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The HSV 1 genome in quiescently infected NGF differentiated PC12 cells can not be stimulated by HSV superinfection

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This study reports that quiescent herpes simplex virus (HSV) type 1 genomes, persisting in long-term infected nerve growth factor (NGF) differentiated PC12 cells, were not stimulated by superinfection with a HSV-1. We have previously shown that HSV-1 can establish long term, quiescent infections in NGF differentiated PC12 cells. To determine if virion associated factors or virus induced gene products could trans-activate the quiescent viral genomes, long term infected PC12 cell cultures were superinfected at a high moi (moi of 20) with a recombinant HSV 17α47/lacZ that contains the lacZ gene within the alpha 47 locus. Progeny virus and gene expression from the resident 'quiescent' viral genomes were not detected following superinfection with recombinant 17α47/lacZ. The failure to stimulate the quiescent genome appears to be related to the inability of the super infecting virus to induce any gene expression from its own genome following entry into the long term NGF treated PC12 cells. Interestingly, both primary and superinfecting viruses could be stimulated from the quiescently infected cultures following cocultivation with inducer cells. These data suggest that (i) HSV genomes in quiescently infected PC12 cells are unable to be stimulated by incoming virion associated factors and (ii) NGF differentiated PC12 cells maintained in tissue culture for longer than 3 weeks became completely refractory to viral gene expression. The possibilities that these results are reflective of populations of neural cells, *in vivo* in mouse central nervous system, which are completely refractory to virus gene expression, yet accommodating to the maintenance of viral genomes and thus favor 'latency', are discussed. *Journal of NeuroVirology* (2000) **6,** 341 – 349.

Keywords: cell culture model; HSV neuronal latency; reactivation

Introduction

Herpes Simplex Virus type 1 (HSV-1) is a neurotropic herpesvirus that causes a variety of human infections. Primary HSV-1 infection occurs mainly at the epithelia. After initiation of primary (productive) infection, HSV-1 travels by retrograde transport within the axon to the corresponding sensory nerve ganglion in which latent infection is established. In productively infected cells in culture, HSV-1 gene expression proceeds in a temporally regulated cascade (Honess and Roizman, 1974, 1975). Immediate-early (IE) genes are expressed first and can be transcribed in the absence of *de novo* viral protein synthesis. IE gene expression is followed by early (E) genes and late (L) genes, resulting in viral amplification and cell death. In contrast, latent infection is characterized by the absence of infectious virus in tissues, usually the neurons of the peripheral nervous system (reviewed by Roizman and Sears, 1996). Latently infected cells contain viral DNA and extremely limited (if any) productive-cycle gene expression yet express the latency-associated transcripts (LATs) (Stevens *et al*, 1987; Deatly *et al*, 1987; reviewed by Wagner and Bloom, 1997).

HSV can undergo sporadic reactivation from the peripheral nervous system to produce recurrent mucocutaneous lesions at/or near peripheral sites innervated by the infected ganglia (reviewed by Stevens, 1989; Fraser *et al*, 1991). Reactivation stimuli range from mechanical or pharmacological

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insults to the neuron and surrounding tissue to systemic changes in immune modulators and neurotransmitters (reviewed by Fraser and Valyi-Nagi, 1993). Superinfection has been used *in vitro* to reactivate the latent HSV genome when latency was established with the replication defective viruses or wild-type virus (Harris *et al*, 1989; Russel and Preston, 1986; Lieb *et al*, 1989; Stow and Stow, 1989; Harris and Preston, 1991; Zhu *et al*, 1990).

In previous studies, a two-cell system for the stimulation of HSV from an *in vitro* long-term tissue culture model was characterized (Su *et al*, 1999). Rat pheochromocytoma (PC12) cells, in response to NGF treatment, cease division, extend long processes that have been shown to support action potentials, and acquire many biochemical properties characteristic of the peripheral nervous system (Greene and Tischler, 1976; Greene and Rukenstein, 1989). HSV-1 can establish a long-term, quiescent infection of NGF-differentiated PC12 cells which in some respects resemble *in vivo* latency (Block *et al*, 1994; Su *et al*, 1999; Danaher *et al*, 1999). Here, we report that unlike other *in vitro* systems (Harris *et al*, 1989; Harris and Preston, 1991; Russel and Preston, 1986; Stow and Stow, 1989), superinfection with wild-type HSV-1 strain 17 failed to reactivate the quiescent HSV-1 DNA in PC12 culture. Furthermore, HSV-1 failed to replicate in long-term NGF differentiated PC12 culture, but succeeded in establishing a long-term, quiescent infection.

