JOURNAL OF VIROLOGY, Nov. 2007, p. 11781–11789 Vol. 81, No. 21
0022-538X/07/$08.00+0 doi:10.1128/JVI.01234-07
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Reactivation of Expression from Quiescent Herpes Simplex Virus Type 1 Genomes in the Absence of Immediate-Early Protein ICP0[▽]

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Received 6 June 2007/Accepted 10 August 2007

Model systems have previously been developed in which herpes simplex virus (HSV) is retained in human fibroblasts in a nonreplicating state known as quiescence. The HSV type 1 (HSV-1) immediate-early (IE) protein ICP0, an important activator of gene expression, reactivates the quiescent genome and promotes the resumption of virus replication. Previous studies reported that infection with ICP0-null HSV-1 mutants fails to reactivate quiescent HSV, even when the mutant itself undergoes productive replication, leading to the hypothesis that quiescent genomes exist in a silent configuration in which they are shielded from *trans*-acting factors. I reinvestigated these findings, using HSV-1 mutants with lesions in the transcription activators VP16, ICP0, and ICP4 to establish quiescent infection at high efficiency. Superinfection with ICP0-null HSV-1 mutants at a low multiplicity of infection (MOI), so that individual plaques were formed, reactivated expression from the quiescent genome, demonstrating that the requirement for ICP0 is not absolute. The previously reported failure to observe reactivation by ICP0-null mutants was shown to be a consequence of either a low initial MOI or a high superinfecting MOI. Competition between viral genomes at the level of gene expression and virus replication, especially when ICP0 was absent, was demonstrated during reactivation and also during normal infection of human fibroblasts. The results show that the multiplicity-dependent phenotype of ICP0-null mutants limits the efficiency of reactivation at low MOIs and that competition between genomes occurs at high MOIs. The conclusion that quiescent HSV genomes are extensively silenced and intrinsically insensitive to *trans*-acting factors must be reevaluated.

Infection with wild-type herpes simplex virus type 1 (HSV-1) usually results in productive virus replication and death of the host cell. Viral genes are expressed in a coordinated cascade characterized by three phases, immediate-early (IE), early, and late. IE genes are the first to be transcribed, and the IE proteins, particularly ICP4, ICP0, and ICP27, play crucial roles in the synthesis of early and late proteins. The ICP4 protein is essential for productive infection, stimulating early and late RNA synthesis through interactions with basal cellular transcription factors (7, 52). Protein ICP0 is not essential for virus replication, but infection in its absence is initiated inefficiently in many cell types (12, 42, 45, 46). Human fibroblasts are particularly restrictive for replication of ICP0-null mutants, but in contrast, the human osteosarcoma line U2-OS is fully permissive (51). ICP0 is a ubiquitin E3 ligase, suggesting that it acts by stimulating proteolysis of strategic cellular targets (4, 15). The tegument protein VP16 strongly stimulates IE transcription shortly after entry of the virion into the host cell, providing a further level of gene regulation (6, 35). Virus mutants that specify nonfunctional VP16 are phenotypically similar to ICP0-null mutants, exhibiting a cell type-dependent defect in initiating productive infection (2, 44).

After infection of humans or experimental animals, some neuronal cells survive infection and harbor HSV-1 in a latent state for the lifetime of the host (10, 25, 48). The mechanisms that silence the viral genome to prevent gene expression during latency are poorly understood, although recent studies have demonstrated that chromatin structure is probably an important factor, since lytic promoters are marked by histone modifications characteristic of nontranscribed cellular genes whereas the genome region that specifies the latency-associated transcript shows modifications associated with actively transcribed genes (3, 26, 27, 49).

To gain insight into the mechanisms of gene silencing, tissue culture systems that mimic aspects of latency have been developed. In the earliest of these, interferon-treated human fibroblasts were infected with HSV-1 and incubated for 7 days in the presence of an inhibitor of viral DNA synthesis to prevent lytic infection and cytopathology (50). The cultures were then transferred to 40.5°C and maintained without detectable virus replication, with reactivation achievable by downshift to 37°C or by superinfection with human cytomegalovirus (HCMV). This system was modified by increasing the initial incubation temperature to 42°C and avoiding the use of inhibitors (40). Human fibroblasts were infected with HSV-1 or HSV-2 at MOIs up to 0.03, and after incubation for 6 days at the supraoptimal temperature, the cultures could be transferred to the permissive temperature of 37°C and maintained without virus production. The quiescent virus resumed replication if the cultures were superinfected with HSV-1, HSV-2, or HCMV. Investigation of the functions required to reactivate quiescent HSV-2 demonstrated that superinfection with a range of HSV-1 mutants resulted in the resumption of HSV-2 replication, with the exception of the HSV-1 mutant *dl*1403 (41). This virus lacks the coding region for ICP0, suggesting a requirement for this IE protein in reactivation of quiescent HSV. The results were substantiated by the observation that ICP0 expressed by adenovirus recombinants was also able to reactivate quiescent HSV-2, whereas ICP0 mutant proteins

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▽ Published ahead of print on 22 August 2007.

that were inactive for stimulation of gene expression were nonfunctional for reactivation (19, 53). The nature of the block to virus replication imposed by incubation at 42°C is not known, and further developments of the quiescent system used HSV-1 mutants at the normal temperature of 37°C. It was found that *dl*1403, and the VP16 mutant *in*1814, could be retained in human fibroblasts for many days in a quiescent state and that superinfection of cultures with wild-type HSV-1 or HCMV resulted in reactivation (20, 45). In the studies with *in*1814, in which cultures were initially infected at an MOI of 0.03, superinfection with *dl*1403 again failed to reactivate expression from the quiescent genomes (20).

From these early investigations, it was concluded that ICP0 is required to reverse the quiescent state and reactivate viral-gene expression. Furthermore, reactivation was not detected even under conditions in which *dl*1403 itself replicated, suggesting that the quiescent genome was unaffected by the plethora of transcription and replication factors produced by the superinfecting *dl*1403. In turn, this observation led to the hypothesis that the quiescent genome is organized into a compact structure, possibly related to heterochromatin, and that reversal of this state uniquely requires ICP0.

