Establishment of latency \textit{in vitro} by the herpes simplex virus type 1 mutant \textit{in} 1814

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The herpes simplex virus type 1 (HSV-1) mutant \textit{in}1814 possesses an insertion mutation that abolishes trans-activation of immediate early (IE) transcription by the virion protein Vmw65. Interactions between \textit{in}1814 and the host cell were examined by use of an \textit{in vitro} latency system which relies on infection of human foetal lung (HFL) cells at 42 °C to prevent lytic growth of virus. Mutant \textit{in}1814 was retained in HFL cells after infection at low m.o.i, and incubation at 42 °C, and was reactivated by superinfection of monolayers with viruses that express the HSV-1 IE protein Vmw110. Moreover, latency was established by \textit{in}1814 in an analogous manner at 37 °C. The low cytotoxicity of \textit{in}1814 enabled an investigation of latency after infection at high m.o.i. (five particles per cell) to be undertaken. At 42 °C, or at 37 °C in the presence of an inhibitor of DNA synthesis, \textit{in}1814 DNA was maintained at low abundance (one to eight copies per infected cell) in a non-linear configuration. The absence of trans-activation by Vmw65 therefore predisposes HSV to latency, as opposed to lytic growth, in HFL cells, resulting in the retention of the genome in a form resembling that found \textit{in vivo}.

Introduction

After infection of an organism with herpes simplex virus (HSV), the viral genome is maintained in a latent state in neuronal cells. Periodically, in response to various stimuli, virus can be reactivated from latency, often resulting in the development of a lesion at the appropriate peripheral site (reviewed by Roizman & Sears, 1987; Stevens, 1989). During latency, viral transcription can be detected only from the long repeat segments of the genome, giving rise to products known as the latency-associated transcripts (LATs) (Croen et al., 1987; Rock et al., 1987; Spivack & Fraser, 1987; Stevens et al., 1987). Latent HSV DNA is maintained in a non-linear configuration, probably as a circular episome, and is assembled into a chromatin-like structure (Rock & Fraser, 1983, 1985; Efstatiou et al., 1986; Mellerick & Fraser, 1987; Deshmane & Fraser, 1989). Thus, during latency, the transcription pattern and physical organization of the HSV type 1 (HSV-1) genome is very different from that found during productive infection.

To simplify molecular studies on the establishment, maintenance and reactivation stages of latency, tissue culture \textit{in vitro} models have been developed. The systems with the most obvious relevance to latency in animals and humans utilize foetal neurons from rats or primates (Wigdahl et al., 1983, 1984b; Wilcox & Johnson, 1987, 1988; Wilcox et al., 1990). Infection of cultured rat foetal neurons with HSV is non-productive provided an inhibitor of DNA synthesis is present for a few days after infection and nerve growth factor (NGF) is included in the culture medium. Removal of NGF, or addition of agents that antagonize its effect, results in reactivation of latent HSV (Wilcox & Johnson, 1987, 1988; Wilcox et al., 1990). Despite its obvious attractions, extensive use of this system is hindered by the limited quantity of neurons that can readily be obtained. An alternative approach is to use non-primary tissue culture cells, and human foetal lung (HFL) fibroblasts have been employed for this purpose (Wigdahl et al., 1981, 1982). We have developed a latency system in which HFL cells are infected at low m.o.i. with HSV type 2 (HSV-2) and incubated at the supraoptimal temperature of 42 °C for 6 days. During this treatment virus replication is not detected but cell monolayers remain intact. Cultures can subsequently be downshifted to 37 °C and maintained without production of virus, and HSV-2 is therefore considered to be latent (Russell & Preston, 1986). HSV-1 mutants that express only the immediate early (IE) proteins at 38.5 °C or 42 °C establish latency efficiently, showing that little, or no, viral gene expression is required for this event (Russell et al., 1987). Latent virus can be quantitatively reactivated by superinfection with HSV or human cytomegalovirus (Russell & Preston, 1986; Russell et al., 1987). Furthermore, the HSV-1 IE protein Vmw110 is necessary and sufficient for reactiva-
tion (Russell et al., 1987; Harris et al., 1989; Zhu et al., 1990). Therefore, in the HFL latency system, it appears that the viral genome is maintained in a quiescent state from which it can be reactivated only by a specific type of trans-activating protein, exemplified by Vmw110.

