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Heat stress activates production of herpes simplex virus type 1 from quiescently infected neurally differentiated PC12 cells

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We have previously described a novel *in vitro* model of a non-productive herpes simplex virus type 1 (HSV-1) infection in neurally differentiated (Nd)-PC12 cells that allows for inducible virus replication upon forskolin treatment. In this study, we further characterized the quiescent state of infection and examined the ability of heat stress (HS) to induce virus from this non-productive state. These studies demonstrated that (i) the quiescent state is characterized by the absence of cell-associated virus, capsids, and viral antigens; (ii) HS (43°C, 3 h) efficiently activated virus from quiescently infected Nd-PC12 (QIF-PC12) cells; (iii) the rate of virus production was significantly greater following HS than forskolin treatment, and the rates of both were dependent on MOI; (iv) forskolin and HS appeared to affect pathways of viral activation from a quiescent state as they did not enhance viral growth in Nd-PC12 cells; (v) viral α4 gene and host HSP72 gene transcription were rapidly induced in QIF-PC12 as soon as 3 h post-HS initiation; (vi) induction of the viral α27 gene followed that of representative β and γ genes, U_L30 and U_L18, respectively, and (vii) HS induced asynchronous HSV-1 replication from QIF-PC12 cells with 1 : 400 to 1 : 22 000 positive foci detected as rapid as 24 h post-induction when established at MOIs of 30 and 3, respectively. These findings provide evidence that α4 may be involved in the switch from quiescence to productive infection. Furthermore, this model has the potential to advance our understanding of how HS initiates the HSV-1 productive cycle from a cryptic viral genome.

Keywords: cell culture model; heat stress; herpes simplex virus; viral latency; reactivation

Introduction

Herpes simplex virus type 1 (HSV-1) is a neuroepitheliotropic DNA virus capable of lytic and latent infection. During the typical primary infection, virus multiplies in orofacial epithelium, invades local nerve endings, and travels by retrograde axonal transport (Johnson, 1964; Wildy, 1976) to neuronal cell bodies in regional sensory ganglia (Hill *et al*, 1972; Stevens and Cook, 1971). Although some sensory neurons appear to permit productive infection and are destroyed, most sensory neurons survive the infection and retain the virus in a latent state. During latency, infectious virus and viral antigens are not detected and virus evades immune surveillance (Baringer and Swoveland, 1973; Valyi-Nagy *et al*, 1991). A prominent characteristic of HSV-1 is its ability to reactivate from latency and cause viral shedding and/or recurrent disease. Although extensive knowledge of the lytic cycle of the virus exists, factors that regulate viral latency and reactivation remain largely unknown.

HSV-1 reactivation involves either a spontaneous or inducible switch from viral latency to an active replicative state within the neuron. Many stimuli that induce reactivation *in vivo* and *in vitro* have been identified, however the molecular target of these agents has not been well characterized. In experimental animals and humans, inducible reactivation has been reported to occur following

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axonal injury (Carton and Kilbourne, 1952; McLennan and Darby, 1980; Walz *et al*, 1974), traumatization of peripheral tissues (Hill *et al*, 1978; Valyi-Nagy *et al*, 1991), tooth extraction (Openshaw, 1982), iontophoresis with epinephrine, timolol and dexamethasone (Gordon *et al*, 1986; Hardwicke and Schaffer, 1997; Hill *et al*, 1987; Kwon *et al*, 1981), administration of prostaglandins (Blyth *et al*, 1976), IL-6 (Kriesel *et al*, 1997), ultraviolet light radiation (UVR) (Laycock *et al*, 1991; Perna *et al*, 1987; Stanberry, 1989; Wheeler, 1975), cadmium (Fawl and Roizman, 1993), transient hyperthermia (Halford *et al*, 1996; Moriya *et al*, 1994; Sawtell and Thompson, 1992) and immunosuppression with cyclophosphamide and prednisolone (Hurd and Robinson, 1977). Of these factors, heat stress (HS) has a record of efficient reactivation of HSV-1 from latently infected sensory ganglionic neurons *in vivo* (Sawtell and Thompson, 1992, 1997; Sawtell *et al*, 1998), and *ex vivo* (Halford *et al*, 1996; Moriya *et al*, 1994).

Recently, we reported that neurally differentiated (Nd)-PC12 cells can harbor HSV-1 in a quiescent, yet reversible state (Danaher *et al*, 1999). These quiescently infected Nd-PC12 cultures (QIF-PC12) demonstrate forskolin-inducible virus production in a high percentage (50–100%) of cultures for up to 8 weeks after infection, whereas mock-induced cultures maintain the quiescent viral state in the majority of infected cultures. In this study we used the inductive effects of HS to demonstrate that: (i) HS activates HSV-1 replication from QIF-PC12 cells; (ii) HS is a more efficient inducer of virus production than forskolin; (iii) the proportion of cultures that activate virus production is dependent upon the multiplicity of infection (MOI); (iv) activation is a rare event limited to only a minority of the QIF-PC12 cell population (1 : 400–1 : 22 000) when established at MOIs of 30 and 3, respectively, and measured 24 h post-induction, and (v) expression of HSV-1 productive genes (i.e., $\alpha 0$, $\alpha 4$, $\alpha 27$, U_L30 and U_L18) switched from undetectable or very low levels to detectable or elevated levels in a sequential order by 24 h post-HS with $\alpha 4$ induction occurring as early as 3 h after initiation of HS. Commensurate with the above findings, viral antigen and infectious virus became detectable in cells as early as 24 h post-HS. These findings suggest that HS is an efficient agent for the induction of HSV-1 replication from a non-productive state in QIF-PC12 cells.