Further manipuation of HSV-1 resulted in the isolation of mutants with greater impairment of IE gene expression and reduced cytotoxicity, which are suitable for the establishment of cultures containing quiescent genomes after initial infection at a high MOI. Mutants derived from *in*1814 and additionally impaired for the expression of ICP0 and ICP4 were used to obtain cultures in which most cells contained at least one quiescent genome (14, 37, 38). Even greater reduction in cytotoxicity was achieved by inactivating all IE genes, resulting in mutants that established quiescent infections in human fibroblasts and Vero cells (21, 23, 43). Mutant KM110 lacks the ICP0 coding region and the C-terminal activating domain of VP16, and although potentially capable of replication, this mutant is sufficiently impaired to establish quiescent infection at high efficiency in human fibroblasts without cytotoxicity (34). In each of these systems, provision of ICP0 reactivated the quiescent genome (21, 31, 37, 38, 43).

Recently, the inability of ICP0-null viruses to reactivate quiescent HSV-1 was reinvestigated by Minaker et al. (31). Based on the observation that the HSV-1 mutant KM110 can induce an antiviral response in human fibroblasts that is neutralized by ICP0 (11, 32, 33), these authors questioned whether cultures harboring quiescent HSV-1 were truly permissive for replication of ICP0-null mutants. By carrying out superinfection with viruses expressing enhanced green fluorescent protein as a marker, they demonstrated that, at an MOI of 30, ICP0-null viruses replicated as efficiently as wild-type HSV-1 at an MOI of 10 and that the mutant virus failed to reactivate expression from quiescent genomes (31). These experiments therefore supported the accepted view that quiescent genomes are strictly silenced and can be reactivated only by ICP0.

One disconcerting aspect of the studies described above concerns the complex phenotype of ICP0-null mutants, which exhibit cell type dependence in the ability to initiate productive replication. The human osteosarcoma line U2-OS permits normal virus replication in the absence of ICP0, whereas human fibroblasts represent the most impaired tissue culture examples, being more than 1,000-fold less permissive than U2-OS. Recent experiments suggest that U2-OS cells lack a repression mechanism that is active in human fibroblasts (18). Therefore, after infection at a low MOI, most ICP0-null genomes themselves become quiescent in human fibroblasts (13, 45). Interestingly, some genomes express distinct subsets of viral genes during the first few days of infection, suggesting that silencing is a random process (13). The crucial properties of the small number of cells in a human fibroblast culture that are permissive for replication are unknown at present. Most previous studies with ICP0-null mutants have reasonably emphasized that the defect in initiation of infection is greatest at low MOIs (less than 1), leading to the proposal that the absence of ICP0 is less important during infection at high MOIs (5, 8, 13, 46).

The experiments reported here were stimulated by a surprising observation that quiescent HSV-1 genomes could indeed be reactivated by superinfection with *dl*1403 under appropriate conditions. I therefore reinvestigated the requirements for reactivation of quiescent HSV-1.

MATERIALS AND METHODS

Plasmids. Plasmid pCP58016 was constructed by excising the enhanced yellow fluorescent protein (YFP) coding region from pEYFP-C1 (Clontech) as a HindIII (end filled)/AgeI fragment and inserting it into pCP1802 (22) cleaved with NotI (end filled) and AgeI. This procedure results in a plasmid with YFP controlled by the HCMV major IE promoter (MIEP) and the simian virus 40 polyadenylation sequences, all embedded in the HSV-1 thymidine kinase (TK) coding region. Plasmid pCP376 has the *Escherichia coli lacZ* region, controlled by the HCMV MIEP, inserted into the coding sequences of the nonessential UL43 open reading frame, as described previously (37). Plasmid pMC17 consists of the HSV-1 VP16 coding region cloned into pUC9 (1). Plasmid pGX152 contains the HSV-1 EcoRI B fragment cloned into the EcoRI site of pBR328.

Cells and viruses. Human fetal foreskin fibroblasts (HFFF2) and U2-OS cells were propagated in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 5% (vol/vol) fetal calf serum and 5% newborn calf serum, with 100 units of penicillin and 100 μg of streptomycin per ml (D5 + 5). Syrian hamster fibroblasts (BHK-21) were propagated in Glasgow modified Eagle medium (Invitrogen) supplemented with 10% (vol/vol) newborn calf serum and 10% (vol/vol) tryptose phosphate broth plus 100 units of penicillin and 100 μg of streptomycin per ml. Wild-type HSV-1 was strain 17, and the derived mutants *ts*K (containing a temperature-sensitive mutation in the ICP4 coding region) and *dl*1403 (with most of the ICP0 coding region deleted) have been described previously (9, 36, 46). Mutant *in*1312 has mutations in the coding sequences for VP16, ICP0, and ICP4 (39). Mutant *in*1374 is *in*1312 with an insertion of pCP376 (37). Mutant *in*1330 was derived from *in*1312 by repair of the VP16 mutation, using methods described by Marshall et al. (28), and therefore, this virus lacks ICP0 and encodes a temperature-sensitive ICP4. Mutant *dl*1403Y was constructed by cotransfection of *dl*1403 DNA with ScaI-cleaved pCP58016 and subsequent plaque purification and propagation of TK-negative isolates. The genome structure and absence of parental virus were confirmed by Southern hybridization analysis of viral DNA, as described previously (24). Rescue of the ICP0 mutation of *dl*1403Y was achieved by cotransfection of *dl*1403Y DNA with EcoRI-cleaved pGX152, followed by purification and propagation of isolates that contained an intact ICP0 coding region, determined by Southern hybridization (46). Mutant *dl*1403/β-gal was constructed by cotransfection of *dl*1403 DNA with ScaI-cleaved pCP376, followed by selection and purification of a recombinant with the HCMV MIEP-*lacZ* insertion at UL43, using methodology described previously (37). Viruses were titrated on U2-OS cells at 31°C in the presence of 3 mM hexamethylene bisacetamide to overcome the effects of the VP16, ICP0, and ICP4 mutations (28, 29). Particle-to-PFU ratios were determined as described by Everett et al. (13); the values for the virus stocks used were 32 for *dl*1403, 11 for *dl*1403Y, and 9 for *dl*1403YR.