The concept that the establishment of latency does not require viral gene expression has received support from studies with the HSV-1 mutant in1814. The mutant possesses a 12 bp insertion in the gene encoding Vmw65, the virus structural protein that trans-activates transcription of IE genes (Ace et al., 1989). As a consequence of the mutation, trans-activation cannot be detected and production of IE proteins is reduced by up to 10-fold. The reduction impairs the ability of in1814 to initiate infection at low m.o.i., particularly in HFL cells, resulting in a high observed particle:p.f.u. ratio for virus stocks. The ratio on HFL cells is typically between 1 × 10^4 and 5 × 10^4, compared with values between 10 and 50 for wild-type (wt) HSV-1. When analysed in the mouse eye latency model, in1814 did not replicate detectably in the eye or trigeminal ganglion, yet latency was established efficiently and the virus was reactivated by ganglion explant (Steiner et al., 1990). The properties of in1814 in tissue culture and animal systems demonstrate that Vmw65 is important for the initiation of infection, especially at low m.o.i., and that the lack of trans-activation of IE genes does not prevent the establishment or reactivation of latency. The results support the attractive, but unproven, hypothesis that the block leading to latency is at the level of IE gene transcription (Kristie & Roizman, 1988; Kemp et al., 1990).

The HSV-1 mutant dl1403, which possesses a deletion spanning most of the coding sequences for Vmw110, exhibits a multiplicity-dependent failure to initiate infection and a consequent high particle:p.f.u. ratio, especially on HFL cells (Stow & Stow, 1986, 1989). Mutants in1814 and dl1403 are therefore apparently similar in their growth characteristics in tissue culture cells. Many of the dl1403 particles that enter cells but do not proceed to productive infection can nevertheless enter a latent state at 37 °C and can be reactivated by superinfection with human cytomegalovirus or varicella-zoster virus (Stow & Stow, 1989). Combining this observation with our previous studies utilizing incubation at 42 °C to establish latency, we describe here the use of in1814 to achieve high efficiency latency in HFL cells at either 37 °C or 42 °C.

### Methods

**Cells and viruses.** HFL (Flow 2002) cells were obtained from Flow Laboratories, and grown as described previously (Russell et al., 1987). The HSV-1 mutant in1814, and a rescued ‘revertant’ 1814R (Ace et al., 1989) were grown in BHK cells. Virus released into the culture medium was pelleted by centrifugation, and virus particle concentrations were determined by electron microscopy. Mutants dl1403 and tsK were grown in and titrated on BHK cells. Ultraviolet light inactivation of tsK, to reduce its titre at 31 °C by a factor of 5 × 10^5, was carried out as described by Notarianni & Preston (1982).

**In vitro latency.** Infection of HFL cells and incubation at 42 °C were performed as described previously (Russell & Preston, 1986). After infection at 37 °C, cytosine arabinoside (AraC) at 50 μg/ml, or aphidicolin at 5 μg/ml, was added, where appropriate, and maintained in culture medium. Medium was changed every 2 days.

**Reactivation of latency in1814.** Monolayers containing latent in1814 were superinfected with wt HSV-1 (strain 17) or dl1403, progeny virus was amplified by growth in BHK cells, and cytoplasmic nucleic acids were prepared, using methods described previously (Russell et al., 1987). The nucleic acid fraction was cleaved with BamHI in the presence of 30 μg/ml ribonuclease A.

Electrophoresis and Southern blot hybridization were carried out as described by Russell et al. (1987). The probe was a radiolabelled 1.6 kb HindIII/EcoRI fragment from mMC17 (Ace et al., 1988), containing the coding sequences for Vmw65.