Results

Heat stress activates HSV-1 production from QIF-PC12 cells

A variety of factors have been shown to reactivate virus from *in vitro* and *in vivo* HSV-1 latency models. To determine if some of these factors provide similar activation of HSV-1 from QIF-PC12 cells, non-productive cultures were subjected to forskolin treatment, NGF-withdrawal, or heat stress (HS, 43°C, 3 h). QIF-PC12 cultures were established by infecting NGF-differentiated (Nd)-PC12 cells with HSV-1 strain 17^+ at MOI of approximately 1.0 on days 7–12 post-plating in the presence of ACV as previously described (Danaher *et al*, 1999). Cultures were evaluated for virus production throughout the study using culture supernatants in plaque forming assays on Vero cells. After ACV treatment for 9–10 days, cultures were maintained in the absence of ACV for 7–9 days prior to treatments, with media changes every 2–3 days. Virus was infrequently detected (1.6% of cultures) during the 7–9 days following ACV withdrawal. Subsequently, non-productive cultures were treated as indicated and monitored for virus production. The results of three separate activation experiments are shown in Table 1. On average, HS and forskolin resulted in virus production from 97 and 92% of cultures, respect-ively. In contrast, mock- and DMSO-induced cultures resulted in virus production from 5.2 and 4.3% of cultures, respectively. These data indicate that HS activates

Table 1. Activation of virus production from QIF-PC12 cells

Treatment	*Number of cultures producing virus*			
	Experiment #1	*Experiment #2*	*Experiment #3*	*Total (mean)*
Pre-treat	0/48 (0%)	1/72 (1.4%)	2/72 (2.8%)	3/192 (1.6%)
Mock	0/12 (0%)	2/11 (18%)	1/35 (2.9%)	3/58 (5.2%)
Forskolin	12/12 (100%)	12/12 (100%)	9/12 (75%)	33/36 (92%)
DMSO		1/12 (8.3%)	0/11 (0%)	1/23 (4.3%)
−NGF		2/12 (17%)		2/12 (17%)
+Serum, −NGF		0/12 (0%)		0/12 (0%)
+Serum		0/12 (0%)		0/12 (0%)
Heat stress	24/24 (100%)		11/12 (92%)	35/36 (97%)

Cells were infected with 17^+ at MOI of approximately 1 on days 7–9 post plating. ACV (100 μM) was maintained for 9–10 days post infection. Cultures were treated on days 7–9 post ACV withdrawal. Virus production was monitored for 8 days following treatment by the direct plaque assay from culture supernatants. All cultures were maintained in serum free media supplemented with NGF unless indicated.

HSV-1 production from QIF-PC12 cells and that DMSO (i.e., carrier of forskolin) treatment does not induce virus production above background levels.

In our previous report (Danaher *et al*, 1999) we demonstrated that NGF-withdrawal had no effect on virus production from non-productive QIF-PC12 cells. This is in direct contrast to the findings of others (Block *et al*, 1994; Wilcox and Johnson, 1987; 1988). Because our previous study employed only serum free conditions, the ability of NGF withdrawal to induce virus production in the presence of serum was addressed. In experiment 2 of Table 1, NGF withdrawal from cultures maintained in the absence of serum (−NGF) failed to activate virus production in a significant number of cultures compared with mock-induced control cultures. Similarly, cultures treated with serum both in the presence and absence of NGF remained free of detectable levels of virus throughout the assay period. These data are consistent with our previous observations that NGF withdrawal does not induce significant levels of virus production (Danaher *et al*, 1999) and further indicate that serum-induced resumption of cell division does not facilitate virus production from QIF-PC12 cells.

Activation of HSV-1 by heat stress is dependent on MOI

To determine whether the degree of HSV-1 activation from QIF-PC12 cells was associated with MOI, Nd-PC12 cell cultures were infected with HSV-1 strain 17^+ at MOI's of 15, 1.5 and 0.15 and maintained in ACV for 10 days to establish a quiescent infection. Ten days after ACV removal, cultures harboring a quiescent infection were subjected to forskolin (50 μM), HS or mock-treatment. Cultures were monitored for virus production throughout the study as described above. During the early quiescent phase (i.e., 1−10 days following ACV withdrawal) spontaneous activation of virus occurred in less than 5% of cultures established at all three MOIs (data not shown). Figure 1 shows the cumulative percent of cultures that produced virus following mock-, forskolin-, and HS-treatment of the QIF-PC12 cultures. The activation rate induced by HS was 2−3 days faster than that induced by forskolin treatment at all MOIs examined. At MOI of 15, all treated cultures produced virus. However at MOIs of 1.5 or less, HS treatment activated virus replication in 20−40% more cultures than forskolin treatment. Virus activation in mock-induced QIF-PC12 control cultures was significantly less ($P<0.005$, χ^2) occurring in only 9% (2/22), 4% (1/24) and 0% (0/24), (MOI=15, 1.5, and 0.15, respectively). These data indicate that virus was produced more rapidly and efficiently from QIF-PC12 cells exposed to HS than forskolin, and that the kinetics and efficiency of HSV-1 activation in the cultures are dependent on MOI.