Infection of HFFF2 monolayers. To establish cultures containing quiescent HSV-1, HFFF2 cells on 60-mm-diameter plates were infected; overlaid with Dulbecco's medium supplemented with 2% (vol/vol) fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin per ml; and propagated at 38.5°C, as described previously (37). After incubation for 7 days, the monolayers were trypsinized and cells were seeded on 24-well plates. Superinfection was carried

out after incubation at 38.5°C for a further 24 h. In many cases, cells were acid washed by incubation for 30 seconds at room temperature with pH 3 buffer, as described by Minaker et al. (31).

Analysis of infected cell DNA. Total DNA was prepared from cultures, cleaved with BamHI, transferred to nitrocellulose membranes, and analyzed by Southern hybridization, using the VP16-coding fragment of pMC17 as a probe.

β-Gal assays. Histochemical assay for β-galactosidase (β-Gal)-expressing cells, using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate and counterstaining, were carried out as described by Jamieson et al. (24). Quantification of β-Gal in cell extracts, using 4-methylumbelliferyl β-D-galactoside as a substrate, was carried out by fluorometric analysis as described previously (38).

YFP assays. Cell extracts were prepared as for β-Gal assays. Extracts were placed in microtiter plates with blackened edges, and fluorescence was determined by use of a Hidex Chameleon plate reader with excitation at 485 nm and emission at 535 nm.

RESULTS

Reactivation by superinfection with *dl*1403. Cultures of HFFF2 cells were infected with 3 PFU of the HSV-1 mutant *in*1374 per cell and incubated at 38.5°C for 8 days to enable full establishment of quiescent infection (37). After this treatment, no β-Gal-positive cells were detectable upon histochemical staining of cultures, as reported previously (37). Superinfection of cultures with 2 PFU of the ICP4 mutant *ts*K per cell resulted in approximately 50% of the cells expressing β-Gal, demonstrating that a substantial proportion of the cells harbored quiescent virus. Cultures were superinfected with the ICP0 deletion mutant *dl*1403 at various multiplicities and stained for the presence of β-Gal after incubation at 38.5°C for 2 days in the presence of 2% human serum to prevent secondary spread of virus. Surprisingly, and in contrast to the conclusions of previous studies, most plaques were β-Gal positive at the lowest multiplicities of *dl*1403 that resulted in plaque formation (Fig. 1A). The majority of β-Gal-positive plaques were uniformly stained, suggesting that reactivation occurred at the initial, or first few, rounds of replication. A minority, however, contained a mixture of β-Gal-positive and -negative rounded cells (not shown), presumably representing reactivation at a later stage in plaque formation. At an MOI of 0.1 (in terms of PFU on U2-OS cells, representing three *dl*1403 particles per cell), approximately 30 plaques formed on HFFF2 monolayers of 2×10^5 cells. As the MOI of *dl*1403 was increased, the total number of plaques rose sharply in a nonlinear fashion characteristic of infection of human fibroblasts with ICP0 mutants (12, 13, 45), and an increasing proportion did not express β-Gal (Fig. 1B). The numbers of β-Gal-positive plaques appeared to reach a maximum at a *dl*1403 MOI of 0.5, but it was not possible to obtain accurate data because discrete plaques could not generally be discerned within the extensive cytopathic effect (CPE) that developed during incubation at 38.5°C for 2 days (Fig. 1C). This experiment showed that β-Gal expression was readily activated in response to infection with *dl*1403 and that the effect was most obvious when superinfection was carried out at a low MOI. As expected, superinfection with wild-type HSV-1 yielded plaques that were uniformly β-Gal positive at all multiplicities tested (results not shown). Superinfection at 38.5°C with the UL26 mutant *ts*1201 resulted in cross-complementation with quiescent *in*1374, as described by Samaniego et al. (43), and the plaques obtained were also β-Gal positive (results not shown). In view of the findings shown in Fig. 1, the conclusion that quiescent genomes remain silent during productive infection with *dl*1403 clearly requires qualification.

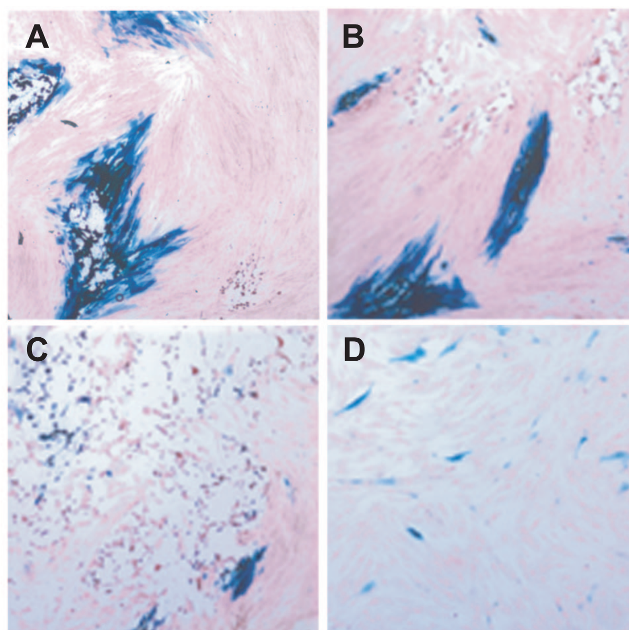

FIG. 1. Reactivation of β-Gal expression by superinfection with *dl*1403. HFFF2 cultures containing quiescent virus were established after initial infection with 3 PFU of *in*1374 per cell. At 8 days p.i., the cultures were superinfected with *dl*1403 at an MOI of 0.1 (A), 0.3 (B), or 0.5 (C) (based on U2-OS cell titers) and incubated for a further 2 days at 38.5°C in D5 + 5 with 2% (vol/vol) human serum added. Cultures superinfected with *dl*1403 at an MOI of 10 and incubated for 20 h in D5 + 5 with 50 μg/ml AraC added are shown in panel D.

