JOURNAL OF VIROLOGY, Apr. 2006, p. 3360–3368 Vol. 80, No. 7
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.7.3360–3368.2006

ICP0 Is Not Required for Efficient Stress-Induced Reactivation of Herpes Simplex Virus Type 1 from Cultured Quiescently Infected Neuronal Cells

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Received 23 October 2005/Accepted 19 January 2006

Viral genes sufficient and required for herpes simplex virus type 1 (HSV-1) reactivation were identified using neuronally differentiated PC12 cells (ND-PC12 cells) in which quiescent infections with wild-type and recombinant strains were established. In this model, the expression of ICP0, VP16, and ICP4 from adenovirus vectors was sufficient to reactivate strains 17^+ and KOS. The transactivators induced similar levels of reactivation with KOS; however, 17^+ responded more efficiently to ICP0. To identify viral transactivators required for reactivation, we examined quiescently infected PC12 cell cultures (QIF-PC12 cell cultures) established with HSV-1 deletion mutants R7910 (ΔICP0), KD6 (ΔICP4), and *in*1814, a virus containing an insertion mutation in VP16. Although growth of these mutant viruses was impaired in ND-PC12 cells, R7910 and *in*1814 reactivated at levels equivalent to or better than their respective parental controls following stress (i.e., heat or forskolin) treatment. After treatment with trichostatin A, *in*1814 and 17^+ reactivated efficiently, whereas the F strain and R7910 reactivated inefficiently. In contrast, KD6 failed to reactivate. In experiments with the recombinant KM100, which contains the *in*1814 mutation in VP16 and the n212 mutation in ICP0, spontaneous and stress-induced reactivation was observed. However, two strains, V422 and KM110, which lack the acidic activation domain of VP16, did not reactivate above low spontaneous levels after stress. These results demonstrate that in QIF-PC12 cells ICP0 is not required for efficient reactivation of HSV-1, the acidic activation domain of VP16 is essential for stress-induced HSV-1 reactivation, and HSV-1 reactivation is modulated uniquely by different treatment constraints and phenotypes.

Herpes simplex virus type 1 (HSV-1) is a large enveloped DNA virus that causes considerable human illness. Key to its pathogenic success is the dual life cycle of the virus in its animal host. Following the primary infection in epithelium, HSV-1 enters sensory ganglionic neurons, wherein the virus exists long-term in a cryptic and mostly silent state (42, 83, 84). During its latent existence within the neuron, HSV-1 DNA forms an episome (73) and the majority of the genome is transcriptionally inactive (27, 84; also reviewed in reference 68). In this quiescent or latent state, the virus evades immune surveillance yet remains poised to resume its productive cycle when appropriate signals are received.

Increasing evidence suggests that the viral transactivator genes VP16, ICP0, and ICP4 may be important in mediating regulatory steps between latency and reactivation (32, 33, 38, 50, 53, 87). This premise is based on *trans* expression studies in vitro and ex vivo (33, 37, 97) as well as latency studies involving HSV-1 recombinants deleted or mutated in the viral transactivators. In the latter, reactivation efficiency is altered compared with that of their parental wild-type strains (7, 34, 50, 89). However, recombinant viruses possessing mutations in VP16, ICP0, and ICP4 are also deficient in growth to various degrees (2, 3, 10, 18, 67, 90). Thus, it is difficult to establish equivalent levels of latency with such recombinants (7, 35, 92) and interpret the results of these studies. As a result, investigators have devised novel approaches for studying HSV reactivation.

One such approach uses neuronally differentiated PC12 cells (ND-PC12 cells), derived from rat pheochromocytoma, in which quiescent HSV-1 infections are established (4, 11–14, 55, 85, 86). This model offers several advantages for the study of the molecular events involved in reactivation within individual neuronal cells. These include the ability to (i) maintain viral quiescence for weeks in the absence of antiviral agents and induce reactivation with diverse physiological and biological stimuli (11–14), (ii) focus on neuronal events per se, without other cell types, such as immune cells and their products, contributing to the regulation of viral replication and infected cell survival (6, 64, 88), and (iii) effectively utilize mutant viruses in reactivation studies by directly infecting cultured neuronal cells (55). The latter feature allows for the establishment of quiescent infections in neuronal cultures that contain equivalent viral genome copy numbers of mutant and wild-type viruses.

To identify viral gene products sufficient and required for HSV-1 reactivation, we examined the phenotype of VP16, ICP0, and ICP4 in quiescently infected PC12 cells (QIF-PC12 cells) (11–14, 55). Our analyses revealed that the expression of the viral transactivors (i.e., VP16, ICP0, or ICP4) in *trans* resulted in HSV-1 reactivation. However, efficient stress-inducible reactivation in vitro was dependent on VP16 and ICP4 but not ICP0. In addition, we present evidence suggesting that the acidic activation domain of VP16 contributes to the ability

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of HSV-1 to reactivate above mock-treatment (i.e., spontaneous) levels after stress.

MATERIALS AND METHODS

Cells and viruses. Rat pheochromocytoma (PC12) and African green monkey kidney (Vero) cells were grown as previously described (12). U2OS cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). 293, 911, and E5 cells (17) were grown in Dulbecco's minimum essential medium supplemented with 10% FBS. All cells were obtained from the American Type Culture Collection (Rockville, Md.), except E5 cells, which were provided by D. Bloom, University of Florida, Gainesville. All cells were maintained at 37°C in 5% CO_2.