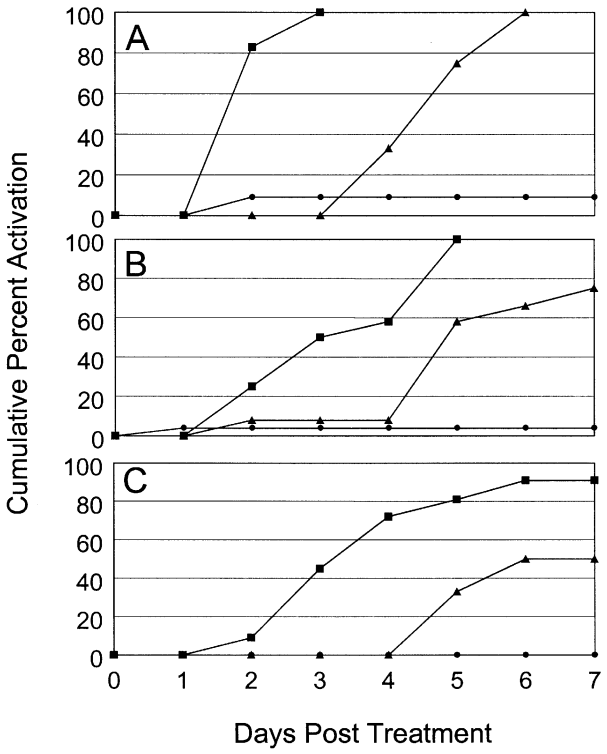

Figure 1 The frequency of HSV-1 activation from QIF-PC12 cells depends on MOI and inducing agent. PC12 cells were plated at 1.1×10^5 cells/3.8 cm^2 well as described in Materials and methods. QIF-PC12 cultures were established with strain 17^+ at MOIs of 15 (**A**), 1.5 (**B**), and 0.15 (**C**) and treated on day 10 post-ACV withdrawal (20 days p.i.). Mock (●), 50 μM forskolin (▲), and HS (43°C, 3 h) (■) treated cultures were evaluated for virus production throughout the assay by the direct plaque assay. The treatment and mock-treatment groups consisted of 11 to 12 and 22 to 24 cultures respectively, at each MOI.

Non-productive QIF-PC12 cultures are free of cell-associated virions and rapidly produce virus following heat stress

To further examine the kinetics of HS-induced virus activation, whole cell lysates were analyzed for virus during the quiescent and activation phases. QIF-PC12 cultures were established at an MOI of 3. On days 3 and 7 post-ACV withdrawal, individual 12-well cultures were harvested, freeze thawed 3×, and the entire cell lysates were screened for infectious particles by direct plaque assay. Virus was not detected from any of these lysates (data not shown). Virus also was not detected from QIF-PC12 culture supernatants during the first week post-ACV withdrawal. Figure 2 shows that following HS-treatment on day 7 post-ACV withdrawal (17 days p.i.), virus was detected in the majority of cell culture lysates (7/12) and supernatants (15/24) within 1 and 2 days, respectively. This compares with virus detection in 8.3% (5/60) of supernatants by day 5 post-treatment of mock-induced cultures. Of the mock-induced cultures free of detectable

virus in supernatants, virus also was not detected in lysates (0/12) on day 5 post-treatment. Long term QIF-PC12 cell cultures 32 days p.i (i.e., 22 days post-ACV withdrawal) maintained the ability to produce virus following an activation signal, as HSV-1 was produced in 58% (7/12) of culture supernatants subjected to HS compared with 5.6% (1/18) of mock-HS treated cultures by day 4 post-HS.

To determine whether QIF-PC12 cell cultures contained viral particles during the non-productive state, parallel cultures were established at an MOI of 30 and monitored by transmission electron microscopy. Virions were readily detectable in the cytoplasm and juxtaposed to the nuclear membrane at 24 h p.i., however virus was not detected at the time of ACV withdrawal (i.e., initiation of the quiescent phase). In contrast, 50% of the QIF-PC12 cells contained nucleocapsids or virus in the cytoplasm within 72 h after HS (data not shown). These results indicate that (1) the block in the viral lytic cycle conferring quiescence in QIF-PC12 cells is not associated with viral uncoating or capsid maturation, (2) cell associated viruses are not present in QIF-PC12, and (3) activation from quiescence is more rapid than is indicated by assays of supernatants, as virus is detectable at least 24 h sooner when assayed by whole cell lysates.

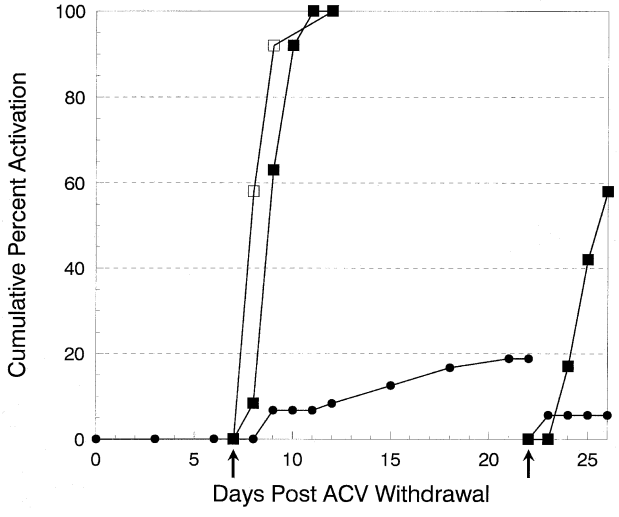

Figure 2 Virus activation from short-term and long-term QIF-PC12 cell cultures as measured by supernatants and whole cell lysates. QIF-PC12 cultures were established with strain 17^+ at MOI of 3 and subjected to HS as indicated by arrows. Percentage of cultures producing virus as detected in mock-(●) and HS-treated cultures (■) using culture supernatants, and in HS-treated cultures (□) using whole cell lysates by direct plaque assay. Virus was not detected in whole cell lysates derived from infected cultures on days 3 and 7 post-ACV withdrawal and day 5 post-mock treatment (data not shown). The experiment was initiated with 120 QIF-PC12 cultures. Thirty-six and 60 cultures were HS- and mock-treated, respectively on day 7 post-ACV withdrawal. Twelve and 18 cultures were HS- and mock-treated, respectively on day 22 post-ACV withdrawal.