The resumption of β-Gal expression could be due to a transcriptional activation of the HCMV MIEP that controls *lacZ* in *in*1374 or to recombination between the superinfecting and quiescent genomes. To determine whether DNA replication was required for stimulation of β-Gal expression, cultures containing quiescent *in*1374 were superinfected with *dl*1403 in the presence of cytosine arabinoside (AraC) and maintained at 38.5°C for 20 h. Upon histochemical staining, many β-Gal-positive cells were observed (Fig. 1D). Because CPE was much reduced during the shorter time when AraC was present compared with incubation for 2 days with 2% human serum, as in Fig. 1A to C, it was possible to detect positive cells at MOIs of *dl*1403 up to 10 (Table 1 and Fig. 1D). At an MOI of 10, approximately 0.5% of cells were β-Gal positive.

To investigate further the functions of the superinfecting virus required to stimulate β-Gal production, mutant *in*1330, which has deletion of ICP0 plus the temperature-sensitive mutation from *ts*K in ICP4, was tested (Table 1). Cultures containing quiescent *in*1374 were superinfected with *dl*1403 or *in*1330 in the presence of AraC and maintained at 38.5°C for 20 h or 31°C for 60 h. Both viruses activated β-Gal expression at 31°C, but only *dl*1403 was effective at 38.5°C over a range of multiplicities. The expression of β-Gal upon superinfection with *dl*1403 is therefore dependent on functional ICP4 but not on DNA replication, strongly suggesting that initially the effect represents transcriptional activation of the quiescent genome rather than recombination with the superinfecting virus. After

TABLE 1. Reactivation by *dl*1403 and *in*1330[a]

MOI	No. of β-Gal-positive cells after superinfection			
	*dl*1403 (31°C)	*dl*1403 (38.5°C)	*in*1330 (31°C)	*in*1330 (38.5°C)
0	5	0	4	0
0.1	5	7	7	0
0.15	9	7	10	1
0.3	24	75	27	2
0.5	38	125	37	2
1.0	79	155	77	1
1.5	93	171	90	0
2.5	106	243	100	1

[a] HFFF2 cultures were infected with 3 PFU of *in*1374 per cell and incubated at 38.5°C for 8 days. After trypsinization and reseeding, the cells were superinfected with *dl*1403 or *in*1330 and incubated for a further 20 h at 38.5°C or 60 h at 31°C with 50 μg/ml AraC present. The numbers of β-Gal-positive cells per culture of 2×10^5 cells are expressed as the means of duplicate determinations.

initial reactivation has occurred, reactivated and superinfecting viruses undergo recombination in the normal manner.

The above-mentioned findings apparently contradict previous studies, which showed that superinfection with ICP0-null mutants failed to reactivate quiescent HSV-2, *in*1814, or KM110 (20, 31, 41). There are differences in the experimental details of the previous studies and those represented in Fig. 1. Initial infections with HSV-2 or *in*1814 could not be performed at MOIs greater than 0.03, due to loss of cultures through escape virus replication or cytopathology (20, 31, 40). Mutant KM110 was sufficiently impaired to permit an initial MOI of 6, but superinfection was carried out only at high MOIs, 10 for wild-type virus and 30 for the ICP0-null mutant (31). The effects of altering initial and superinfecting MOIs on reactivation were therefore investigated to determine whether the differences in experimental protocols were responsible for the apparent discrepancies in results.

Construction of YFP-expressing *dl*1403 and rescuant. Many of the studies described below involve quantifying reactivation by *dl*1403 and wild-type HSV-1. This is potentially problematic, because *dl*1403 preparations have a high particle-to-PFU ratio on HFFF2 cells, and thus comparison, even on the basis of titers on U2-OS cells, might result in the delivery of different functional genome loads to infected cells. To obtain appropriate virus stocks for superinfection studies, mutant *dl*1403Y, which is *dl*1403 with an insertion of YFP controlled by the HCMV MIEP at the TK locus, was constructed. A rescuant with the ICP0 deletion repaired, *dl*1403YR, was then produced. These viruses had similar particle-to-PFU ratios when titrated on U2-OS cells (11 for *dl*1403Y and 9 for *dl*1403YR). To ensure that infections with these preparations were comparable in human fibroblasts, two further tests were carried out. First, HFFF2 monolayers were infected at an MOI of 10 or 30, cells were acid washed to remove nonpenetrated virus, and viral-DNA levels were measured at 1.5 h postinfection (p.i.) by Southern blotting and hybridization. As shown in Fig. 2A, equivalent quantities of input DNA were delivered to cells at each MOI tested. Second, cultures of HFFF2 cells were infected with various amounts of *dl*1403Y or *dl*1403YR, coinfected with 10 PFU of wild-type HSV-1 per cell, and incubated overnight in the presence of AraC. YFP levels in the cultures were determined and shown to be very similar for the two viruses (Fig. 2B). Thus, infection at equal MOIs (as measured by titers on U2-OS cells) with *dl*1403Y or *dl*1403YR resulted in the delivery of equivalent numbers of potentially competent genomes to cells. Although this virus pair was used in most of the following experiments, the results were reproduced where possible (but are not presented) using *dl*1403 and wild-type HSV-1.

Comparison with previously published results. To investigate the effects of altering the initial MOI on the efficiency of reactivation of quiescent virus, HFFF2 monolayers were infected with *in*1374 at an MOI of 3, 0.3, or 0.03 and maintained at 38.5°C for 8 days. Superinfection was carried out with *dl*1403Y or *dl*1403YR at an MOI of 0.1, and cultures were maintained at 38.5°C until widespread CPE was observed. This reproduced the methodology used by Russell et al., (41) and additionally enabled analysis at a higher initial MOI through the use of the severely impaired mutant *in*1374. Intracellular DNA was extracted for analysis by Southern hybridization (Fig. 3). At all multiplicities, infection with *dl*1403YR resulted in the presence of DNA containing the VP16 mutation, identified by the existence of a novel BamHI site in the BamHI f fragment derived from replication of reactivated quiescent *in*1374 (Fig. 3, lanes 4 to 6) (2). As expected from the experiments shown in Fig. 1, cultures initially infected with 3 PFU of *in*1374 per cell and superinfected with *dl*1403Y also contained DNA with the VP16 insertion (Fig. 3, lane 1). At initial infection with 0.3 PFU of *in*1374 per cell, however, superinfection with *dl*1403Y did not yield replicated DNA containing the VP16 mutation (Fig. 3, lane 2), with one exception in five independent determinations (results not shown). At an initial MOI of 0.03, DNA containing the VP16 mutation was never detected. Therefore, the findings from the published experiments with quiescent HSV-2 or *in*1814 were reproduced, since