HSV-1 strains were obtained from the following persons: 17^+ and *in*1814 from N. Fraser of the Wistar Institute, Philadelphia; KOS from R. Thompson, University of Cincinnati; F and R7910 from B. Roizman, University of Chicago; KD6 from D. Bloom, University of Florida, Gainesville; and KM100, KM110, and V422 from J. Smiley, University of Alberta, Edmonton. Viral stocks of wild-type HSV-1 strains were propagated in Vero cells and virus titers were determined as described previously (12). R7910 was propagated in U2OS cells, and virus titers were determined. KD6 was propagated in E5 cells, and virus titers were determined. The remaining HSV-1 recombinants were propagated in U2OS cells supplemented with N,N′-hexamethylenebisacetamide (HMBA), and virus titers were determined. Recombinant adenovirus (Ad) vectors (with the E1 region deleted) Ad.T-OBP, Ad.T-gD, Ad.T-ICP0, Ad.T-ICP4, Ad.T-VP16, Ad.T-LAT2.6, Ad.T-null, Ad.T-n212, Ad.T-VP16Δ, and Ad.C-rtTA were provided by W. Halford of Montana State University, Bozeman (33). Ad.C-rtTA constitutively expresses the reverse tetracycline-regulated transactivator (rtTA). Ad.T-gD, Ad.T-OBP, Ad.T-ICP0, Ad.T-ICP4, Ad.T-VP16, and Ad.T-LAT2.6 are inducible tetracycline-responsive (TRE) vectors. In the presence of doxycycline (DOX) and Ad.C-rtTA, Ad.T-gD expresses HSV-1 glycoprotein D, Ad.T-OBP expresses the HSV-1 origin binding protein (OBP), Ad.T-ICP0 expresses the promiscuous transactivator ICP0, Ad.T-ICP4 expresses ICP4, Ad.T-VP16 expresses VP16, and Ad.T-LAT2.6 expresses the first 2.6 kb of LAT, thus spanning the splice donor site that defines the 5′ end of the 2.0-kb LAT intron (33). Ad.T-n212 and Ad.T-VP16Δ contain mutations in the ICP0 and VP16 encoding regions, respectively, and are described elsewhere (33). Adenovirus vectors were propagated in 293 cells at 0.03 PFU/cell in Dulbecco's modified Eagle's medium supplemented with 10% FBS and incubated at 37°C for 5 to 6 days. Adenovirus vector-infected cells were pelleted by low-speed centrifugation, resuspended, and subjected to freeze-thawing and centrifugation to produce clarified lysates. Virus titers ranged from 8×10^8 to 1×10^{11} as determined by plaque assay with 911 cells. All viral stocks were maintained at −85°C.

Neuronal differentiation. PC12 cells were dissociated by passage through a 22-gauge needle and plated in RPMI 1640 containing 0.1% fraction V bovine serum albumin (BSA) in tissue culture dishes coated with rat tail collagen type 1 (Becton Dickinson, Franklin Lakes, NJ) at 2.2×10^5 cells/ml. Cells were differentiated and maintained in RPMI 1640 supplemented with 0.1% BSA and 50 ng/ml of 2.5S mouse nerve growth factor (NGF) (Becton Dickinson) (maintenance medium) beginning on the day of plating. Morphological differentiation was confirmed by microscopic visualization of dendritic processes.

Viral growth analysis. PC12 cells were seeded at 5.5×10^5 cells/35-mm-diameter collagen-coated dish, neuronally differentiated for 1 week, and then inoculated at a multiplicity of infection (MOI) of 5 for 2 h at 37°C. Cells were rinsed with citrate buffer, pH 3, and twice with maintenance medium and then incubated in maintenance medium at 37°C. Cultures were frozen at various times postinoculation, and virus titers were determined in duplicate by plaque assay with U2OS cells in medium supplemented with HMBA, except KD6, whose virus titer was determined with E5 cells.

Establishment of a quiescent infection and reactivation of HSV-1. Quiescent HSV-1 infections were established in ND-PC12 cells in 12-well plates at MOIs of 5 to 10 as previously described (12, 55) (Fig. 1). Briefly, ND-PC12 cells were cultured in maintenance medium for 4 days postplating. On days 4 through 6, cells were cultured in maintenance medium supplemented with 10% horse serum and 5% FBS instead of BSA. On day 6, cells were cultured in maintenance medium supplemented with 100 μM acycloguanosine (ACV) (Sigma, St. Louis, MO) and then inoculated with virus the following day. Infected cells were cultured in maintenance medium supplemented with ACV for 10 days. After ACV withdrawal, a quiescent state was maintained for 7 days prior to experimental stress treatment. In all QIF-PC12 cell experiments, cultures free of detectable infectious virus in culture supernatants were stress treated to activate virus on day 17 after infection, unless otherwise indicated, by subjecting the cells to heat stress (HS) (43°C for 3 h) or to maintenance medium supplemented with 50 μM forskolin (Sigma) or 400 ng/ml trichostatin A (TSA) (Sigma) as previously described (11–13). In transinduction assays, quiescently infected cultures were superinfected with adenoviruses at an MOI of 40 in maintenance medium containing Ad.C-rtTA (MOI of 10) for 2 h on day 17 after HSV-1 infection, and then maintenance medium containing DOX (10 μM) was placed on cultures for 2 days. QIF-PC12 cell culture supernatants were monitored for virus production by plaque-forming assay performed on monolayers of Vero, E5, or U2OS cells as previously described (55).