Results

Wild-type strain 17 and 17α47/lacZ establish long term infections of NGF differentiated PC12 cells

To determine if the quiescent HSV genome(s) in the long term infected PC12 cells could be stimulated by HSV-1 superinfection, two phenotypically distinct HSV-1 were desired. Thus, one strain could be used for establishment of the quiescent culture and the other genetically distinct strain could be used for superinfection. Therefore, a recombinant HSV-1 strain (17α47/lacZ) containing the lacZ (β-galactosidase) gene within the alpha 47 locus was constructed, as described in Materials and methods. Figure 1 shows the map of 17α47/lacZ virus. The alpha 47 locus was chosen as the site of the lacZ insertion, since alpha 47 mutants have been shown to establish and reactivate from latent infection in the murine model with kinetics similar to that of wild-type virus (Nishiyama *et al*, 1993).

Plaque purified 17α47/lacZ mutant virus was characterized as below. CV-1 monolayers were infected with wild-type virus strain 17 or mutant virus 17α47/lacZ at a moi of 0.1 followed by Sodium Citrate buffer (pH 3) treatment. The infected cells were collected and infectious virus content was determined as shown in Figure 2A. 17α47/lacZ and its parent, strain 17, grew with similar kinetics and to similar titers in productively infected CV-1 cells. Thus, the recombinant virus is not growth impaired in CV-1 cells. Next, the virus plaque phenotype was determined by the X-gal staining as described in Materials and methods. As expected, 17α47/lacZ formed blue plaques when incubated with X-gal as a result of its production of β galactosidase (Figure 2B), whereas virus strain 17 plaques remained clear. Although alpha 47 null mutants have been shown to be able to establish latent infection in animals (Nishiyama *et al*, 1993), it is necessary to determine if 17α47/lacZ can establish quiescent infections in NGF differentiated PC12 cells. Therefore, PC12 cells were differentiated with NGF for 10 days and infected with either wild- type 17 or 17α47/lacZ at a moi of 20 by methods described in Su *et al* (1999). As shown in Figure 2C, both viruses behaved similarly with respect to long term infection of NGF differentiated PC12 cells. That is, as reported previously (Su *et al*, 1999), the infectious cycle can be characterized by two phases: (i) an initial period of approximately 6 days immediately following infection, during which progeny virus can be detected in the culture medium, and (ii) a quiescent phase occurring approximately 10 days after infec-

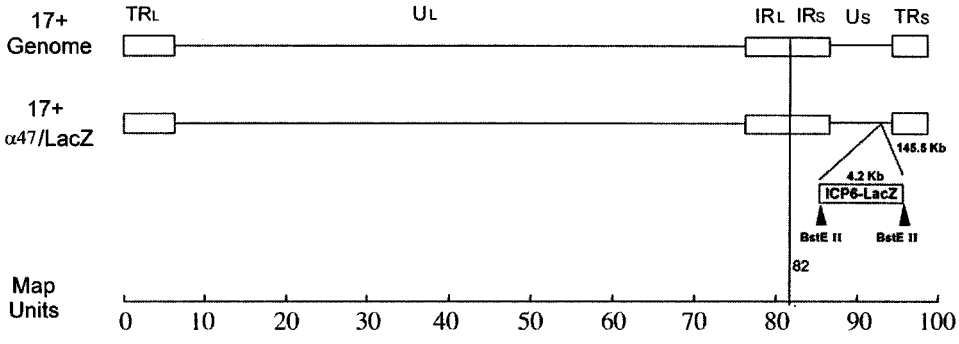

Figure 1 Schematic of the 17α47/lacZ genome. The genome of HSV-1 is a linear double-stranded DNA molecule consisting of two unique sequence regions bounded by inverted terminal repeat sequences. This structure is shown in the upper line of the Figure. The position of the 4.2 kb ICP6-LacZ DNA fragment insertion in the genome of recombinant 17α47/lacZ is shown in the middle line of the figure. The bottom line of the figure shows the map units of the genome.

tion, during which there is no detectable virus in the culture medium. Significantly, both quiescent strain 17 and 17α47/lacZ genomes can be stimulated from long-term infections by cocultivation with 'inducer' HepG2 cells. Thus, the kinetics of infection and induction were similar for both viruses and similar to that which was reported in Su *et al* (1999).