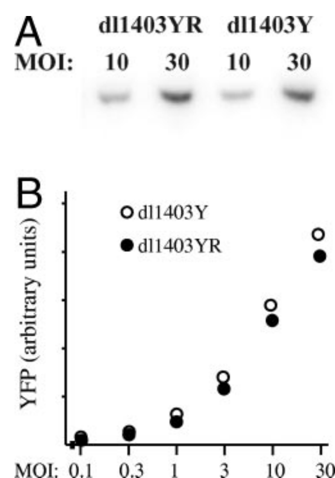

FIG. 2. Comparison of *dl*1403Y and *dl*1403YR. (A) HFFF2 monolayers were infected with *dl*1403Y or *dl*1403YR at an MOI of 10 or 30. After a 1-h adsorption period, the cells were acid washed and incubated at 37°C for a further 30 min. Cellular DNA was prepared and cleaved with BamHI. A Southern blot was probed with radiolabeled VP16 coding region from pMC17. (B) HFFF2 monolayers were infected with *dl*1403Y or *dl*1403YR at various MOIs and coinfected with wild-type HSV-1 at an MOI of 10. The monolayers were incubated at 38.5°C for 20 h in D5 + 5 with 50 μg/ml AraC. YFP levels were determined in cell extracts.

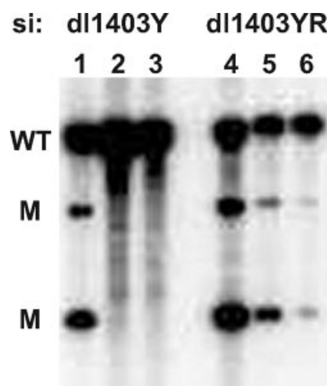

FIG. 3. Reactivation at low superinfecting MOIs. HFFF2 cultures containing quiescent virus were established after infection with *in*1374 at an MOI of 3 (lanes 1 and 4), 0.3 (lanes 2 and 5), or 0.03 (lanes 3 and 6). At 8 days p.i., the cultures were superinfected (si) with *dl*1403Y (lanes 1 to 3) or *dl*1403YR (lanes 4 to 6) at an MOI of 0.1 and incubated at 38.5°C until extensive CPE developed. Cellular DNA was prepared and cleaved with BamHI. A Southern blot was probed with radiolabeled VP16 coding region from pMC17, revealing a band of 8 kbp from wild-type virus (WT) and bands of 5 kbp and 3 kbp derived from genomes containing the BamHI site that gives rise to the VP16 mutation of *in*1374 (M).

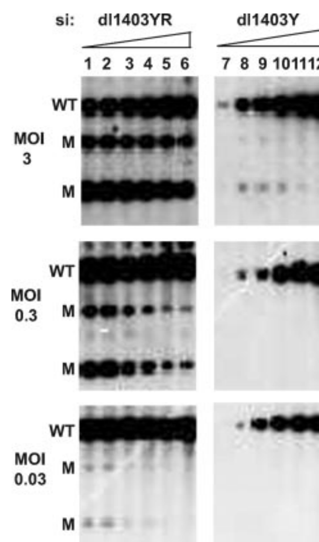

FIG. 4. Reactivation by superinfection. HFFF2 cultures containing quiescent virus were established after infection with *in*1374 at an MOI of 3 (top), 0.3 (middle), or 0.03 (bottom). At 8 days p.i., the cultures were superinfected (si) with *dl*1403YR (lanes 1 to 6) or *dl*1403Y (lanes 7 to 12). After a 1-h adsorption period, the cells were acid washed and incubated at 38.5°C for 22 h. Cellular DNA was prepared, cleaved with BamHI, and probed with radiolabeled VP16 coding region from pMC17, as described in the legend to Fig. 3. The MOI of the superinfecting virus was 0.1 (lanes 1 and 7), 0.3 (lanes 2 and 8), 1 (lanes 3 and 9), 3 (lanes 4 and 10), 10 (lanes 5 and 11), or 30 (lanes 6 and 12).

reactivation by superinfection with the ICP0-null virus *dl*1403Y was detected reliably only in cultures initially infected with 3 PFU of *in*1374 per cell and never in cultures initially infected at an MOI of 0.03, the highest value used in the earlier studies.

The apparent discrepancy with the findings of Minaker et al. was also addressed. Cultures were infected with 3 PFU of *in*1374 per cell, maintained at 38.5°C for 8 days, and superinfected with *dl*1403Y or *dl*1403YR at different MOIs. After further incubation at 38.5°C for 22 h, the intracellular DNA was analyzed (Fig. 4). At all multiplicities tested, superinfection with *dl*1403YR resulted in the presence of replicated viral DNA containing the VP16 mutation resident in *in*1374 (Fig. 4, lanes 1 to 6). As the *dl*1403YR MOI increased above 0.3, however, the recovery of mutant DNA declined, indicating a degree of competition between genomes during replication. At an initial *in*1374 MOI of 0.3 or 0.03, superinfection with *dl*1403Y did not yield detectable amounts of replicated mutant DNA at any superinfecting MOI tested. At an initial *in*1374 MOI of 3 and multiplicities of *dl*1403Y greater than 0.1, DNA containing the VP16 mutation was present (Fig. 4, top, lanes 8 to 10). As the superinfecting multiplicity increased to 10 and 30, the amount of replicated mutant DNA decreased sharply (Fig. 4, top, lanes 11 and 12). At an MOI of 30 (lane 12), DNA containing the VP16 mutation was hardly detectable, in agreement with the results of Minaker et al. (31).

Therefore, it appears that superinfection with ICP0-deficient mutants imposes two limitations on reactivation and replication of quiescent virus. At low MOIs, progression into lytic replication is restricted by the well-characterized defect in initiating productive infection. At high MOIs, competition for replication occurs between genomes, resulting in reduced levels of *in*1374-derived genomes even though the total amount of replicated DNA is high.