Activation occurs in minority of QIF-PC12 cells

Immunocytochemistry (ICC) was used to determine whether QIF-PC12 cells expressed viral antigens and the percentage of cells that activate virus following HS. QIF-PC12 cell cultures were prepared in parallel with those described above and infected with HSV-1 strain 17^+ at MOI of 3 and MOI of 30. On day 22 p.i., QIF-PC12 cell cultures were induced to activate virus by HS. Cells were examined for viral antigens by ICC at 0, 24 and 48 h post-HS. Immunoreactive staining for HSV-1 antigen was evident at 24 h post-HS (Figure 3A). In cultures infected at MOI of 3, one immunoreactive focus was evident per 22 000 cells at 24 h post-HS and increased to 1 in 2200 at 48 h post-HS. In cultures infected at MOI of 30, one immunoreactive focus was seen per 400 cells at 24 h post-HS, and increased to one in 40 cells at 48 h post-HS. In contrast, immunostaining was not present in six independent mock-induced QIF-PC12 cell cultures (Figure 3B) and mock-infected PC12 cell cultures

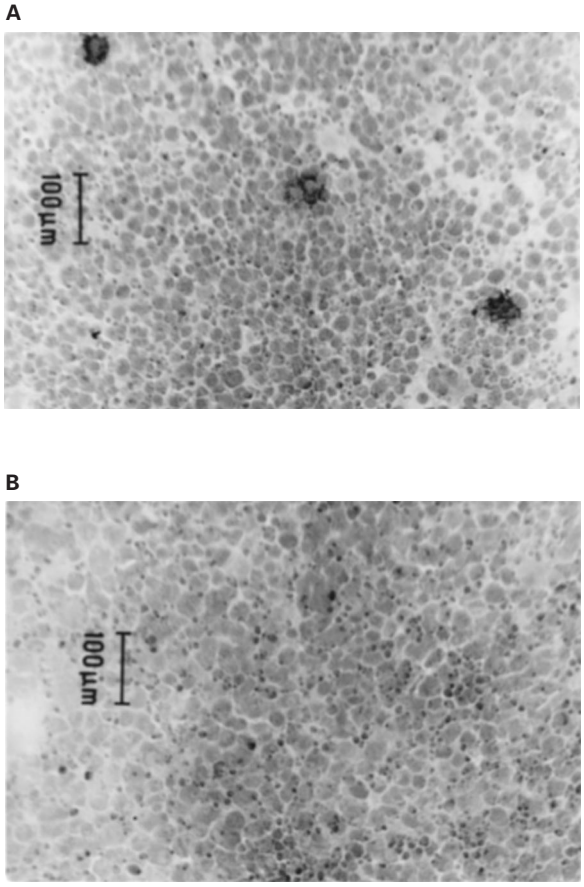

Figure 3 Detection of viral antigens following heat stress induced activation from QIF-PC12. QIF-PC12 cultures were established with strain 17^+ at an MOI of 3. On day 22 p.i., QIF-PC12 cell cultures were induced with heat stress (43°C, 3 h) to activate virus. Cells were examined for viral antigens by ICC at 24 h p.i. as described in the Materials and methods. Cells subjected to HS (**A**) or mock treatment (**B**).

(data not shown). These findings indicate that viral antigens are not expressed during the quiescent phase of QIF-PC12 cells, and the cells activating HSV-1 represent a minority of the infected cell population.

Gene expression during activation from QIF-PC12

We previously reported (Danaher *et al*, 1999) that the level of lytic HSV-1 gene expression drops dramatically during the establishment phase of the quiescent infection in Nd-PC12 cells, to levels that will not support productive viral growth, and continue to decline following the withdrawal of ACV. To examine the rate of gene induction from quiescence following HS treatment, QIF-PC12 cultures were established in 35 mm dishes in parallel with those described for Figure 1. The plating density was increased fourfold to allow for sufficient RNA to be used in RT–PCR assays and quiescent infections were established at an MOI of 0.5. RNA was isolated from mock-treated QIF-PC12 cell cultures immediately following treatment, 48 h post mock-treatment, and from heat-stressed (43°C for 3 h) cultures at 0, 2, 8, 12, 24 and 38 h post-recovery at 37°C. RT–PCR was performed with gene specific primers and analyzed by Southern blot analysis for HSV-1 or ethidium bromide staining for host genes. Figure 4 shows representative RT–PCR products of the genes examined and Table 2 shows the average cDNA copies derived from multiple data points. The sensitivity in all cases was greater than 0.1 target sequences per cell, with a range of 0.0058–0.093 represented by U_L18 and α27, respectively. On day 20 p.i., prior to heat induction, RT–PCR product was not detected for α0, α4, U_L30, or the non-stable 8.3 kb latency associated transcript (LAT), whereas low levels of product were detected for α27 and U_L18 in QIF-PC12 cultures. The most prominent RT–PCR product during the quiescent phase was the stable intron of the LAT (S-LAT) transcript. These levels of expression are consistent with our previous report for quiescent cultures (Danaher *et al*, 1999) and indicate that these levels are insufficient for the virus to resume its lytic cycle.

