfu/ml (data not shown), which is similar to that reported previously for the NGF-PC12 system and corresponds to approximately 1 pfu/cell. These results may be explained by the fact that this low level of replication may not be detectable when total intracellular viral DNA is examined.

Replication and DNA repair occurs in primary rat CNS neurons

Although NGF differentiated PC12 cells exhibit many properties consistent with the peripheral nervous system, PC12 cells are derived from a rat pheochromocytoma, a malignantly transformed tissue (Greene and Tischler 1976). It is unclear to what extent the results with these cells can be attributed to their transformed state, as opposed to their neuronal like properties. It was therefore of interest to investigate HSV DNA repair in a more authentic neuronal cell setting.

Since peripheral nervous tissue is difficult to maintain, in culture, we prepared cultured embryonic rat CNS cells of the hippocampus, a class of tissue to which we had access. Two-week-old rat hippocampal neuronal cultures were infected with HSV-1 and viral titer was tested in the culture supernatants daily for the next 8 days after infection (Fig. 6a; see Materials and methods). HSV titers, as measured by pfu, rose slowly for the first 2 days and peaked 3 to 4 days post-infection, thereafter declining. Interestingly, the titer in the culture medium peaked at approximately 1 pfu/cell at day 3, similar to that observed in the NGF-differentiated PC12 cell infections (data not shown; Su et al. 1999).

However, in contrast to the NGF-PC12 system, intracellular viral DNA accumulated 30-fold from time 0 to day 3 (Fig. 6b) and significant cytopathic effect (CPE) was observed in the primary CNS cultures, but not in the NGF-PC12 cell cultures (data not shown). Although this is substantially less increase in HSV DNA then was seen in Vero cells (Fig. 3), in the CNS cultures, by 7–8 days after infection the primary rat