Superinfection of long-term strain 17 infected NGF differentiated PC12 cells does not reactivate quiescent HSV genomes

With genetically and phenotypically distinguishable strains of HSV in hand, it was possible to examine the ability of one strain to 'induce' the other from long-term quiescent PC12 infection. Therefore, a long-term quiescent infection of NGF differentiated PC12 culture was established using strain 17. Briefly, after PC12 cells were differentiated with NGF for 10 days, cultures were infected with strain 17 at a moi of 20. Figure 3 shows that after an initial period of virus growth, no virus was detected in the culture medium (time points after 11 days). However, HSV was present, but quiescent, in the long-term cultures, since virus could be induced following cocultivation of one set of cultures with HepG2 cells (Figure 3A). The cultures were then

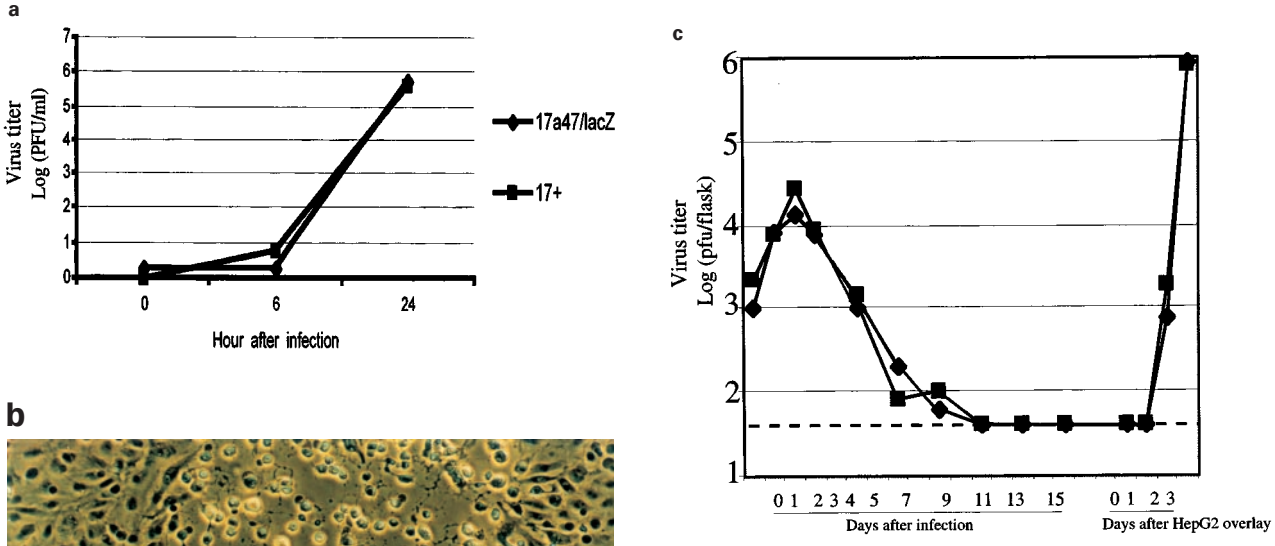

Figure 2 Characterization of mutant 17α47/lacZ. (**A**) Viral growth kinetics on CV-1 culture. CV-1 monolayers were infected with wild-type strain 17 (■–■) or mutant 17α47/lacZ (◆–◆) at a moi of 0.1 followed by Sodium Citrate Buffer (pH 3) treatment. On given time intervals, the infected cells were collected and assayed for infectious virus content by a standard plaque assay on CV-1 monolayer. (**B**) 'Blue plaque formation' of mutant virus by X-gal staining. Plaques formed by wild-type virus strain 17 (top (I)) or mutant virus 17α47/lacZ (bottom (II)) on CV1 monolayers were stained with X-gal as described in Materials and methods. (**C**) Establishment and reactivation of long-term infected PC12 cultures. Ten-day NGF differentiated PC12 cultures prepared as described in Materials and methods were infected with wild type virus strain 17 (■–■) or mutant virus 17α47/lacZ (◆–◆) at a moi of 20. Following 1 h infection at 37°C, infected cultures were treated with citrate buffer (pH 3) to activate residual inoculum. At indicated time after infection, culture medium was collected and assayed for infectious content on CV1 monolayer. On day 18 after infection, long-term infected cultures were overlaid with HepG2 cells. Culture medium was collected at 0, 1, 2, and 3 days after HepG2 overlay and assayed for infectious progeny by a standard plaque assay.