**Analysis of latent in1814 DNA.** Replicate monolayers of HFL cells, amounting to a total of 1.5 × 10^7 cells, were infected with five particles of in1814 per cell and maintained at 42 °C or at 37 °C in the presence of AraC or aphidicolin for up to 4 days. For harvesting, monolayers were washed with ice-cold phosphate-buffered saline (PBS), scraped into PBS and collected by low speed centrifugation. Cells were lysed by addition of 1 ml of lysis buffer (10 mM-Tris-HCl pH 7.5, 2 mM-MgCl₂, 10 mM-NaCl, 0-5% NP40) and incubated at 4 °C for 5 min with intermittent mixing. Nuclei were pelleted by centrifugation at 13000 g for 2 min, the supernatant was discarded, and nuclei were resuspended in 10 ml of a solution containing 20 mM-Tris HCl pH 7.5, 2.5 mM-EDTA, 0.5% SDS and 250 μg/ml proteinase K. Incubation was continued at 37 °C for 16 h, and NaCl was added to 0.3 M. Nucleic acids were purified by two extractions with phenol:chloroform (1:1 v/v), one extraction with chloroform and precipitation by addition of 2 volumes of ethanol. DNA was collected by centrifugation, washed by the addition of ethanol and recentrifugation, and dried thoroughly. Pellets were resuspended in a buffer suitable for digestion with BamHI, according to the manufacturer’s specification, containing 50 μg/ml ribonuclease A. Samples were incubated at 37 °C for 16 h, and digestion was continued for a further 3 h at 37 °C after addition of more BamHI. Digested samples were added to a mixture of water and solid CsCl to give approximately 18 ml of a solution of density 1.708 g/ml, and centrifuged in a Sorvall TV865B vertical rotor at 40000 r.p.m, for 16 h at 15 °C. Gradients were fractionated and refractive indices were measured. Fractions corresponding to densities between 1.719 g/ml and 1.740 g/ml were pooled, 20 μg of Escherichia coli tRNA was added, and samples were dialysed at 4 °C against two changes of 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA. Nucleic acids were concentrated by shaking with an equal volume of butan-2-ol, butanol was thoroughly removed by evaporation, and nucleic acids were precipitated after addition of sodium acetate to 0-3 M followed by 2.5 volumes of ethanol. Precipitated nucleic acids were electrophoresed on a 0.6% agarose gel, blotted onto GeneScreen Plus nylon membrane (New England Nuclear) and hybridized with a radiolabelled DNA fragment as described previously (Russell et al., 1987; Harris et al., 1989). The probe used was purified HSV-1 BamHI k, radiolabelled with 32P by the random primer extension method (Feinberg & Vogelstein, 1983).
Results

In vitro latency with in1814

The latency system described previously (Russell & Preston, 1986) is limited by the fact that only a low initial multiplicity can be used. Thus, addition of more than 3 x 10^4 p.f.u. of HSV-1 or HSV-2 to a monolayer of 10^6 HFL cells results in cell degeneration during incubation at 42 °C. The same problem arises after infection with dl1403, which has previously been shown to remain latent after infection of HFL cells at low multiplicity (Stow & Stow, 1989). We reasoned that cytotoxicity is due, at least in part, to viral gene products expressed after infection, and therefore investigated the properties of in1814, which has a defect in IE transcription, the earliest stage of viral gene expression.

HFL cell monolayers were infected with 0.1 particles of in1814 or 1814R (a rescued 'revertant', essentially equivalent to wt HSV-1) per cell and incubated at 42 °C for 6 days followed by 37 °C for 4 days, the standard in vitro latency protocol (Russell & Preston, 1986). Monolayers were then mock-infected or superinfected with tsK, overlaid with medium containing neutralizing human serum (EHu5), and incubated at 38.5 °C for 2 days. Cells were then stained and plaques counted. The results (Table 1) show that both in1814 and 1814R were retained in a non-infectious state and could be reactivated by superinfection with tsK in a multiplicity-dependent manner. Although the number of p.f.u. of in1814 added (5) was much lower than that of 1814R (5500), on the basis of particles the two viruses established latency with comparable efficiencies.