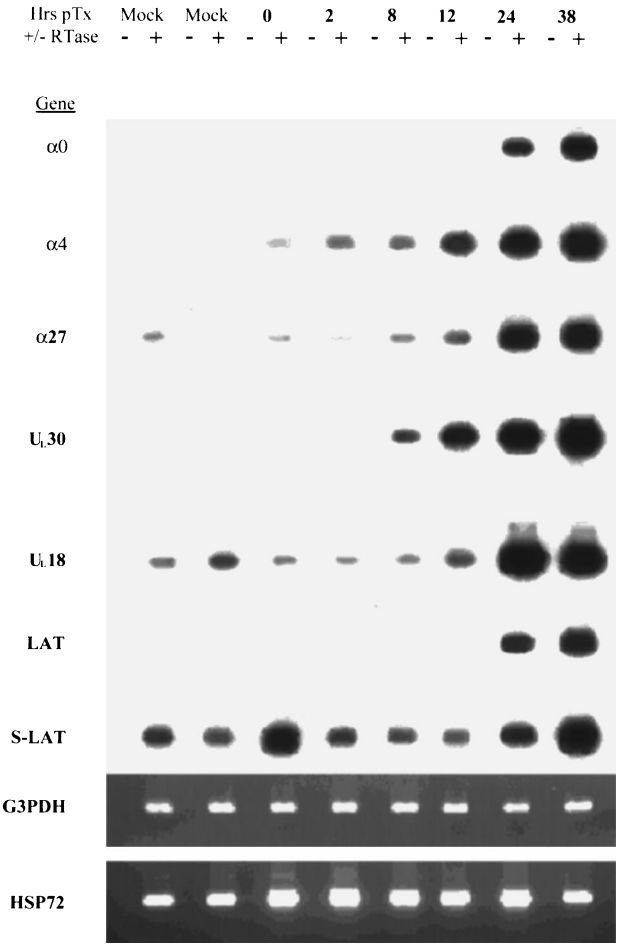

Figure 4 RT–PCR analysis gene induction following heat stress treatment. PC12 cells were plated at 1.1×10^6 cells/9.6 cm^2 dish, differentiated with NGF and maintained in parallel with those described in Figure 1. QIF-PC12 cultures were established at an MOI of 0.5. On day 10 post-ACV withdrawal (20 days p.i.) cultures were subjected to heat stress (43°C, 3 h). Analysis by RT–PCR was performed at the times indicated as described in the Materials and methods. pTx=post termination of treatment. RTase=reverse transcriptase.

As shown in Table 2, following HS-treatment the levels of the host G3PDH gene transcript remained at the level of mock-induced cultures at all time points examined, indicating that equivalent efficiencies of cDNA synthesis occurred. In contrast, the host HSP72 gene expression was induced 50-fold by the end of the 3 h HS-treatment period (0 h), peaked at approximately 150-fold induction by 2 h, and returned to control levels by 38 h post-HS. Furthermore, HS resulted in induction of all viral genes examined within 24 h of HS, in a pattern resembling a sequential order type mechanism.

Of the viral genes examined, HS induced α4 most rapidly. Expression of α4 was characterized by the absence of detectable levels of RNA from cultures at the initiation of HS, and the consistent detection of transcript by the end of the 3 h HS-treatment period. In contrast, α4 transcripts were not detected in duplicate QIF-PC12 cell cultures at 48 h post mock-induction (data not shown). This indicates that α4 transcription initiates rapidly during HS treatment, and increased at least twofold above the limit of detection within the first 3 h following initiation of HS. After the initial induction of α4, transcript levels continued to increase throughout the assay period, achieving levels of at least 5- and 47-fold above the limit of detection by 8 and 24 h post-HS, respectively. In contrast, levels of α27 transcript did not increase above the levels of mock-induced control samples until 12 h following the termination of HS. After this time its levels continued to increase as much as 80-fold compared to control

Table 2 Gene induction during heat-stress induced reactivation from QIF-PC12 cells

Gene	*Average cDNA copies/cell at indicated times post treatment (+/− s.d.)*								
	Mock #1	*Mock #2*	*0 h*	*2 h*	*8 h*	*12 h*	*24 h*	*38 h*	*Limit*
α0	nd	nd	nd	nd	nd	nd	0.56[a] (0.80)	0.40[b] (0.334)	0.059
α4	nd	nd	0.17[b] (0.15)	0.17 (0.027)	0.48[b] (0.48)	0.54 (0.26)	3.9 (0.84)	12 (7.2)	0.082
α27	0.11 (0.017)	0.10 (0.003)	0.10 (0.007)	0.099 (0.007)	0.11 (0.023)	0.28 (0.041)	9.3 (5.6)	9.1 (1.2)	0.093
U_L30	nd	nd	nd	nd	0.041[a] (0.058)	0.038[a] (0.053)	3.4 (2.2)	0.97 (0.66)	0.033
U_L18	0.0088[b] (0.009)	0.0062[a] (0.008)	0.0081[b] (0.008)	<[a]	0.021 (0.002)	0.15 (0.005)	1.8 (1.1)	3.8 (0.60)	0.0058
LAT	nd	nd	<[c]	nd	nd	<[c]	0.32 (0.045)	1.7 (0.42)	0.018
S-LAT	0.34 (0.061)	0.26 (0.068)	1.4 (0.28)	0.45 (0.068)	0.23 (0.069)	0.30 (0.069)	2.8 (1.4)	3.3 (1.0)	0.018
G3PDH	24 (2.5)	19 (1.6)	19 (1.7)	21 (2.1)	20 (2.0)	17 (1.4)	16 (1.9)	22 (0.97)	
HSP72	58 (14)	87 (19)	4600 (1700)	13000 (3100)	1600 (420)	1000 (300)	2400 (790)	69 (17)	