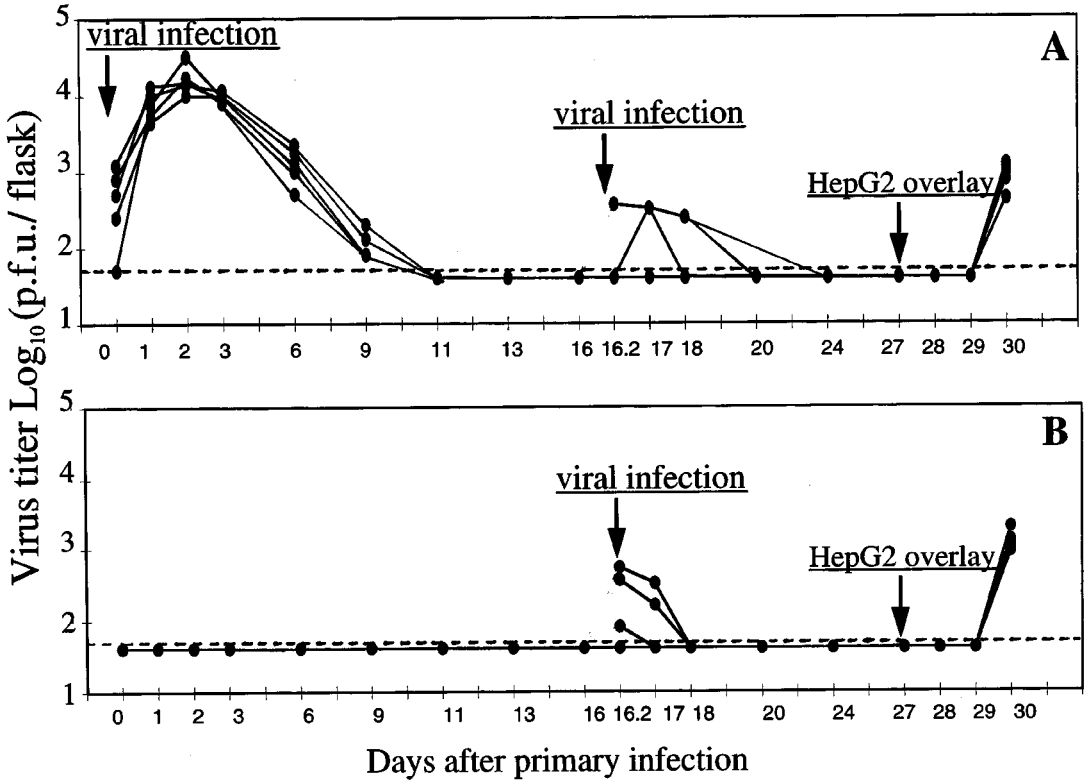

Figure 3 Superinfection failed to reactivate long-term infected PC12 culture. PC12 cells were seeded on a poly-L-ornithine coated flask and treated with NGF. On day 10 after NGF treatment, PC12 cultures (**A**) were infected with strain 17 at a moi of 20. Following 1 h of incubation at 37°C infected cultures were treated with Sodium Citrate Buffer (pH 3) as described in Materials and methods. Four hours after infection, 0.5 ml of culture medium was collected as day 0 sample. 'Control group' (**B**) shows mock infected. At the indicated times after infection culture medium were removed and the infectious content was determined by a standard plaque assay on a monolayer of CV-1 cells. On day 16 after mock (**B**) or primary infection (which is 26 days of NGF treatment), both long-term infected or mock infected cultures were infected with mutant virus 17α47/lacZ at a moi of 20, and followed by sodium citrate buffer (pH 3) treatment. Culture medium was collected as the indicated times thereafter. Culture medium was collected as the indicated times thereafter. On day 27 after primary infection (which is 37 days of NGF treatment), all cultures were co-cultivated with HepG2 cells to induce reactivation. Each value is presented as total p.f.u. in the medium of each flask. The dashed line represents the minimum level of detection by the standard plaque assay which is 40 p.f.u./flask.

considered to be 'long-term, quiescently infected' with HSV-1, as defined in Su *et al* (1999).

The ability of strain 17α47/lacZ to induce the quiescent strain 17 wild-type genome in the NGF-differentiated PC12 culture was determined as follows. On day 16 after infection with the primary virus (strain 17) (Figure 3A), which is 26 days of NGF treatment and a time at which no virus was detected in the medium, one set of cultures was 'superinfected' with 17α47/lacZ. The amount of HSV-1 (wild-type and 17α47/lacZ) in the culture medium were then followed as a function of time following superinfection. The total amount of virus in the culture medium declined steadily after infection with 17α47/lacZ. This suggests that no new viral progeny was being made. Presumably, the decline reflected the disappearance of superinfecting inoculum. Indeed, no wild-type virus (clear plaques) was detected in the culture medium following superinfection, as determined by X-gal staining of virus plaques. Since the blue plaque formed by mutant virus 17α47/lacZ is dominant over the clear plaque formed by the wild-type virus strain 17, it is possible that a low level of reactivated virus (forming clear plaques) were masked by the residual superinfected viruses (blue plaques). Therefore, a second experimental series was performed in which a long-term infection with mutant virus 17α47/lacZ was established and superinfected with wild-type virus strain 17. As expected, no blue plaques were detected by the X-gal staining when CV-1 cell monolayer was infected with the culture medium collected following superinfection with wild-type virus strain 17. Thus, superinfection with either strain 17 or mutant virus 17α47/lacZ failed to induce the resident wild-type, or mutant 17α47/lacZ viral genome into production of progeny.