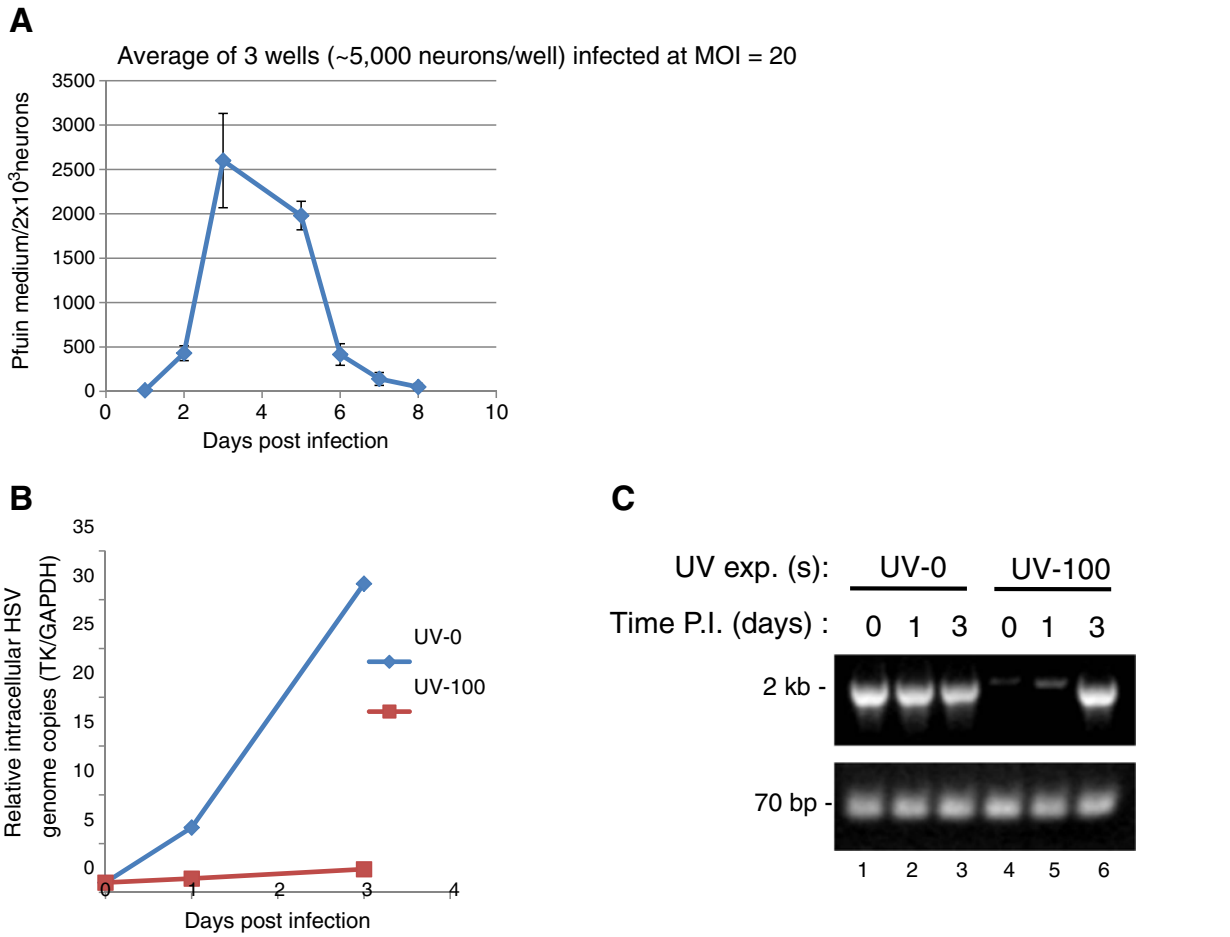

Fig. 6 HSV-1 infection of embryonic rat hippocampal neurons shows evidence of repair of UV-damaged HSV-1. **a** Primary rat hippocampal neurons (4×10^4 cells per infection) were infected with unirradiated (UV-0) or irradiated (UV-100) HSV-1 at an MOI of 20. Samples of culture supernatants were titered for infectious HSV-1 at the indicated times post-infection. Data represents the average of three infected cultures with standard deviation. **b** At 0, 1 and 3 days post-infection, the neuron cultures were collected and DNA was prepared and tested for HSV-1 TK DNA by SYBR green real-time PCR using standard primers (TK1.70). The data shows viral TK DNA normalized to cellular GAPDH DNA. **c** DNA was diluted as necessary to equalize HSV-1 DNA amounts and a long-range (2-kb amplicon) and short-range (70-bp amplicon) endpoint PCR was performed using TK1.2000 primer and TK2.70 primers, respectively. PCR products were separated on 2 % agarose gels and stained with ethidium bromide

CNS neurons infected with HSV-1 showed gross CPE, whereas uninfected neurons appeared normal under phase contrast light microscopy, albeit at a reduced (2- to 4-fold) cell number by visual approximation (data not shown). Neurons infected with UV-irradiated virus (HSV-UV 100) also appeared mostly healthy by light microscopy.

HSV-UV100 intracellular DNA accumulated little to not at all in the rat CNS neurons (Fig. 6b), similar to the results seen in Vero cell infections (Fig. 3a) and NGF-differentiated PC12 cells (Fig. 5a). Therefore, this level of UV-derived damage severely blocked replication in most infected neurons. HSV-UV100 DNA showed a slow increase in the long amplicon PCR, attaining the levels observed in unirradiated viral infections (Fig. 6c, compare lanes 4–6 to lanes 1–3). The kinetics of restoration of the long amplicon signal from HSV UV-100 was significantly slower (indicating less efficiency) in the cultured neurons than in the Vero cells (compare the 24-h signals in Fig. 5c with the 24-h signals in Fig. 2b), although more efficient than that observed in the NGF-differentiated PC12 cells. As with the Vero cell HSV-UV 100 infections, the apparent repair of the damaged viral DNA deduced from the PCR assay could be explained by replication of minimally damaged subset of the inoculum. However, in the case of the primary CNS neurons, it is more likely that we observed real repair of existing UV damage based on the maximal replication rate observed with undamaged virus (30-fold genome copy increase; Fig. 6b).

Discussion

In this report, a method to detect HSV DNA containing UV-induced damage, and its repair, in the infected cell, in

culture is described and demonstrated. Although there is a growing number of studies showing a role for DNA repair functions in the replication of HSV and that HSV can induce and influence the host DNA repair system (Lilley et al. 2005, 2010; Mohni et al. 2010, 2011; Wilkinson and Weller 2004, 2006), our report is to our mind the first that shows directly that HSV damaged DNA can be repaired by cellular functions. Indeed, DNA repair has been repprted to be important in the replication of gamma herpes viruses as well as HSVs (Lilley et al. 2005; Mohni et al. 2011; Tarakanova et al. 2007) Although our results are also consistent with a role for the viral replication machinery in mediating optimal repair, the results also suggest that HSV pol independent repair of viral DNA in the host cell can occur. Confirmation of that repair functions are responsible for the return of the PCR ability of the damaged DNA we used in our experiments, described here, and corroboration of the role of repair in the HSV life cycle will come from use of cell mutants in which DNA repair functions have been impaired, and/or pharmacological inhibition of repair functions.

UV-induced damage may not be the most common cause of damage to the HSV genome, in the course of herpes virus pathogenesis. It was used here, primarily as a model. Having said that, in the course of natural infection, viral DNA may also be exposed to damaging agents including UV radiation from the sun and chemical mutagens.