cDNA samples were subjected to amplification by the PCR and analyzed by Southern blot in duplicate for the non-stable LAT (LAT) and in triplicate for all other HSV-1 genes. All samples derived from parallel cultures treated on day 20 pi. Times indicate recovery time at 37°C following heat-stress. [a]Product detected in 1/3 samples. [b]Product detected in 2/3 samples. [c]Product detected in 1/2 samples. <Indicates that the average cDNA copies was less than the limit of detection [Limit]. Nd, not detected. S-LAT refers to stable latency associated transcripts. Limit, limit of detection [target sequences/cell].

levels, by 24 h post-HS. α0 transcripts, which like α4 were not detected in mock-induced control cultures, were first detected at 24 h post-HS. The representative β gene, U_L30, transcript was not detected in mock-induced control cultures but was detected at 8 h post-HS. By 24 h post-HS, the level of U_L30 rose 100-fold above the level of detection. Transcription of the representative γ gene, U_L18, was induced twofold by 8 h following HS treatment and increased to more than 200 times the level of mock-induced control cultures by 24 h post-HS.

The level of the S-LAT intron was enhanced greater than fourfold by the end of HS treatment compared to mock-induced controls and returned to control levels following 2 h of incubation at 37°C. Since the level of the non-stable LAT (LAT) transcript remained undetectable until 24 h post-HS, the early enhancement of S-LAT appears to be the result of altered stabilities of RNA during HS treatment rather than LAT transcription. These data indicate that both the HSV-1 α4 gene and the host HSP72 gene respond rapidly to heat stress, whereas expression of α27, α0, and LAT remain constant or undetectable until after β (i.e., U_L30), and γ (i.e., U_L18) gene induction has occurred.

Discussion

Data presented in this report extend our earlier findings concerning the basic cellular and molecular characteristics of the quiescent and activation stages of the QIF-PC12 cell culture model. In this model a quiescent state between HSV-1 and Nd-PC12 is established with the transient use of ACV. Although this state is established with the transient use of ACV, inhibitory levels are not present in QIF-PC12 cell cultures at the time of virus induction as measured by viral growth curves (data not shown). The maintenance phase of the quiescent infection is characterized by the absence of virions, capsids and viral antigens, nevertheless virus can be rapidly induced by physical (i.e., HS) and chemical (i.e., forskolin) means. Although HS and forskolin induce virus production from quiescence, neither agent enhanced viral growth rates or yields in Nd-PC12 cells (data not shown). This indicates that these agents act at the level of induction, rather than altering cell permissivity. Furthermore, the absence of viral structures prior to induction suggests that the block in the lytic pathway is between viral uncoating and capsid maturation. Thus, QIF-PC12 cells allow for the study of important stages of virus activation from a non-productive state that is maintained in the absence of inhibitory viral growth conditions that are necessary with other cell culture models (Hammer *et al*, 1981; Harris and Preston, 1991; O'Neill *et al*, 1972; Russell *et al*, 1987; Scheck *et al*, 1989; Wigdahl *et al*, 1982).

The inductive effects of forskolin and HS demonstrate similarities and differences in QIF-PC12 cells and with latency models. While both forskolin and HS induced HSV-1 activation in QIF-PC12 cells, HS resulted in a greater rate and frequency of activation. The rapid production of virus following HS correlates with the kinetics of HS-induced virus

reactivation from animal and *in vitro* explant models induced by heat stress (Halford *et al*, 1996; Moriya *et al*, 1994; Sawtell and Thompson, 1992). The data are consistent with evidence that the kinetics of HSV reactivation varies by induction protocols (Halford *et al*, 1996; Sawtell and Thompson, 1992; Shimeld *et al*, 1990; Smith *et al*, 1992). The contrasting properties of HS and forskolin suggest that more than one pathway leads to activation of virus, or they act at different stages of a common pathway. In addition, the absence of immune based cells in our cultures indicates that induced activation of HSV-1 within a neural cell can occur independent of immunological signals (Kriesel *et al*, 1997). Thus, this model may allow for the distinction between the role of neural and accessory cells in viral activation induced by physical and chemical agents *in vitro*.

HS activated the production of virus from QIF-PC12 cells established at low MOI (i.e., <1). Thus, QIF-PC12 cells established at low MOI provide sufficient viral load for activation studies and activation characteristics similar to reactivation in explant HSV-1 latency models (Halford *et al*, 1996). It is also apparent that QIF-PC12 cells, established at high MOI, yield only moderate increases in the level of spontaneous activation, and that these levels are low enough not to preclude our ability to study activation events in this cell culture model. The data clearly demonstrate that increasing the MOI enhances the activation efficiency of the model. These findings suggest that a direct correlation of MOI, viral genome copies per infected cells and activation efficiency exists in this model, as has been reported in animal models of latency (Sawtell *et al*, 1998).