Competition between genomes during coinfection. To investigate the prediction that competition for DNA replication occurs at high MOIs of *dl*1403, a reconstruction experiment was performed. HFFF2 cells, mock infected but incubated at 38.5°C for 8 days, were infected with 3 or 0.3 PFU of *in*1374 per cell, together with different amounts of *dl*1403Y or *dl*1403YR. The mock treatment was carried out for comparability with quiescent infection, although freshly seeded cells gave equivalent results (not shown). After incubation for 22 h, intracellular DNA was analyzed for the presence of the VP16 mutation (Fig. 5). For coinfection with *dl*1403YR, increasing the MOI from 0.1 to 1 resulted in a consistent proportion of replicated genomes containing the VP16 mutation (Fig. 5, lanes 1 to 3), as

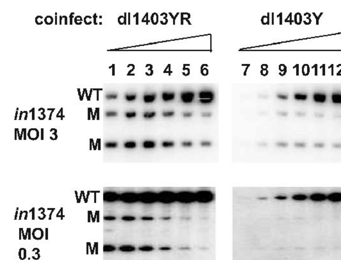

FIG. 5. Coinfection of *in*1374 with *dl*1403Y or *dl*1403YR. HFFF2 monolayers were incubated at 38.5°C for 8 days, infected with *in*1374 at an MOI of 3 (top) or 0.3 (bottom), and coinfected with *dl*1403YR (lanes 1 to 6) or *dl*1403Y (lanes 7 to 12). After a 1-h adsorption period, the cells were acid washed and incubated at 38.5°C for 22 h. Cellular DNA was prepared, cleaved with BamHI, and probed with radiolabeled VP16 coding region from pMC17, as described in the legend to Fig. 3. The MOI of the coinfecting virus was 0.1 (lanes 1 and 7), 0.3 (lanes 2 and 8), 1 (lanes 3 and 9), 3 (lanes 4 and 10), 10 (lanes 5 and 11), or 30 (lanes 6 and 12).

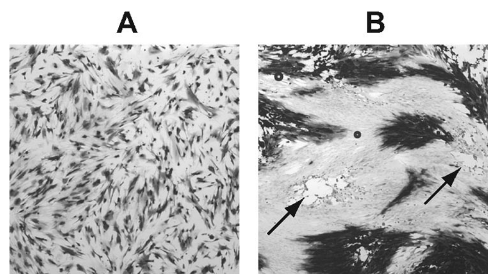

FIG. 6. Competition between *dl*1403 genomes during coinfection. HFFF2 monolayers were infected with *dl*1403 and *dl*1403/β-gal, mixed in a 1:3 ratio on the basis of PFU, at a total MOI of 1. The cultures were coinfected with 3 PFU of wild-type HSV-1 per cell and incubated at 37°C for 22 h with 50 μg/ml AraC present (A) or incubated at 37°C for 40 h in D5 + 5 containing 2% human serum (B). Two plaques that did not contain β-Gal-expressing cells are marked with arrows.

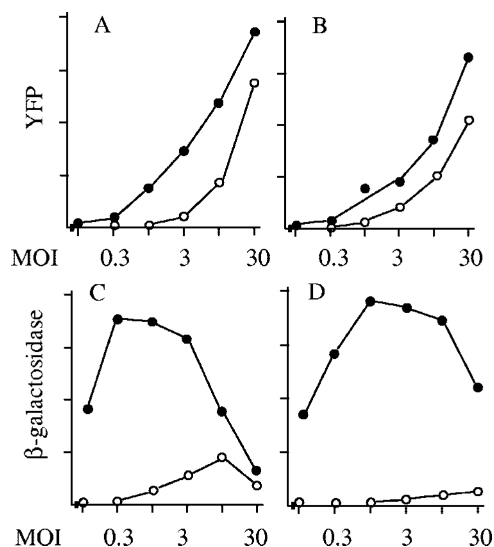

FIG. 7. YFP and β-Gal expression during coinfection or superinfection. For coinfection (A and C), HFFF2 monolayers were incubated at 38.5°C for 8 days and then infected with *in*1374 (MOI, 0.5) and coinfected with *dl*1403YR (filled circles) or *dl*1403Y (open circles). After incubation at 38.5°C for 22 h with 50 μg/ml AraC, YFP (A) or β-Gal (C) levels were determined in cell extracts. For superinfection (B and D), HFFF2 monolayers were infected with *in*1374 (MOI, 3) and maintained at 38.5°C for 8 days. The cultures were then infected with *dl*1403YR (closed circles) or *dl*1403Y (open circles), and after incubation at 38.5°C for 22 h with 50 μg/ml AraC, YFP (B) or β-Gal (D) levels were determined in cell extracts.

predicted, since replication of *in*1374 is possible only when a *dl*1403YR genome is also present. As the amount of *dl*1403YR increased further, the recovery of *in*1374-derived genomes declined, especially when *in*1374 was initially present at an MOI of 0.3 (Fig. 5, lanes 4 to 6). In the case of coinfection with *dl*1403Y, replication of both viruses increased up to an MOI of 1, as expected from the multiplicity-dependent phenotype of ICP0-null mutants at low MOIs (Fig. 5, lanes 7 to 9), but after this point the proportion of genomes containing the VP16 mutation declined, until at a *dl*1403Y MOI of 30, this value was very low, especially at an initial *in*1374 MOI of 0.3 (Fig. 5, lanes 10 to 12). Tellingly, *in*1374-derived genomes were always present at much lower levels when the coinfecting virus was *dl*1403Y than with *dl*1403YR, until the MOI was 30. Therefore, the reconstruction experiment demonstrated that competition for DNA replication of coinfecting viruses occurs at multiplicities of dl1403Y and dl1403YR greater than 1, in a manner similar to that observed during superinfection.