Curiously, it was noticed that superinfection of the long-term infected cultures with 17α47/lacZ did not result in the usual initial 1-week period of productive virus appearance in the culture medium. That is, although infection of NGF differen-

tiated cells with either strain 17 or 17α47/lacZ had been shown to result in an initial transient period of virus production (see Figure 3A), this was not seen during superinfection (comparing virus titers shown in Figure 3A between 0 and 9 days and after superinfection, between 16 and 20 days). Rather, the added superinfecting inoculum in the culture medium of the superinfected culture steadily declined after infection without a transient period of 'progeny' production. This could have been the result of interference from the resident wild-type virus genome. Alternatively, since the superinfecting virus was introduced into the cultures after cells had been exposed to NGF for several weeks (unlike the primary virus), it is possible that the longer exposure to NGF had resulted in cultures with increased refractoriness to productive HSV infection. These possibilities were distinguished by infecting NGF differentiated cultures with strain 17 virus for the first time at a time equivalent to that used for the superinfection in the experiment shown in Figure 3A. Therefore, PC12 cells that had been differentiated with NGF for 26 days which are longer than those routinely used were infected with 17α47/lacZ virus and the amount of progeny detectable in the culture medium was followed as a function of time. Figure 3B shows that the steady decline of virus following infection is similar to that seen in the superinfection segment of the experiments shown in Figure 3A: there was a steady decline in virus titer (presumably the decline of inoculum) and an absence of a productive phase. These data suggest that the PC12 cells had become progressively more refractory to productive virus infection after an additional 16 days of NGF treatment.

Virus gene expression following superinfection

Although infectious strain 17 virus was not detected following superinfection of the long-term quiescently infected PC12 cells described in Figure 3, it is possible that virus gene expression had been induced. That is, superinfection with 17α47/lacZ may have been sufficient to stimulate gene expression from its own or the resident viral genomes, but insufficient to induce production of infectious viral progeny. This possibility was explored by testing for the appearance of key viral RNA transcripts in the long-term quiescently infected cells as a function of time followed by infection.

Briefly, as shown in Figure 4, RNA prepared from tissue cultures was reverse transcribed and amplified by polymerase chain reaction, using primers specific for either a cellular RNA, glyceradehyde-3-phosphate dehydrogenase (gapd), or viral transcripts representing the immediate early (alpha 27), early (thymidine kinase, tk) or late (gC) kinetic classes. Analysis of the amplification products by agarose gel electrophoresis shows that all kinetic classes were produced 6 h after productive infection

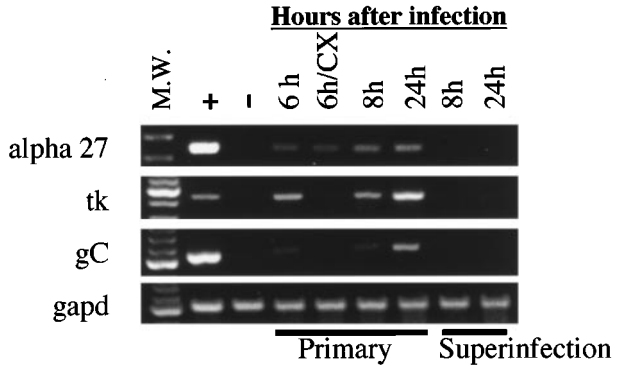

Figure 4 HSV-1 gene expression during primary and superinfection in NGF differentiated PC12 culture. PC12 cells were seeded onto poly-L-ornithine coated flask (1×10^5 cells/flask) and differentiated with NGF. On day 10 after NGF treatment, cultures were infected with HSV-1 wild-type strain 17 at a moi of 20 (as primary infection) with or without cycloheximide as indicated. After 16 days of primary infection, long-term infected cultures were superinfected with mutant virus 17α47/lacZ at a moi of 20. RNA was harvested at indicated time points after primary or superinfection and assayed for IE (alpha 27), E (tk), and L (gC) transcripts by RT–PCR. '+' represents RNA from 17 infected PC12 cultures at 18 h after infection, and '−' represents RNA harvested from mock infected PC12 cultures.