One obstacle to directly measure DNA repair in our system, is that the specific replication of any surviving "non-damaged" DNA, or degradation of "damaged" DNA may complicate interpretation, since replication of an "undamaged" subset of DNA present in the irradiated population could appear as repaired DNA. In the studies here, we controlled for replication by using a replication defective DNA polymerase-mutant virus, HP66 (Marcy et al. 1990) and we examined degradation by quantifying viral DNA by standard real-time PCR. Therefore, the "improvements" (increase in PCR ability) in irradiated viral DNA quality, measured by large amplicon PCR in HP66-infected cells (Fig. 4b) are unlikely to be due to the presence of nascently synthesized viral genomes. However, HP66 DNA (irradiated or not) in infected cells decreased in quantity over a period of 48 h (Fig. 4a), suggesting complete degradation of approximately 50 % to 60 % of viral genomes. Previous work has shown that cellular repair pathways are important for replication of UV-irradiated virus (Muylaert and Elias 2007, 2010). Thus, CPD removal and repair is highly likely to be occurring in our system and a portion of the increased DNA quality observed at later times post-infection is due to cellular nucleotide excision repair mechanisms.

It was interesting that we observed significantly different results with NGF-differentiated PC12 cells, compared with Vero cells. Unlike Vero cells, there was no increase in viral DNA integrity using the PCR processivity assay for up to 3 days post-infection, indicating a failure of repair. One concern in Vero cells about the possibility of targeted degradation of highly damaged virions, if true, was obviously insufficient in the PC12 cells to alter the surviving pool of DNA sufficiently for us to detect the changes. Also, this was despite the fact that we used the replication competent HSV-1 strain 17+ in these studies in which replication of undamaged genomes could have increased the total DNA quality without any repair, but did not. Overall, the NGF-differentiated cells appear to take up undamaged and damaged virus, after which approximately 60 % to 80 % of the genomes are degraded, respectively. The genomes that persist appear to remain in an inactive state with respect to their ability to act as substrates for virus and host repair systems.

In a previously published report, we showed an accumulation of viral DNA damage during the quiescent HSV-1 infection in NGF-differentiated PC12 cells (Millhouse et al. 2010). We suggested that the observed phenomenon could have resulted from impaired DNA repair in these cells, or alternatively from increased DNA damage rates. The types of damage that would occur in the long term infected NGF differentiated PC12 cells is not likely to be the same as the UV-derived damage we used in this report. However, common repair pathways can be used to deal with these different damage types. With the evidence reported here, it seems possible that the HSV damage detected in the long term infected NGF-PC12 cells may involve a failure of the cellular repair systems in these cells. It may be interesting to attempt to rescue the DNA repair defect of the NGF-PC12 system to see if the introduction of specific repair activities in these cells can stimulate a more productive infection and block the formation of quiescence.

Although NGF-differentiated PC12 cells possess several neuronal characteristics, they are derived from chromaffin cells of a neuroendocrine tumor. Because we were interested to see if the repair defect was a neuronal characteristic, we wanted to examine DNA repair in primary rat neurons as a comparison. Neurons, in general, have been shown to possess less robust DNA repair activity. This has been described as a reduction in global DNA damage surveillance and greater utilization of transcription dependent DNA repair mechanisms (Fishel et al. 2007; McMurray 2005; Nouspikel and Hanawalt 2000, 2002). To examine the infection of primary neurons, we utilized hippocampal neurons derived from embryonic rats. Our results show that UV-damaged HSV-1 was repaired by 1 to 3 days post-infection with relatively small changes in the total viral DNA content. This result stands in some contrast to the NGF-PC12 system. That is, repair appears to be occurring. However, the repair occurred at a much slower, less efficient rate than was seen in Vero cells. It is possible that the DNA repair proficiency of these neurons may be related to the embryonic or hippocampal phenotype. Most neurogenesis occurs early in

development and the hippocampus is one of a few regions that can support neurogenesis later in life (Kuruba et al. 2009). Neural stem cells might be expected to be DNA repair proficient, although little is known about them and their numbers are likely to be very low.

An additional consideration is that the PC12 cells may have a serious defect in DNA repair independent of the neuronal characteristics of the cells. Undifferentiated PC12 cells are still restrictive to HSV-1 replication and the repair of UV-damaged HSV-1 was also not observed when undifferentiated cells were examined in parallel experiments similar to that shown in Fig. 5 (data not shown). If true, this could be an interesting model to study the role of DNA repair in HSV-1 replication and the establishment of viral quiescence if the DNA repair deficiency leads to quiescence. More studies are required to exam the importance of neuron age and type as a function of DNA repair activity and susceptibility to HSV-1 replication.

Acknowledgments Dr. Douglas Brenneman is thanked for help in preparation of primary neuronal cultures. This work was supported by National Institute of Health Grant NS 33768 and an appropriation from The Commonwealth of Pennsylvania through the Institute for Hepatitis and Virus Research.

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