A further demonstration that competition can result in some genomes failing to replicate at high MOIs of ICP0-null virus was obtained by carrying out an alternative coinfection experiment. The HSV-1 mutants *dl*1403 and *dl*1403/β-gal were mixed at a ratio of 1:3 in terms of PFU and used to infect HFFF2 monolayers. At a total MOI of 1, almost all cells contained a genome capable of expressing β-Gal, as shown by coinfection with wild-type HSV-1 in the presence of AraC and histochemical staining after 22 h (Fig. 6A). Significant CPE developed when cultures were incubated at 37°C for 40 h with 2% human serum present, but plaques were observed in certain areas of the monolayers and staining revealed that some did not express β-Gal (Fig. 6B). The plaques consisted of significant clusters of β-Gal-negative cells, virtually all of which (by extrapolation from the results of superinfection with wild-type HSV-1 [Fig. 6A]) contained a *dl*1403/β-gal genome. Thus, in these instances, the *dl*1403/β-gal genome failed to replicate, and furthermore, the HCMV MIEP (and presumably the entire *dl*1403/β-gal genome) was inactive even in cells that supported replication of *dl*1403. It is possible that the β-Gal-negative plaques arose from a cell initially infected with *dl*1403 alone, but cells subsequently infected during plaque formation must have contained *dl*1403/β-gal.

Gene expression during coinfection and superinfection. The experiments described above suggest that during ICP0-null infections, some viral genomes failed to replicate even in cells supporting active virus production. In other words, the interpretation of reactivation experiments also applies to coinfection. I pursued this finding to investigate whether similar effects could be observed at the level of gene expression. HFFF2 cells, incubated at 38.5°C for 8 days, were infected with a fixed amount (0.5 PFU per cell) of *in*1374, together with different amounts of *dl*1403Y or *dl*1403YR. As stated above, freshly seeded cells gave equivalent results (not shown). Cultures were maintained at 38.5°C for 22 h, with AraC present to ensure that gene expression represented the activities of input genomes without complications resulting from DNA replication. Cell extracts were analyzed for YFP and β-Gal content (Fig. 7A and C). The results showed that YFP production was much lower for *dl*1403Y than *dl*1403YR at a low total MOI, as expected from the known properties of ICP0-null mutants. At high MOIs, 30 PFU per cell of *dl*1403Y and 10 PFU per cell of *dl*1403YR produced similar amounts of YFP, confirming the results of Minaker et al. (31). The expression of β-Gal, however, was much higher during coinfection with *dl*1403YR, until at an MOI of 30, the values were comparable. At an MOI of 30, however, competition clearly occurred in *dl*1403YR-infected cells, since YFP levels were high but β-Gal expression was well below the maximum that was observed at MOIs of 0.3 to 3. Therefore, competition at the level of gene expression occurred in *dl*1403YR-infected cells at MOIs greater than 3. Maximal expression of *dl*1403Y (as judged by YFP production)

occurred at MOIs of 30 or greater, but at this value, competition reduced the expression of β-Gal.

The experiment shown in Fig. 7A and C demonstrated that expression of β-Gal from *in*1374 genomes is restricted during coinfection with *dl*1403Y, due to inefficient entry into lytic infection at low MOIs and competition at high MOIs. To make a quantitative assessment of whether the events observed during coinfection can account for the reduced reactivation of quiescent genomes upon superinfection with *dl*1403Y, an analogous experiment was carried out by superinfecting cultures containing quiescent *in*1374 (Fig. 7B and D). The production of YFP followed the pattern observed during coinfection, with equivalence between *dl*1403Y and *dl*1403YR almost reached at an MOI of 30 (Fig. 7A and B). Expression of β-Gal after superinfection with *dl*1403YR followed a pattern similar to that observed during coinfection, although competition was observed mainly at an MOI of 30 (Fig. 7C and D). Upon superinfection with *dl*1403Y, β-Gal expression was very low, detectable only at MOIs of 3 or greater. In relative terms, the maximum expression level during coinfection with *dl*1403Y was 22% of that reached during coinfection with *dl*1403YR, whereas for reactivation, this value was 2.5%. Therefore, this experiment suggests that gene expression during reactivation by *dl*1403Y is relatively less efficient than during coinfection.

DISCUSSION

Previous studies, using a variety of methodologies, have concurred that infection with ICP0-null HSV-1 mutants does not reactivate quiescent HSV. In the experiments described here, I demonstrated that by alteration of the experimental protocol it is possible to reach a different conclusion, namely, that reactivation does occur under conditions in which the superinfecting virus replicates from an initial low MOI. In view of these results, it is necessary to reassess the role of ICP0 in stimulating quiescent genomes to resume gene expression and, particularly, to review the hypothesis that quiescent genomes are shielded from *trans*-acting factors. The assertion that ICP0 alone is sufficient for reactivation in human fibroblasts, however, is not in doubt. Infection of cultures with adenovirus recombinants that express ICP0 results in reactivation of quiescent genomes, even when only small amounts of ICP0 are produced (19, 21, 53).

The results of previous investigations have essentially been reproduced here; thus, discrepancies with published conclusions are not due to experimental inconsistencies. After initial infection at a low MOI of 0.03, it was not possible to detect reactivation even when superinfection with *dl*1403 was at a low MOI and the virus was permitted to replicate and spread through the culture. The reasons for this observation are not entirely clear, since a significant proportion of cells (3%) should contain a quiescent genome, and the most likely explanation is that the rare initial infection that resulted in replication of *dl*1403 would be unlikely to occur in a cell containing a quiescent genome. Presumably, infection through secondary spread was effectively at high MOIs, resulting in competition that prevented either reactivation itself or detection of reactivated quiescent genomes. In addition, the *dl*1403 genomes that did not form plaques initially would become quiescent, competing with *in*1374 during superinfection. As a further consideration, the initiation of infection from incoming genomes may be more rapid than from quiescent genomes; if this is the case, competition from superinfecting virus would be exacerbated. The coinfection experiment shown in Fig. 6 confirmed that competition effects of this type can occur.

The failure to detect reactivation when both initial and superinfecting MOIs are high, reported by Minaker et al., was reproduced. At lower superinfecting MOIs, however, it was possible to detect reactivation by histochemical staining of plaques or analysis of DNA. Competition at high MOIs of *dl*1403Y accounted for the failure to detect reactivation, since this effect was also observed, albeit less dramatically, when superinfection was carried out with *dl*1403YR at high MOIs.