of NGF treated PC12 cells. Similarly, although all kinetic classes were present in NGF treated PC12 cells 24 h following primary infection with strain 17 (corresponding to the day 1 time period in Figure 3A), there was no detectable HSV transcripts in long-term infected NGF differentiated PC12 cells following superinfection with 17α47/lacZ (corresponding to the day 17 time period in Figure 3A). Parenthetically, cycloheximide incubation at and during the time of infection prevented the appearance of tk, and gC, but not alpha 27 transcripts during productive infection. Cycloheximide prevents protein synthesis and has been used to characterize the kinetic classes of HSV transcripts (Honess and Roizman, 1974). The presence of the immediate early transcript, alpha 27, which is transcribed in the absence of viral protein synthesis, suggests that this is an infection in which typical lytic cycle viral gene regulation in tissue culture is occurring.

The failure to detect any alpha 27, tk or gC transcripts following superinfection of long-term infected PC12 cells suggested that little or no viral gene expression occurred from both the incoming (superinfecting) virus and the resident, quiescent viral genome.

Localization of HSV DNA in the long term differentiated PC12 cells after infection

It is possible that inability of superinfecting HSV to induce viral gene expression (as shown in Figure 4) was due to a failure of the superinfecting virus to penetrate the long term NGF differentiated PC12 cells. That is, long term differentiated PC12 cells

might be refractory to the translocation of virus into the nucleus. In this case, infecting virus would be expected to be unable to reach the nuclei of the long term differentiated PC12 cells. Thus, an experiment was performed to determine if viral DNA could be recovered from the nucleus of long term differentiated PC12 cells at various times after infection. Briefly, PC12 cells, differentiated with NGF for 26 days ('long term differentiated') were infected with HSV strain 17 at a moi of 20. At 1 and 6 h after infection, cells were harvested and divided into three fractions: cytoplasmic (C), nuclear associated (Na) and nuclear (N). The amount of viral DNA associated with each fraction was determined by a PCR (see Materials and methods). As expected, cellular genomic DNA (amplified by gapd primers) was readily detected in the nuclear fraction, and not the cytoplasmic fraction (shown in Figure 5, gapd row). The Na (nuclear associated) fraction is the material recovered from the supernatant of nuclei incubated with deoxycholate and is assumed to include nuclear membrane proteins, but be depleted of the nuclear matrix and chromosomal DNA (as in Materials and methods and le Maire *et al*, 1976). Consistent with this assumption is that the majority of gapd (a marker of a host chromosomal gene) is recovered in the nucleus and not the Na. The Na fraction is included to determine if virus has penetrated the nucleus as opposed to being 'stuck' on the nuclei membrane. As shown in Figure 5, 6 h postinfection, the majority of viral DNA had reached the nucleus and not the nuclear associated fraction. Thus, the failure of HSV gene expression to occur in long term NGF differentiated PC12 cells is not due to an inability of the cells to translocate the virus into the cell nucleus.

Discussion

Previous characterization suggested the long-term, quiescently infected NGF-differentiated PC12 culture provides a feasible model to study the mechanism of HSV gene stimulation from a quiescent genome. Here we report that, superinfection of the wild-type HSV-1 strain 17 at a moi of 20 was not sufficient to stimulate previously established long-term quiescent HSV-1 genomes in the PC12 culture.

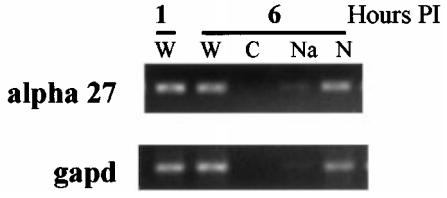

Figure 5 Sub cellular fractionation of HSV DNA following infection of long-term NGF-differentiated PC12 cell. Long-term NGF differentiated PC12 cells (26 days after NGF treatment) were infected with HSV-1 strain 17 at moi of 20 at 37°C. One and 6 h post infection (PI), cells were harvested and DNA present in intact, whole cells (W), cytoplasmic (C), nuclear associated (Na), or nuclear (N) fractions were prepared and determined as in Materials and methods. DNA of each fraction was isolated and analyzed by PCR with primers specific for HSV-1 gene alpha 27 or cellular gene gapd.