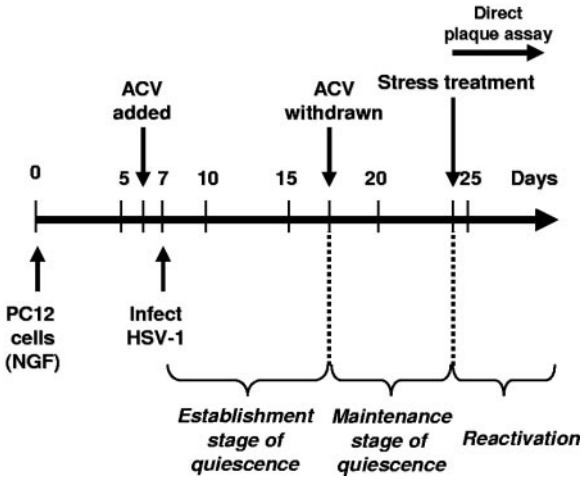

FIG. 1. Outline of QIF-PC12 cell culture model. PC12 cells are neuronally differentiated in medium containing NGF throughout the experiment. ACV is added to the culture medium on day 6, cells are inoculated with HSV-1 the following day, and ACV is maintained in the culture medium through day 16 postplating. Thereafter, HSV-1 remains in a quiescent state in the absence of ACV, until stress treatment on day 17 after infection or spontaneous reactivation occurs.

RESULTS

HSV-1 reactivation from QIF-PC12 cells after *trans* expression of ICP0, VP16, and ICP4. Viral transactivators expressed in *trans* reactivate HSV-1 from latently infected fibroblasts and trigeminal ganglion (TG) tissue cultures (33, 37, 43, 97). Thus, we wished to determine if *trans* expression of the major transactivators would stimulate reactivation from ND-PC12 cells that contained quiescent HSV-1 genomes. QIF-PC12 cell cultures were established with HSV-1, and induction was performed by superinfecting the cells with TRE-regulated adenovirus vectors that express ICP0, ICP4, or VP16. HSV-1 strains KOS and 17^+ were used to assess the contribution of differing reactivation phenotypes (41, 76).

In 17^+-established quiescently infected cultures, HSV-1 reactivated more efficiently after superinfection with Ad.T-ICP0 than after superinfection with Ad.T-VP16 or Ad.T-ICP4 (Fig. 2A). In contrast, reactivation levels from quiescently infected cultures established with KOS were similar after superinfection with Ad.T-ICP0, Ad.T-VP16, and Ad.T-ICP4 (Fig. 2B). Reactivation was not detected above background for any culture superinfected with control Ad.T-OBP (0%), Ad.T-LAT2.6 (3%), or Ad.T-gD (3%). Also, superinfection with Ad.T-n212 or Ad.T-VP16Δ, adenoviruses that encode nonfunctional mutant forms of ICP0 and VP16, respectively, did not result in reactivation from quiescently infected cultures established with 17^+ (data not shown). These data indicate that (i) the expression of ICP0, VP16, and ICP4 in *trans* is

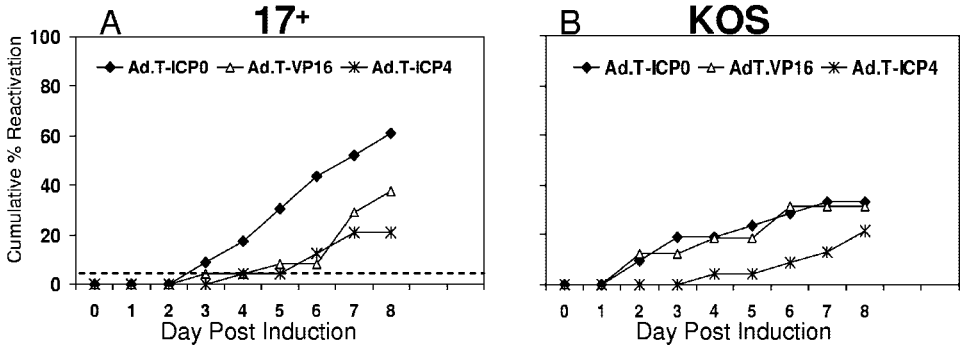

FIG. 2. *trans* expression of ICP0, ICP4, and VP16 from adenovirus vectors reactivates HSV-1 from QIF-PC12 cells. Quiescent infections were established in ND-PC12 cells with strains 17^+ (A) and KOS (B) at an MOI of 5. On day 17 after infection, cultures free of detectable infectious virus were superinfected with the indicated adenoviruses (MOI of 40) and Ad.C-rtTA (MOI of 10) in the presence of 10 μM DOX. Reactivation was determined from plaque assays of culture supernatants performed on Vero cells. Reactivation frequencies are based on two independent experiments ($n = 24$ cultures per group per experiment). Standard deviations for all points were less than 15%. The dashed line indicates the level of spontaneous reactivation for 17^+-established cultures mock treated with maintenance medium. In KOS-established quiescently infected cultures, spontaneous reactivation was not observed.

sufficient to reactivate HSV-1 from neuronal cells that harbor virus in a quiescent state and (ii) the most dramatic difference between 17^+ and KOS was in response to the expression of ICP0 in QIF-PC12 cells.