The possibility of establishing latency at 37 °C, a physiological temperature, was also investigated. HFL monolayers were infected with in1814 and maintained at 37 °C, with or without AraC in the growth medium, or at 42 °C. At various times, cells were then infected with tsK or u.v.-irradiated tsK, or mock-infected, incubated at 38.5 °C for 2 days under EHu5, and stained (Table 2). At the start of the experiment, co-infection with either irradiated or non-irradiated tsK complemented in1814, as expected from previous results showing that provision of Vmw65 complements in1814 (Ace et al., 1989). After 1 day, however, in1814 could be reactivated efficiently by superinfection with tsK but not irradiated virus, suggesting that the genome was no longer sensitive to transactivation by Vmw65. Continued incubation for up to 4 days gave equivalent results, with the exception that virus was detected after incubation for 4 days at 37 °C without AraC. Breakthrough of this type is probably due to replication from a single plaque, as occasionally occurs after establishment of latency at 42 °C (Russell & Preston, 1986). A significant drop in the amount of virus that could be reactivated by superinfection with tsK was noted after incubation of cultures maintained at 37 °C for 1 day, although the reasons for this observation are not clear.

Therefore, at low initial m.o.i., in1814 is maintained at 37 °C in a quiescent state and can be reactivated by superinfection, like in1814 at 42 °C or dl1403 at 37 °C (Stow & Stow, 1989). The inclusion of AraC in the growth medium did not affect the retention of latent virus at 37 °C.

Table 1. Latency with in1814 and 1814R

<table>
<thead>
<tr>
<th>Superinfection</th>
<th>M.o.i.†</th>
<th>Mock</th>
<th>1814R</th>
<th>in1814</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tsK</td>
<td>0-1</td>
<td>0</td>
<td>ND‡</td>
<td>200§</td>
</tr>
<tr>
<td>tsK</td>
<td>0-01</td>
<td>0</td>
<td>12</td>
<td>19</td>
</tr>
</tbody>
</table>

* Monolayers were initially infected at 0.1 particle/cell and incubated at 42 °C for 6 days followed by 37 °C for 4 days.
† Multiplicity of superinfection, p.f.u./cell.
‡ ND, No data.
§ Plaque numbers after superinfection, expressed as values from three separate determinations.

Table 2. Reactivation of latent in1814

<table>
<thead>
<tr>
<th>Time of superinfection (days)</th>
<th>P.f.u. reactivated per 10^5 particles of in1814 after superinfection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Latency at 42 °C</td>
<td></td>
</tr>
<tr>
<td>0†</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0-1</td>
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<tr>
<td>2</td>
<td>&lt;0-1</td>
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<tr>
<td>3</td>
<td>0-5</td>
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<tr>
<td>4</td>
<td>&lt;0-1</td>
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<tr>
<td>B. Latency at 37 °C with AraC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt;0-1</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0-1</td>
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<tr>
<td>3</td>
<td>&lt;0-1</td>
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<tr>
<td>4</td>
<td>&lt;0-1</td>
</tr>
<tr>
<td>C. Latency at 37 °C without AraC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt;0-1</td>
</tr>
<tr>
<td>2</td>
<td>0-5</td>
</tr>
<tr>
<td>3</td>
<td>0-1</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
</tr>
</tbody>
</table>

* HFL monolayers were initially infected with 10^6, 10^5 or 10^4 particles of in1814 per monolayer of 10^6 cells. At various times, superinfection was carried out with 0.1 p.f.u. of tsK or u.v.-irradiated tsK (based on the titre before u.v. treatment) per cell. Numbers represent the means of duplicate determinations.
† The day 0 values are the same for parts A, B and C.
Reactivation of latent *in1814* requires Vmw110

Reactivation of latent HSV-2 after incubation of cells at 42 °C requires the HSV-1 IE protein Vmw110 (Russell *et al.*, 1987; Harris *et al.*, 1989; Zhu *et al.*, 1990). To determine whether this is also the case for reactivation of *in1814*, HFL cells harbouring latent *in1814* were superinfected with either wt HSV-1 or *dl1403*. Progeny were grown for one cycle in BHK cells, and virion DNA was analysed by Southern blot hybridization. *In1814* DNA possesses an additional *BamHI* site, due to the inserted linker, thus *BamHI* cleavage results in the production of novel bands of approximately 5 kbp and 3 kbp from the 8 kbp *BamHI* f. As shown in Fig. 1, infection with wt HSV-1, but not *dl1403*, reactivated latent *in1814* after establishment of latency at 42 °C or 37 °C, irrespective of the presence of AraC. Therefore, *in vitro* latency with *in1814* is stable to superinfection with *dl1403* but not with wt HSV-1.