Our analysis of gene expression by RT–PCR permitted the detection of as few as nine to 140 target sequences per 1500 cells, yet gene products of $\alpha 0$, $\alpha 4$, U_L30 could not be detected during the quiescent infection (i.e., day 20 p.i.). In contrast, low levels of $\alpha 27$ and U_L18 were detected during the quiescent infection. The presence of low levels of some α and β gene RNAs during quiescence is not surprising as Kramer and Coen (1995) have reported similar findings in murine trigeminal ganglia latently infected with HSV-1. While the importance of this finding remains to be determined, our previous report (Danaher *et al*, 1999) indicates that these levels diminish with time.

During the response to HS (Figure 4), the immediate early HSV-1 $\alpha 4$ and host HSP72 genes demonstrated induced expression within the first 3 h of treatment (0 h recovery time). Unlike the expression of the host HSP72 gene, which peaked at 2 h post-HS and dropped to control levels by 38 h post-HS, the level of $\alpha 4$ transcription continually increased throughout the assay period. The level of transcription of other HSV-1 genes was not enhanced during the HS treatment period but increased at various times following recovery at 37°C. Representatives of the β and γ class genes (i.e., U_L30 and U_L18, respectively) were induced by 8 h following HS, whereas increased expression of $\alpha 27$ was not detected until 12 h post-HS. Both the levels of the non-stable LAT and $\alpha 0$ remained below the levels of detection until 24 h post-HS. Since the level of $\alpha 0$ message prior to HS treatment was below the level of detection, it is unknown whether this gene was induced following HS, and remained below the level of detection, or was not induced until late in the activation cycle. It is important to note, a similar pattern of gene induction following explant-induced virus reactivation has been reported. In the murine explant model, detectable levels of γ gene expression immediately follow $\alpha 4$ and precede $\alpha 27$ gene expression (Devi-Rao *et al*, 1994), and induction of $\alpha 27$ (Halford *et al*, 1996) and $\alpha 0$ (Minagawa *et al*, 1994) are not detectable until 24 h post-explant. These data implicate $\alpha 4$ in the early role of coordinating reactivation from latency and argue against an early role of $\alpha 27$, $\alpha 0$ and the non-stable LAT in this process. In contrast, $\alpha 27$ has been detected as early as 4 h following epinephrine-induced reactivation in rabbit models (Bloom *et al*, 1994), and $\alpha 4$ has not always been detected rapidly post-explant of latently infected murine ganglia (Minagawa *et al*, 1994). Different levels of assay sensitivity, inducing agents, host cells and spontaneous reactivation rates may contribute to the altered detection patterns noted.

Consistent with the reactivation findings of *in vivo* models (Sawtell and Thompson, 1992), our ICC data indicate that viral activation occurs in a minority of QIF-PC12 cells. Based on the frequency of reactivation of 1 in 22 000 at an MOI of 3, it can be predicted that less than one in 10^5 of the QIF-cell population activates virus replication at a MOI of 0.5 at 24 h post-HS. This may explain why relatively low levels of HSV-1 gene expression were demonstrated during the inductive phase of activation (Table 2). Whether the level of $\alpha 4$ gene activity detected at 0 h recovery from HS (Table 2) is derived from the cells predicted to activate, or additional cells that initiate the activation event but subsequently abort the process is not known.

In summary, this report presents data that extends our knowledge of the characteristics and utility of the PC12 cell model for study of quiescent HSV-1 infection. We have shown that the quiescent state lacks infectious particles, yet has the capacity to produce infectious virus following chemical and physical stress. The rapid induction of virus from quiescence by heat treatment provides the opportunity for analysis of early molecular events of activation in a well characterized neuronal cell line. The identification of $\alpha 4$ gene induction in response to HS lays the groundwork for examining this gene's regulation and contribution to activation via heat

stress induction *in vitro*. The use of the QIF-PC12 cell culture system may allow for the characterization of the molecular mechanisms involving HS initiation of the productive cycle from a cryptic viral genome, regardless of the model's similarities with latency *in vivo*.

Materials and methods

Virus and cells
Rat pheochromocytoma (PC12) and Vero cells were grown as previously described (Danaher *et al*, 1999). HSV-1 strain 17^+ was a kind gift of N Fraser (Wistar Institute, Philadelphia, PA, USA). Viral stocks were prepared in Vero cells and maintained at -85°C. Virus production was determined using supernatants from infected cultures in a direct plaque assay (DPA) on monolayers of Vero cells as previously described (Miller and Smith, 1991).

Morphologic differentiation
For studies involving differentiated PC12, cells were maintained in RPMI 1640 supplemented with 0.1% bovine serum albumin, fraction V (BSA) and 50 ng/ml of 2.5S mouse nerve growth factor (NGF) (Becton Dickinson) (maintenance media) throughout the studies, unless indicated, beginning on the day of plating. PC12 were plated, following two rinses with RPMI 1640 containing 0.1% BSA and dissociation by passage through a 22-gauge needle, on 12-well tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) coated with rat tail collagen type 1 (Becton Dickinson) at 1.1×10^5 cells/well in maintenance media. Collagen was applied as recommended by the supplier. For RNA studies parallel cultures were established in 6-well dishes at 1.1×10^6 cells/well. Following 4 days of differentiation in maintenance media, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (RPMI+serum), and 50 ng/ml NGF for 2 days. The following day cultures received maintenance media supplemented with 100 μM acycloguanosine (ACV) purchased from Sigma (St. Louis, MO, USA). Morphologic differentiation was confirmed by microscopic visualization of dendritic processes. Media was changed every 3 days unless indicated.