Efficient HSV-1 reactivation from QIF-PC12 cells occurs in the absence of ICP0 but requires ICP4. Since the major HSV-1 transactivators were sufficient to stimulate reactivation from QIF-PC12 cells, it was important to determine if these gene products were required for spontaneous or stress-induced reactivation. We began by comparing the reactivation properties of the ICP0 deletion mutant R7910 with those of its parent F strain (46) and the reactivation properties of the ICP4 deletion mutant KD6 with those of its parent KOS strain (20). Quiescently infected cultures were established, and cultures lacking detectable virus in the supernatant medium were induced with forskolin, HS, or TSA. In these experiments, R7910 and F reactivated efficiently in response to HS (88% and 67%, respectively), moderately after forskolin (54% and 29%, respectively), but poorly following TSA treatment (8% and 21%, respectively) (Fig. 3). In fact, R7910 reactivated more efficiently than its parental strain after HS and forskolin treatments (Fig. 3A and B). However, R7910 did not reactivate significantly above mock-treatment levels after TSA treatment (Fig. 3C). R7910 maintained its ability to reactivate efficiently in long-term quiescently infected cultures that were subjected to heat on day 30 after infection (data not shown). KD6, in contrast, failed to reactivate after treatment with any of the inducing agents tested. In quantitative real-time PCR assays, the viral DNA copy numbers were virtually equivalent for cultures established with the mutants and their parental strains (data not shown), indicating that viral genome copy number did not contribute to the observed effect. In addition, R7910 and KD6 reactivated from QIF-PC12 cultures after superinfection with TRE-regulated adenovirus vectors that express ICP0 and ICP4, respectively (data not shown). Together, these data indicate that in QIF-PC12 cells (i) ICP0 is not required for efficient HSV-1 reactivation, (ii) ICP4 is required for the detection of HSV-1 reactivation when assays involve viral growth, and (iii) heat and forskolin induce HSV-1 reactivation through processes that are distinct from that of TSA.

***in*1814 reactivates efficiently from QIF-PC12 cells.** The contrasting requirements of ICP4 and ICP0 for HSV-1 reactivation led us to examine VP16 for its requirement in reactivation. QIF-PC12 cell cultures were established with the HSV-1 mutant *in*1814 or its parental strain. *in*1814 contains a 12-bp insertion that disrupts the transactivating domain of VP16. Briefly, cultures established with these strains were maintained as quiescent cultures that were free of detectable virus until day 17 after infection, when cultures were subjected to HS, forskolin, or TSA treatment. Figure 4 shows that *in*1814 and 17^+ reactivated efficiently in response to all three stressors. Similar to R7910 and F strains, *in*1814 reactivated better than 17^+ after HS and forskolin treatments. However, unlike R7910, F, or KOS, QIF-PC12 cell cultures established with 17^+ and *in*1814 were efficiently induced with TSA. These data indicate that *in*1814 retains the ability to reactivate from neuronal cells that harbor virus in a quiescent state and that the genetic background of 17^+ appears to confer a reactivation phenotype different from that of strain F in this model.

HSV-1 containing the *in*1814 mutation retains its ability to reactivate in the absence of ICP0. Since HSV-1 lacking normal function of either VP16 or ICP0 reactivated efficiently from QIF-PC12 cells, we analyzed whether a mutation in both genes would impair reactivation efficiency. To investigate this, we established QIF-PC12 cell cultures with KM100, an HSV-1 recombinant containing the n212 mutation in ICP0 and the *in*1814 mutation in VP16. Inasmuch as KM100 is derived from KOS and 17^+ backgrounds, independent control cultures were established with both wild-type strains. All cultures established a quiescent state and were induced with HS, forskolin, or TSA. Figure 5 shows that KM100 reactivated at levels lower than that of 17^+ but as well as the KOS strain. KM100 reactivated more efficiently after HS treatment (Fig. 5A) than after forskolin (Fig. 5B) or TSA (Fig. 5C) treatment. Spontaneous reactivation from KM100 after mock treatment was similar to wild-type levels. These data indicate that HSV-1 containing the *in*1814 mutation retains its ability to reactivate spontaneously and in response to stress in the absence of ICP0.

Acidic activation domain of VP16 is required for the detection of stress-induced reactivation of HSV-1. The observation