Three possibilities might account for the failure of superinfected HSV-1 strain 17 to reactivate the quiescent HSV genomes or replicate itself. First, the pre-existing virus from the primary infection might inhibit the replication of incoming virus introduced by superinfection. This is considered unlikely because the mock-infected culture was also not permissive to 'superinfecting' viruses (Figure 3B). Second, it is also possible that the incoming virus is trapped inside or outside of the cell, and failed to be uncoated or enter the nucleus. In that case, virion-associated proteins would not be expected to enter the nucleus and could not activate the viral genomes already resident. Figure 5 shows that HSV DNA does translocate into the nucleus of long term differentiated PC12 cells. Although these cells were not 'long term infected' and this was not a 'super infection', it shows that the failure of viral progeny to be produced in long term differentiated PC12 cells (see Figure 3B) was not due to a failure of viral DNA to reach the nucleus or be 'trapped' outside the cell. By analogy, it is hypothesized that viral DNA from the superinfecting virus would be capable of reaching the nucleus of a long term infected PC12 cell.

The third possibility is that the PC12 culture becomes progressively less permissive to HSV replication as a function of the time of NGF treatment. After 26 days of NGF treatment, PC12 cells were well-differentiated, neuron-like cells. Thus, the intracellular environment at day 26 is different from that at 10 days post differentiation with NGF, in which the modest replication of HSV was observed after infection. In this model, after 26 days of differentiation by NGF, PC12 cells become less permissive or refractory to HSV replication. A similar phenomenon was observed *in vivo* by Hay *et al* (1995). They showed that excised neonatal TG is more permissive to HSV replication as compared to the excised adult TG when the tissue was infected with HSV-1 *in vitro* in which the host immune system was not involved. Mitchell (1995) also reported that a differential expression pattern of HSV-1 immediate-early gene promoter exists within different neuronal phenotypes and between the same neurons in different ages of mice. An approximately 100-fold-greater number of neurons in the TG expressed ICP4 promoter activity in newborn mice compared with adults. Here, we report that the longer the treatment of NGF differentiated PC12 cells, the less permissive the cell is to HSV infection. After 26 days of differentiation, PC12 cells were no longer able to support detectable viral replication as measured by plaque

assay and RT–PCR. However, the superinfected genomes were present in the quiescent cultures since cocultivation with HepG2 cells resulted in the appearance of both primarily and superinfected viruses.

Interestingly, in this nonpermissive long-term NGF differentiated PC12 culture, virus entered and established quiescent infection (viral DNA was detected in the nuclear fraction of infected cells (Figure 5)) without permitting productive infection (Figure 3B). These observations are consistent with the findings by others (Ace *et al*, 1989; Chiocca *et al*, 1990; Ecob-Prince *et al*, 1993; Sedarati *et al*, 1993) in which it was shown that the establishment of latent infection does not require viral lytic gene expression. HSV-1 infection in long-term NGF differentiated PC12 culture may therefore represent one of 'default' pathways representing a fate that occurs after virus enters into the cells that are non-permissive for the initiation of lytic cycle gene expression.

Superinfection with virus strain 17 did not induce virus appearance in long-term infected cultures described in this report. Previous reports by others (Harris *et al*, 1989; Russel and Preston, 1986; Stow and Stow, 1989) using replication defective mutant virus showed that supernatant with either intact virus or human cytomegalovirus that expressed HSV transcription factor, ICP0, reactivated latent HSV genomes. Since both *in vivo* TG and cultured neonatal neurons are composed of a variety of cells other than neurons, it is possible that virus from the secondary infection can infect different populations of cells. The infection of other supporting cells might result in the release of various cytokines or growth factors. Orchestration of these cellular factors might also contribute to the change of intracellular physiology of latently infected neurons. This, in combination with the introduced gene, such as ICP0, to compensate the defect of mutant virus results in reactivation. However, in our essentially homogeneous population of NGF differentiated neuron-like PC12 culture that does not contain supporting cells, introducing infectious viruses was possibly not enough to excite the relatively steady quiet cells.

In summary, our data demonstrates that superinfection of HSV-1 virus alone was not enough to induce the appearance of progeny from long-term quiescent HSV-infected PC12 culture. Furthermore, the susceptibility of neurons to HSV-1 infection may vary with the age of neurons.

Materials and methods

Virus and cells
CV-1 cells (from ATCC, Rockville, MD, USA) were maintained in Eagle's minimal medium plus 5% calf serum. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Bethesda, MD, USA) supplemented with 10% fetal bovine serum. HSV-1 strain 17 and mutant 17α47/lacZ virus were prepared in CV-1 cells. Virus titer was determined by a standard plaque assay on CV-1 monolayer under methylcellulose. PC12 cells from ATCC were grown in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum ('PC12 media'). Medium used in cocultivations was the medium used for inducer cells.