Establishment of a quiescent infection
Neurally-differentiated PC12 cells (Nd-PC12) were infected with virus at a MOI of 1–3 unless otherwise indicated, in a volume of 0.4 or 1.0 ml/well, for 12- and 6-well plates, respectively, without agitation at the indicated multiplicity of infection (MOI) overnight at 37°C. ACV was maintained in the medium from 1 day prior to infection to the indicated time post-infection (p.i.). After ACV withdrawal, a quiescent state (i.e, free of detectable infectious virus in culture supernatants) was maintained for at least 7 days prior to induction.

Activation stimuli
HSV-1 quiescently infected PC12 cells (QIF-PC12), that were free of detectable infectious virus, were subjected to heat stress (43°C for 3 h in an incubator), RPMI+serum, maintenance media supplemented with 50 μM forskolin (Sigma) prepared in DMSO (Sigma) as recommended by Huang *et al*. (1982), or NGF withdrawal. NGF withdrawal was facilitated by the addition of rabbit anti-NGF (Sigma), as recommended by the manufacturer.

RNA isolation and cDNA synthesis
Cells were harvested for RNA isolation on the days indicated by scraping cell sheets in PBS followed by centrifugation at 2000 r.p.m. for 5 min. Cell pellets were washed with PBS, pelleted as above and stored at -85°C. RNA was isolated using the Qiagen RNeasy Total RNA Kit as recommended by the manufacturer. Three μg of each RNA sample was treated with DNAse I (Boehringer Mannheim, Indianapolis, IN, USA) in 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl, and 10 units of RNase-free DNAse I in a final volume of 20 μl at room temperature for 15 min. DNAse I was inactivated, following the addition of 2 μl EDTA (25 mM, pH 8.0), by heating at 65°C for 10 min. cDNA was generated with SuperScript II reverse transcriptase (Gibco BRL) and random primers (Gibco BRL) as recommended by the supplier. cDNA was stored at -20°C until use.

RT–PCR
In addition to the primers previously described (Danaher *et al*, 1999) primer sets for the host HSP72 gene and the 5′ end of the non-stable LAT, described by Abe *et al* (1995) and Devi-Rao *et al* (1994), respectively, were used. PCR reactions containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 mM of each primer of the indicated set, 2.5 units Taq DNA polymerase (Gibco BRL) and cDNA derived from 1500 cell equivalents were prepared on ice. Samples were denatured at 94°C for 3 min followed by 35 cycles (94°C for 45 s, 55°C for 30 s, 72°C for 90 s, for HSV and host HSP72 target sequences, and 26 cycles for the host glyceraldehyde-3-phosphate dehydrogenase (G3PDH) target. Controls containing tenfold dilutions of HSV-1 DNA ranging from 1.25 fg to 12.5 pg (7.5 to 75 000 genome equivalents) or twofold dilutions of PC12 cellular DNA ranging from 0.5 ng to 4.0 ng (155–1240 haploid genome equivalents), for G3PDH, and 10–80 ng (3100–24 800 haploid genome equivalents), for HSP72, were used. Experimental and control samples were performed in triplicate unless indicated. The specificity of viral specific PCR products

were verified by Southern blot analysis as described previously (Danaher *et al*, 1999). In brief, equivalent amounts of PCR products were applied to 2.0% agarose gels and electrophoresed in 0.5 × TBE at 50 V for 45–60 min. DNA was transferred to Magna NT nylon membranes (Micron Separation Inc., Westboro, MA, USA) by capillary action following denaturation and neutralization. Membranes were hybridized to digoxigenin-labeled probes, internal to the RT–PCR products, and detected using DIG Oligonucleotides 3′ end labeling and DIG Luminescent Detection Kits (Boehringer Mannheim). The autoradiographs were scanned using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed with GPTools software (Biophotonics, Ann Arbor, MN, USA).

Immunocytochemistry
For immunocytochemical studies, QIF-PC12 cells were harvested, suspended in 200 μl of media containing 2% fetal bovine serum and cytocentrifuged on to glass microscopic slides at 500 r.p.m. for 5 min at room temperature. After drying, cells were fixed with 1% (phosphate-buffered) paraformaldehyde for 10 min. Specimens were incubated overnight at 4°C with 1 : 200 dilution of rabbit polyclonal anti-HSV-1 (Biomeda Corp, Foster City, CA, USA) in Tris-buffered saline containing 5% goat serum and 0.25% Triton-X. The antibody detected strain 17^+ in all experiments performed (data not shown). Indirect immunocytochemistry was performed by the avidin-biotin-peroxidase complex method as specified by the manufacturer (Vectastain; Vector laboratories, Burlingame, CA, USA). The chromogen diaminobenzidine was used as the substrate in the presence of nickel to produce a purple-black precipitate in positively stained cells. Photomicrographs were prepared with an Olympus Vanox AH-3 microscope.

Transmission electron microscopy
PC12 cells were prepared for transmission electron microscopy (TEM) as described by Gao *et al* (1997). Briefly, cells were fixed in 3% gluteraldehyde followed by 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Eponate-12 resin (Ted Pella, Redding, CA, USA) on days 1, 2, 5, 10, 20 p.i. and at 24, 48 and 72 h post-HS. After polymerization, ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with an H-7110/STEM electron microscope (Hitachi Inc., Tokyo, Japan) at 75 kV.

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