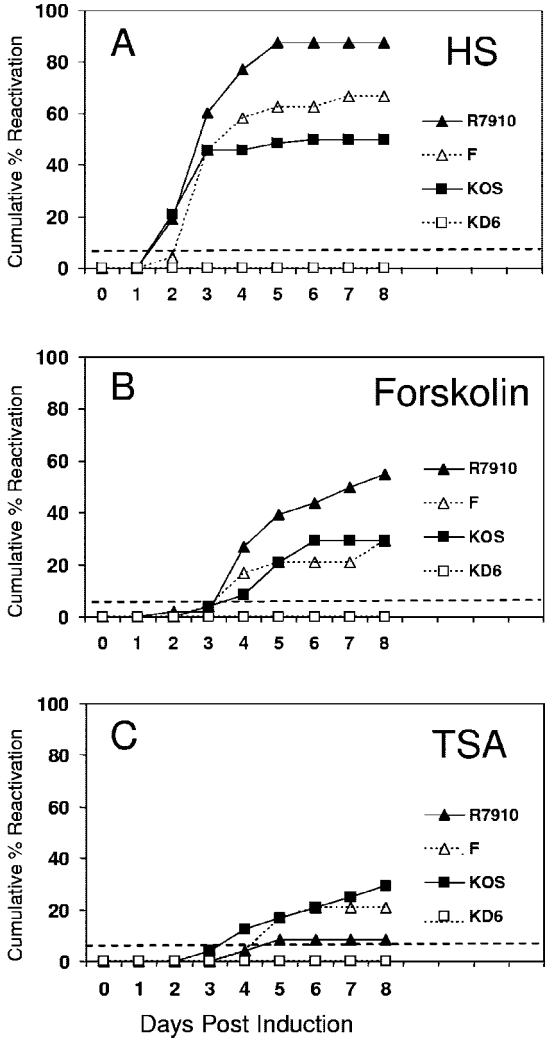

FIG. 3. Reactivation kinetics of HSV-1 recombinants lacking ICP0 or ICP4 in QIF-PC12 cells. Quiescent infections were established with indicated strains in 12-well plates. On day 17 after infection, cultures free of detectable infectious virus were subjected to reactivation stimuli. Reactivation was monitored for 8 days by plaque assay of culture supernatants performed on U2OS cells and E5 cells. Results are plotted based on three independent sets of infections. Standard deviations for all points were less than 15%. The levels of spontaneous reactivation for R7910- and F-established cultures mock treated with maintenance medium were equivalent and are indicated by the horizontal dashed lines. Spontaneous reactivation was not detected in KOS- or KD6-established quiescently infected cultures.

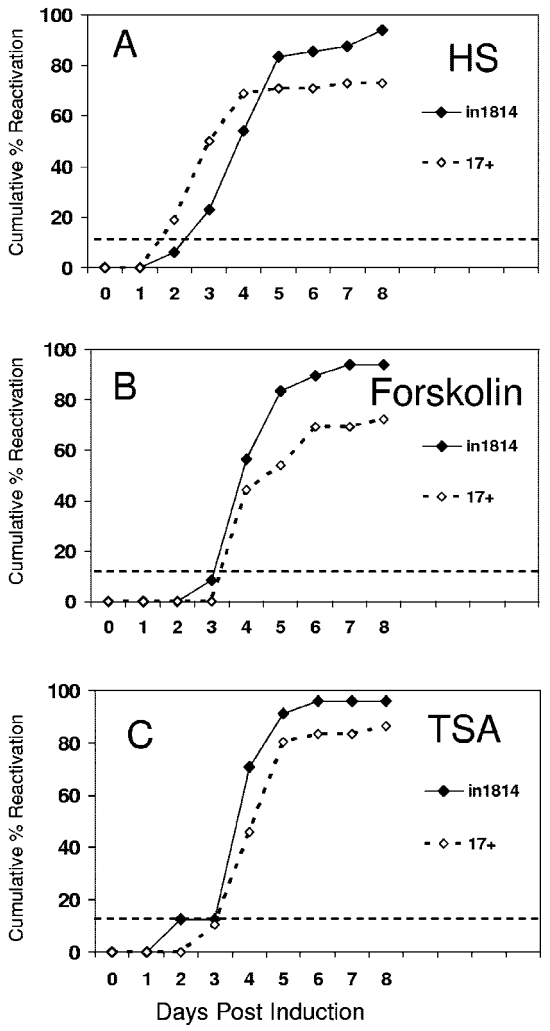

FIG. 4. Reactivation kinetics of *in*1814 and 17^+ in QIF-PC12 cells. Quiescent infections were established with indicated strains in 12-well plates. Cultures were induced to reactivate with indicated stressors ($n = 24$ per treatment group) and monitored on U2OS cells, as described in the legend for Fig. 2. The horizontal dashed lines indicate the levels of spontaneous reactivation for *in*1814-established cultures mock treated with maintenance medium. The level of spontaneous reactivation was 3.4% for mock-treated 17^+-established quiescently infected cultures. Standard deviations for all points were less than or equal to 15%. Similar results were seen with duplicate experiments.

that *in*1814 reactivated efficiently from QIF-PC12 cells is consistent with the possibility that either the *in*1814 mutation retains residual transactivating ability or reactivation does not require VP16 transactivation function (59). To test the role of VP16's activation domain in reactivation, recombinant V422 was used in our model. V422 is truncated in the acidic domain of VP16 after residue 422 and lacks transactivation function (49, 81). Briefly, QIF-PC12 cultures were established with V422 or its parental strain. After treatment with HS, forskolin, or TSA, low levels of reactivation similar to spontaneous reactivation were observed (Fig. 6). In contrast to *in*1814, V422 was incapable of reactivating after stress treatment above the low levels seen with mock-treated cultures. Similar results were observed when V422-established cultures were induced on day 31 after infection (data not shown). In a separate experiment, KM110, which contains both the n212 and the V422 mutation, did not reactivate above low spontaneous levels after stress treatment (data not shown). These data indicate that the acidic domain of VP16 is required for stress-induced reactivation and are consistent with the possibility that *in*1814 retains residual transactivation activity or that the acidic domain of VP16 may possess additional regulatory properties that contribute to altered HSV-1 growth or reactivation, as previously postulated (59, 87).

Relationship of viral growth and HSV-1 reactivation. Since our assay of HSV-1 reactivation requires a round of growth