Construction of recombinant virus 17α47/lacZ
The LacZ gene under the control of the HSV-1 ICP 6 promoter was excised from BPx-4Z4 (Huang *et al*, 1994) using the restriction endonuclease BSTE II. This 4.2 kb fragment was then placed into the BSTE site of the α47 locus. The vector, called α47 Pac βgal-Pac was used to make the virus 17α47/lacZ. Briefly, the vector α47 Pac βgal-Pac was co-transfected into permissive CV-1 cells along with purified HSV-1 strain 17 DNA as described in (Block *et al*, 1993). Recombinant plaques were isolated by dot-blot hybridization for the Lac Z gene and by 'beta-galactosidase staining' as described by Sanes *et al* (1986). Final identification was determined by Southern analysis.

Differentiation of PC12 cells
To differentiate PC12 cells, 1×10^5 cells were seeded on poly-L-ornithine (Sigma Inc., St. Louis, MO, USA) coated 25 cm^2 culture flask. The following day, cells were incubated in 'PC12 media' containing 100 ng/ml of 2.5 s nerve growth factor (NGF) (Collaborative Biomedical Products, Bedford, MA, USA) for 1 week. Medium was replaced every 3 days. On day 7, 20 μM of fluorodeoxyuridine (Flu) (Sigma, St. Louis, MO, USA) was added for 2–3 days to eliminate undifferentiated PC12 cells. Fresh NGF supplemented medium was replaced thereafter.

Establishment of long term HSV-1 infection
Ten days after NGF differentiation, PC12 cultures were infected with either HSV-1 strain 17 or mutant virus 17α47/lacZ, at a moi of 20 (2×10^6 p.f.u./T25 cm^2 flask). After 1 h incubation at 37°C, cultures were treated with 3 ml of Sodium Citrate buffer (Citrate 40 mM, KCl 10 mM, NaCl 135 mM, pH 3.0) for 30 s to 1 min to inactivate residual virus as previously described (Su *et al*, 1999). Buffer was removed and flasks were rinsed with PC12 medium once. After low pH treatment, cultures were incubated with fresh medium containing NGF at 37°C. To monitor for the release of HSV-1 progeny, culture medium was collected and titered on CV-1 cells by a standard plaque assay.

RNA isolation and reverse transcriptase-polymerase chain amplification (RT–PCR)
Total cellular RNA was isolated from cell culture by using the Trizol reagent (Gibco-BRL, Rockville, MD,

USA). RNA was treated with DNase I (Boehringer Mannheim) to eliminate DNA contamination. One μg of RNA was denatured with glyoxal and subjected to electrophoresis on a 1.0% agarose gel to determine the quality of RNA. cDNA was synthesized from 0.5 μg of total RNA isolated from each T25 flask in a total 20 μl volume with the SuperScript Pre-amplification System (Gibco-BRL, Rockville, MD, USA) according to manufacturer's instruction. PCR amplifications were done with 2.5 U of Taq polymerase (Fisher Scientific, Pittsburgh, PA, USA), 0.2 μM of primers and 1 μl of cDNA in a 50 μl of reaction. Primer sequences were described previously (Su *et al*, 1999). PCR amplifications consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min. PCR products were resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining.

Beta-galactosidase staining of HSV plaques
The method for detection of β-galactosidase enzyme was described by Sanes *et al* (1986) and modified as follows. Virus plaques were rinsed with 150 mM NaCl, 15 mM Na phosphate, pH 7.3 (PBS) and then fixed for 5 min at 4°C in 2% formaldehyde plus 0.2% gluteraldehyde in PBS. The plaques were then washed with PBS and overlaid with a histochemical reaction mixture containing 1 mg/ml 4-Cl-5-Br-3-indolyl-β-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. After 2 h incubation at 37°C, the plaques were counted under the microscope.

Subcellular fractionation
PC12 cells were scraped off into culture media, collected, and washed with $1 \times$ PBS. Cell pellet was resuspended in 0.5% NP-40 in NSB lysis buffer (10 mM Tris, pH 7.4, 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM PMSF, 5% sucrose), homogenized by approximately 20 strokes of Dounce Homogenizer. The degree of cell lysis was monitored by trypan blue stain to ensure at least 95% of cell lysis. Nuclei were pelleted down at $750 \times$ g for 5 min at 4°C. Supernatant was further centrifuged at $17\,000 \times$ g to pull down the viral particles in the cytoplasmic fraction. Nuclear pellet was then resuspended 0.1% deoxycholate in NSB to strip off the nuclear membrane protein (le Maire *et al*, 1976) or the viral articles associated outside of the nuclear membrane. After 10 min of incubation at room temperature, deoxycholate treated nuclei were pelleted at $750 \times$ g for 5 min at RT as a nuclear fraction. Deoxycholate treated supernatant was then centrifuged at $17\,000 \times$ g for 50 min to collect viral particles which were associated with nuclear membrane as a nuclear associated fraction (Na).

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