The interpretation of superinfection experiments was supported by reconstructions. Coinfection of cultures with *in*1374 and *dl*1403YR yielded results that were predictable and readily interpretable. Since *dl*1403YR provides the functions that are inactivated in *in*1374, the approach is tantamount to infecting cells with two wild-type viruses that can be differentiated by the presence of distinct marker genes. Although total replication (Fig. 5) and gene expression (Fig. 7) increased with the MOI, the relative contribution from the *in*1374 genome, kept at a fixed level, decreased at high total MOIs. There is a finite capacity within cells for transcription and replication (and for other processes, such as packaging) of viral genomes, and the reduction in contributions from *in*1374 genomes signifies that genomes compete for cellular and viral factors when the total MOI is high. Thus, although overall viral transcription and replication were higher at a *dl*1403YR MOI of 30 than at an MOI of 1, the degree of expression and replication of *in*1374 was much lower at the higher MOI. Indeed, Sacks and Schaffer previously noted competition between ICP0-null genomes and wild-type HSV-1 genomes, although the effect was not investigated further (42).

The situation is more complex during coinfection with *in*1374 and *dl*1403Y. Again, expression and replication of *in*1374 mirror those of *dl*1403Y, since the VP16 and ICP4 defects are complemented by *dl*1403Y. At low MOIs, however, ICP0-null genomes are transcribed and replicated poorly. To achieve efficient productive replication, it is necessary to increase the MOI to 30, at which point (from the observations of coinfection with *dl*1403YR) severe competition limits the transcription and expression of the *in*1374 genome. Indeed, I observed an analogous effect at the DNA synthesis level during coinfection of *dl*1403/β-gal (at a constant MOI of 0.1) with increasing amounts of *dl*1403: replication of *dl*1403/β-gal was severely reduced as the *dl*1403 MOI reached 30 (results not shown). A further demonstration of competition is shown in Fig. 6; the rare β-Gal-negative plaques must consist largely of cells infected with both *dl*1403 and *dl*1403/β-gal.

The findings reported here provide new information on the complexity of the ICP0-null mutant phenotype in restrictive cell types. Previous studies emphasized that the major impairment of gene expression is observed at low MOIs, with the defect overcome, either completely or partially, at high MOIs (5, 8, 12, 13, 42, 46). By including a distinguishable virus at a fixed low MOI, I demonstrated that although total gene expression and replication are similar at high MOIs (10 for *dl*1403YR and 30 for *dl*1403Y), the contributions from individual genomes at these multiplicities are well below maxima

due to competition. It is not clear whether this signifies an overall reduction in expression and replication of all genomes or heterogeneity such that only selected genomes are transcribed and replicated, with the others possibly remaining quiescent.

The results presented here show that superinfection with *dl*1403 can indeed reactivate quiescent HSV, and it is important to consider whether the data completely refute the concept that the quiescent genome is shielded from *trans*-acting factors. Analysis of Fig. 4, 5, and 7 reveals that the relative amount of β-Gal expression or *in*1374 replication was approximately 10-fold lower during superinfection than during coinfection, when the response to *dl*1403Y was compared with the response to *dl*1403YR. The straightforward interpretation is that 90% of quiescent genomes are indeed refractory to superinfection with *dl*1403Y and that only 10% respond. This would imply heterogeneity among quiescent genomes, with some not repressed as tightly as the majority, in agreement with observations made at earlier times after infection (13). It should be noted, however, that all viral genomes appear to be altered in some way during conversion to a quiescent state. Neither the virion protein VP16 nor the chemical hexamethylene bisacetamide affects expression from quiescent genomes, whereas they stimulate transcription of *in*1374 or KM110 if added at the time of infection (18, 37, 38). While heterogeneity among quiescent genomes is the most likely interpretation of the data, some caveats must be recognized. In particular, the events during coinfection and superinfection may not be strictly comparable. Upon coinfection, all genomes are uncoated and transcribed with the same overall timing and through the same mechanisms. During superinfection, however, transcription of quiescent genomes may be delayed compared with that of the superinfecting ICP0-null virus, and if this is the case, the quiescent genome might suffer greater competition than was found for *in*1374 during coinfection. Therefore, although I favor the interpretation that there is heterogeneity in the extents of silencing of individual quiescent genomes, it remains possible that most genomes are potentially susceptible to *trans*-acting factors and that the complex phenotype of ICP0-null mutants precludes efficient reactivation.

A number of studies have addressed the role of ICP0 in reactivation of quiescent, or latent, HSV-1 in neuronal cells. In a system based on infection of rat pheochromocytoma (PC12) cells, quiescent HSV-1 was reactivated by superinfection of cultures with adenovirus recombinants that expressed either VP16, ICP0, or ICP4 (30). Latent HSV-1 in mouse ganglia was also reactivated by infection of dissociated ganglia with adenoviruses expressing VP16, ICP0, or ICP4 (16). Therefore, in these neuron-based systems, reactivation can be initiated by adenovirus-directed expression of VP16 or ICP4 in the absence of ICP0, either through a general effect on the viral genome or by stimulating ICP0 production from the quiescent or latent genome. The observation of ICP0-independent reactivation that I report here, therefore, highlights similarities between quiescence in fibroblasts and latency in neurons.

There is no evidence for the presence of ICP0 in neurons that harbor latent HSV, and thus, the initiation of reactivation must occur in the absence of this protein. Investigation of latency in the mouse has shown that, when careful attention is paid to equalizing the establishment of latency, ICP0-null mutants reactivate less efficiently than wild-type viruses upon explantation of ganglia (17, 47). These experiments indicate that ICP0 has an important role in reactivation in the mouse, but equally, they demonstrate that reactivation can occur in the complete absence of ICP0. Recent experiments have suggested that ICP0 is not required for the initiation of reactivation in vivo after hyperthermic treatment of mice but that the protein is important for later events in virus replication (47). Overall, reactivation in all neuron-based culture or animal model systems is inefficient when the number of viral genomes present is taken into account, with only a very small proportion responding to the stimuli, and it is therefore likely that latent genomes are heterogeneous in terms if their responsiveness to reactivation signals. The findings I report here also suggest heterogeneity in the responsiveness of quiescent genomes to reactivation in the absence of ICP0, signifying a convergence of the fibroblast model with the neuron-based systems discussed above. Studies on the structure and reactivation of quiescent HSV in fibroblasts may thus have direct relevance to latency in vivo.

ACKNOWLEDGMENTS

I thank Mary Jane Nicholl for skilled assistance and Duncan McGeoch, Roger Everett, and Nigel Stow for helpful comments on the manuscript.

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