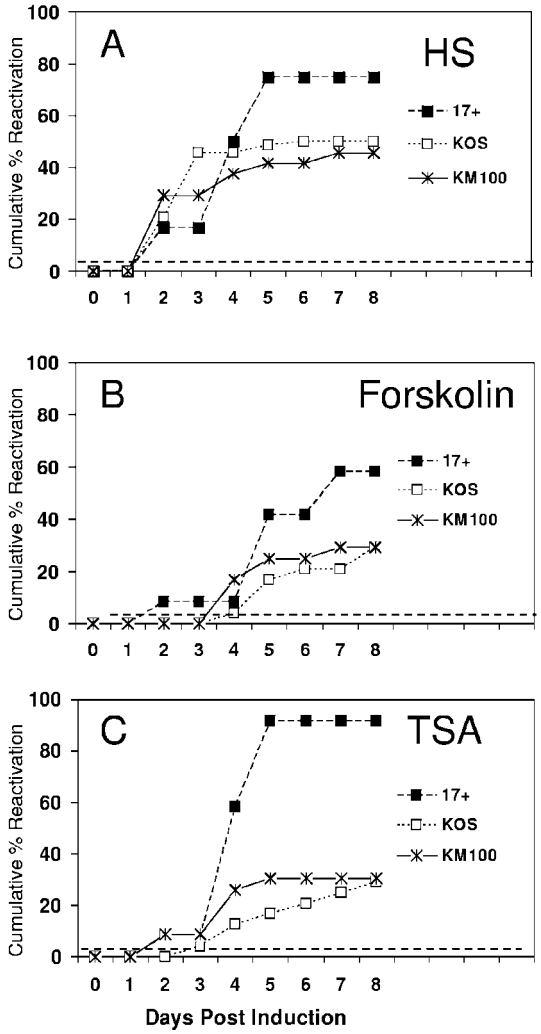

FIG. 5. Reactivation kinetics of KM100 in QIF-PC12 cells. Quiescent infections were established with the indicated viruses in 12-well plates. Cultures were induced to reactivate with indicated stressors ($n = 12$ per group) and monitored for reactivation on U2OS cells, as described in the legend for Fig. 2. The horizontal dashed lines indicate the levels of spontaneous reactivation for KM100-established cultures mock treated with maintenance medium. The level of spontaneous reactivation was 0% for mock-treated KOS-established cultures and 14.4% for 17^+-established quiescently infected cultures. Standard deviations for all points were less than or equal to 15%. Similar results were seen with duplicate experiments.

for progeny to be detected and since some mutants studied were impaired in their ability to reactivate, it was important to determine whether these recombinants were diminished for growth or reactivation in ND-PC12 cells. It was equally important to know, with mutants not impaired in reactivation efficiency, if ND-PC12 cells were capable of complementing the mutations known to impair growth in other cell lines. Growth in PC12 cells that were morphologically differentiated with NGF for 1 week was assessed. Cells were inoculated with R7910, *in*1814, V422, KD6, or their parental wild-type strains, each at an MOI of 5. Virus yields were assessed after several freeze-thawings of the infected cultures by plaque assays on U20S or E5 indicator cells.

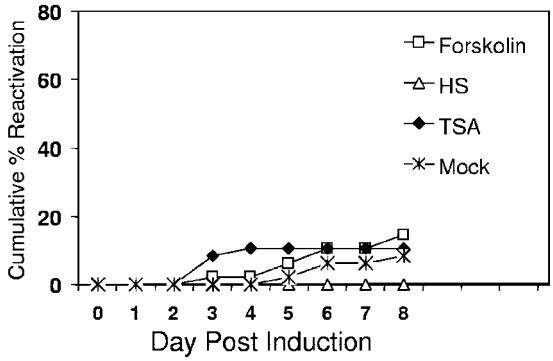

FIG. 6. Reactivation kinetics of V422 in QIF-PC12 cells. Quiescent infections were established with V422 in 12-well plates. Cultures were induced to reactivate with indicated stressors ($n = 24$ per group) and monitored for reactivation by plaque assay using culture supernatants on U2OS cells as described in the legend for Fig. 2. Standard deviations for all points were less than or equal to 10%. The parental control (17^+) reactivated similarly to results shown in Fig. 4. Results obtained from duplicate experiments were similar.

Growth of wild-type strains in ND-PC12 cells was moderate and delayed compared with that reported to occur in permissive cells (10, 22, 59), with a long eclipse phase, 12 to 24 h, observed prior to the synthetic phase (Fig. 7). Strains 17^+ and KOS grew better than strain F, and *in*1814 and R7910 replicated about 1 log less than their parental wild-type strains. Recombinants V422 and KD6 did not grow exponentially and appeared as decay kinetics. These data indicate that growth of HSV-1 in rat neuronal cells is inefficient (60), strain specific, and differentially affected by mutations in the major viral transactivators. The absence of ICP4 and the acidic domain of VP16 significantly reduced viral growth, whereas the absence of ICP0 had less influence on HSV-1 growth. Growth of *in*1814 and R7910 was impaired by 1 order of magnitude, and yet these mutants reactivated as well or better than their parental wild-type strains. This suggests that it is possible to distinguish growth from reactivation when assessed directly in neuronal cells in culture. However, when more-crippling mutations were present, as with ICP4 and VP16, such a distinction was not possible.

DISCUSSION

In vitro neuronal models of HSV-1 quiescence offer certain advantages over animal models of latency for studying mechanisms operating during HSV-1 reactivation. Virus reactivation can be initiated with diverse stimuli (11–14) and examined in neuronal cells under conditions that allow for equivalent viral genome copy numbers of mutant and wild-type viruses. Also, immune cells and the cytokines they may produce, which could alter virus replication and thus the ability to detect reactivation (16, 51, 52), are absent. For these reasons, QIF-PC12 cells were used to investigate the viral genes sufficient and required for HSV-1 reactivation. We found that ICP0, ICP4, and VP16 were sufficient to reactivate HSV-1 when provided in *trans*. However, the major transactivators contributed differentially to HSV-1 spontaneous and inducible reactivation as well as viral growth at the neuronal level in vitro. In addition, stress-induced reactivation was strain specific (Table

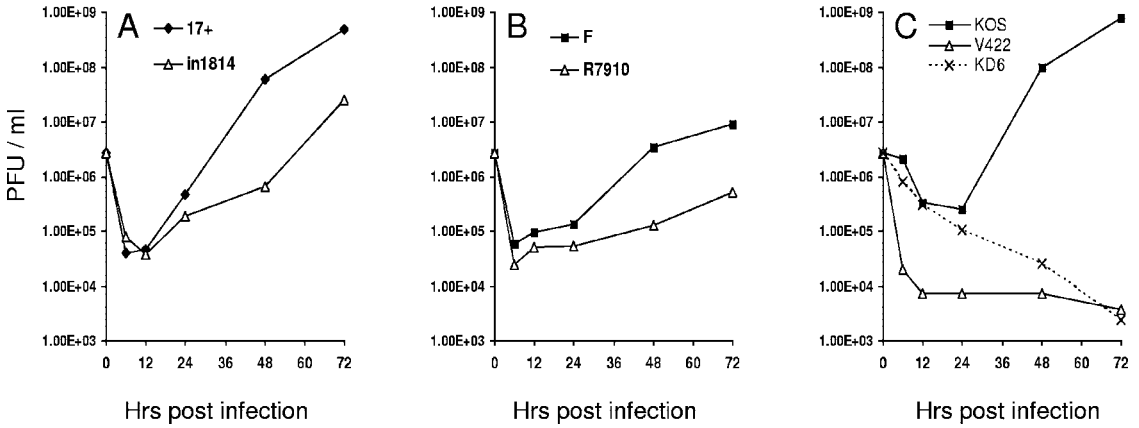

FIG. 7. Growth of HSV-1 recombinants in ND-PC12 cells. PC12 cultures were neuronally differentiated for 7 days and infected at an MOI of 5. At the indicated times, duplicate cultures were harvested, frozen and thawed three times, and viral yields were measured in duplicate by plaque assay on U2OS and E5 cells. Standard deviations for all points were less than or equal to 15%. Similar findings were observed with a duplicate experiment. hrs, hours.

1), similar to that observed with animal models of latency (41, 54, 77).

The *trans* expression of ICP0, ICP4, and VP16 stimulated HSV-1 reactivation in QIF-PC12 cells, whereas expression of LAT, OBP, gD, and nonfunctional mutant forms of ICP0 and VP16 did not. These findings, along with those from a previous report (33), provide strong evidence that the major transactivators, when overexpressed, can transinduce cryptic HSV-1 genomes to reactivate. In our model, strain 17^+ was more reactivation responsive to the *trans* expression of ICP0 than to that of ICP4 and VP16. In contrast, differences between the reactivation abilities of ICP0, ICP4, and VP16 were not readily evident in KOS-established QIF-PC12 cell cultures or KOS latently infected TG cell cultures (33). These results suggest that genetic differences between 17^+ and KOS may have contributed to the distinct responses to ICP0. Although generally similar to the findings with the latently infected TG cell culture model, levels of spontaneous reactivation and transinduced reactivation were lower in QIF-PC12 cells. This might be due to differences in *trans* gene expression levels in specific rat versus murine cells, duration of quiescence, duration of treatment with antiviral drug, and/or the trauma of explant in one system and not the other. Individually, or together, these factors could have allowed HSV-1 to exist in a more repressed transcriptional state in QIF-PC12 cells than in the explanted latently infected TG cultures.

TABLE 1. Summary of reactivation efficiencies of wild-type and mutant HSV-1 strains from QIF-PC12 cells

Virus	Reactivation efficiency[a] after stress treatment		
	Heat stress	Forskolin	TSA
17^+	+++	++(+)	+++
*in*1814	+++	+++	+++
F	++	+	+/−
R7910	+++	++	−
KOS	++	+	+
KD6	−	−	−
KM100	+	+	+
KM110	−	−	−
V422	−	−	−

[a] +++, ≥75%; ++, 50 to 74%; +, 25 to 49%; +/−, 10 to 24%; and −, <10%. ++(+), results ranged from +++ to ++.

ICP0, a promiscuous transactivator that promotes efficient HSV-1 growth and reactivation (5, 8, 23–26, 28, 31, 32, 34, 44), was not required for efficient stress-induced reactivation of HSV-1 in QIF-PC12 cells. This finding is in accord with the observation that the ICP0 promoter is not required for reactivation (15), nor is the gene essential for reactivation from latently infected mouse TG explants, as determined by cocultivation assays (7, 50). In contrast, ICP0 was required for efficient reactivation from explanted latently infected TG (34). One explanation for the observed difference may relate to efficiency issues, in that virtually every QIF-PC12 cell infected at an MOI of 5 maintained a quiescent HSV-1 genome. This could have provided a greater reservoir of viral genomes for reactivation in the QIF-PC12 cell model. Also, ICP0's role in overcoming interferon-induced blocks to virus replication may have contributed (36, 56, 57, 65). Without ICP0, interferon production is likely to be greater in explanted latently infected TG cultures and to have a more pronounced effect (16) than in ND-PC12 cultures that lack immune cells. Since growth of R7910 was impaired in ND-PC12 cells (Fig. 7), a logical interpretation of the data (5, 8, 23–26, 28, 31, 32, 34, 44) is that ICP0 contributes more to efficient viral replication and progression of growth than to efficient reactivation after stress treatment.

The requirement of ICP4 for HSV-1 reactivation was studied using KD6. This ICP4 deletion mutant established a quiescent state in ND-PC12 cells suitable for reactivation in that HSV-1 was biologically retrievable in vitro after superinfection with Ad.T-ICP4. However, KD6 did not reactivate from QIF-PC12 cells either spontaneously or after stress treatments. These findings were not surprising because ICP4 is required for the activation of early and late HSV gene expression and is required for viral growth in permissive cells and neurons (17–20, 25, 28, 29, 62, 63, 67, 72, 75). However, interpretation of the findings is difficult. KD6 does not replicate in ND-PC12 cells and at least one round of viral growth is required for the detection of reactivation in our assay. Thus, it is unclear whether the

absence of ICP4 prevented the initiation of reactivation or processes involved in subsequent viral growth. Although this question remains to be answered, stress-induced cellular factors did not substitute for the functions of ICP4 during reactivation.

This study probed the functions of VP16, an essential functional and structural protein that regulates several processes during HSV-1 infection (49, 58, 66, 78, 80, 91, 96). Although well known for its role in regulating efficient expression of viral immediate-early (IE) genes (9, 61, 66, 69, 75, 91, 93, 95) and replication in permissive cells (49, 82, 87, 96), the role of VP16 in reactivation is less clear. In vivo studies suggest that the expression of VP16 may not influence reactivation (79, 82). However, studies ex vivo (33) and in vitro (Fig. 2) indicate that VP16 is sufficient to reactivate HSV-1. Comparison of the findings for Ad.T-VP16 with those for Ad.T-VP16Δ suggests that a critical transinducing function operating during reactivation lies in the transactivation domain of the protein (33). Thus, to better understand the role of VP16 in reactivation, we studied recombinants possessing mutations in the transactivation domain. *in*1814, which contains a 12-bp insertion that disrupts the interaction of VP16 with host cell factor (HCF, C1) and Oct-1 but preserves the C-terminal acidic domain (2), was observed to reactivate efficiently from QIF-PC12 cells after treatment with diverse stimuli. Similarly, *in*1814 reactivates efficiently from explanted latently infected TG (21, 82). Together, the findings suggest that *in*1814 retains residual transactivation function, possibly within the C-terminal acidic domain of VP16 (1, 2, 30, 39, 58, 59, 87). Consistent with this premise, we found that KM100, which contains the *in*1814 mutation and lacks ICP0 activity, reactivated from QIF-PC12 cells. Thus, even in the absence of ICP0, *in*1814 retains important transactivation abilities.

The importance of the C-terminal region of VP16 was suggested by the in vitro reactivation studies using V422. This recombinant virus contains a truncated VP16 gene at codon 422 (49, 81), making V422 about 100-fold less infectious than its wild-type parental strain and unable to activate viral IE gene expression during lytic infection in cultured cells (49). Without the acidic domain of VP16, IE gene expression is almost completely dependent on ICP0 (59). Although V422 did not grow exponentially in ND-PC12 cells, the mutant was capable of spontaneous reactivation but not stress-induced reactivation. These results suggest that V422 is capable of replicating and reactivating in vitro and that the C-terminal domain modulates the viral reactivation response to stress in QIF-PC12 cells.

It is perplexing that reactivation of R7910 and *in*1814 appeared greater than that of their parental wild-type strains after treatment with HS and forskolin, even though the mutants were impaired for growth. This finding raises the possibilities that ICP0 and VP16 might interfere with certain aspects of reactivation and that their absence could lead to greater reactivation efficiency in QIF-PC12 cells. Alternatively, ICP0 and VP16 could act by inhibiting repression of the herpesvirus transcriptome (31, 40). For example, during viral DNA entry into its host cell, VP16 appears to prevent the deposition of histones on IE viral gene promoters (40). In comparison, ICP0 blocks silencing of viral DNA by dissociating histone deacetylases 1 and 2 from the repressor CoREST/REST complex (31, 74). Thus, a repressed transcriptional state (i.e., HSV-1 latency) can establish efficiently without ICP0 or VP16 (47, 71). This could be important because during the establishment phase of quiescence, viral IE gene products, in the absence of progeny, can be toxic to neuronal cells in vitro (45, 48, 70, 94). Thus, it is intriguing to postulate that more cells survived the initial infection and became latently infected since ICP0 or VP16 were not removing or preventing repression. In this scenario, more latently infected cells would be available for reactivation when virus replication is downregulated by the absence of these transactivators. However, this scenario does not fully explain why the apparent enhanced reactivation efficiency of R7910 and *in*1814 was limited to HS and forskolin treatment and did not occur with TSA treatment. One possibility is that ICP0 and VP16 are required for one or more steps in the TSA-induced pathway of reactivation. This seems logical in view of the roles of ICP0 and VP16 in modulating histone modifications on the viral genome.

In summary, the data presented here demonstrate that (i) VP16 is essential for efficient stress-induced reactivation from QIF-PC12 cells, whereas ICP0 is not, (ii) QIF-PC12 cells display phenotypic differences in reactivation efficiency, and (iii) HSV-1 reactivation is modulated uniquely by different treatment constraints. In addition, we have shown the importance of assays that can distinguish factors involved in HSV-1 growth and reactivation. Further refinements in such models should help illuminate the mechanisms that regulate the activity of these transactivators and HSV-1 reactivation.

ACKNOWLEDGMENTS

We thank N. Fraser, D. Bloom, B. Roizman, W. Halford, and J. Smiley for the viral strains; M. Killen, W. Allen, S. Zimmerman, A. Roe, S. Goodin, and C. Trimby for providing technical assistance; and D. Bloom, J. Hill, B. Roizman, and M. Steiner for helpful discussions.

This research was supported by a grant from the National Institute of Dental Craniofacial Research, no. DE014142 (to C.S.M.).

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