Latent *in1814* DNA is in a non-linear form

During latency in mouse or human ganglia, HSV DNA exists in a non-linear configuration (Rock & Fraser, 1983; Efstathiou *et al.*, 1986), yet only linear molecules were detected in a previous study of latency *in vitro* (Wigdahl *et al.*, 1984a). To investigate the state of latent *in1814* DNA in HFL cells, it was first necessary to increase the initial multiplicity. Observation of monolayers after infection with increasing multiplicities of *in1814* suggested that five, and often 10, particles of virus per cell could be added without detectable c.p.e. Infectious centre assays showed that, at a multiplicity of five particles per cell, 13 to 21% of cells contained a potentially active genome that could be reactivated by superinfection with tsK (results not shown). HFL monolayers were therefore infected with five particles of *in1814* per cell and incubated at 42 °C or at 37 °C in the presence of AraC or aphidicolin. At 5 h, 2 days and 4 days after infection, nuclear DNA was extracted, enriched for HSV sequences by equilibrium density gradient centrifugation, and analysed by Southern blot hybridization (Fig. 2).

Reconstructions with genomic HSV-1 DNA demonstrated the presence of *BamHI* k, the joint-spanning fragment, and *BamHI* q and s, the genomic termini of strain 17. In the samples from infected cells, however, *BamHI* q and s were detected only in DNA extracted at 5 h post-infection, and then at lower concentrations relative to *BamHI* k. At 2 and 4 days post-infection only the joint fragment, *BamHI* k, was detected, irrespective of whether latency was established by incubation at 42 °C or at 37 °C in the presence of AraC or aphidicolin.

Calculation of the amount of *in1814* DNA in latently infected cultures from the intensities of hybridization signals gives quantities of 0· to 0·5 copies per cell at 37 °C and 0·2 to 1·0 copies per cell at 42 °C in a monolayer of 10^6 cells. Comparison of this value with the data from infectious centre assays, which suggested that between 13% and 21% of cells contain a potentially active genome, lead to the conclusion that each infected
cell retains between one and eight genomes of \textit{in1814}. The twofold greater recovery of DNA at 42 °C correlates with the increased reactivation of \textit{in1814} at this temperature (Table 2). Therefore, \textit{in1814} DNA is retained at relatively low abundance in a non-linear configuration during latency \textit{in vitro}.

**Discussion**

Two main points emerge from the studies presented here. First, \textit{in1814} establishes latency \textit{in vitro} in the same way as wt HSV, with the advantage that higher m.o.i. can be used at both 42 °C or 37 °C. Second, the genome is maintained in a non-linear configuration, as found during latency \textit{in vivo} (Rock & Fraser, 1983) and in contrast to previous results with an apparently related \textit{in vitro} system (Wigdahl \textit{et al.}, 1984a).

The ability to carry out medium term experiments on HFL monolayers infected with five particles of \textit{in1814} per cell is thought to be due to the early block to gene expression, which decreases the amounts of IE proteins produced and hence prevents cell destruction. At this multiplicity, however, it would be expected that on average no more than one particle enters each cell since wt virus preparations rarely exhibit a particle : p.f.u. ratio of less than 10. This prediction is borne out by the infectious centre assay, which suggests that 13 to 21% of cells receive a potentially active virus, an effective particle : p.f.u. ratio of 24 to 38.

The fact that \textit{in1814} establishes latency as efficiently as wt viruses on the basis of particles emphasizes that the insertion mutation does not affect entry and uncoating of the genome. Thus, the reduction of five- to 10-fold in IE transcription (Ace \textit{et al.}, 1989) is critical in determining whether lytic or latent infection ensues when one particle enters a cell. Presumably, unless threshold levels of IE proteins are reached to initiate the expression of early and late genes, cellular processes intervene to convert the genome to the latent state. A similar explanation has been proposed for events leading to latency in the mouse (Steiner \textit{et al.}, 1990).

The establishment of latency in HFL cells is facilitated by both the \textit{in1814} mutation and incubation at 42 °C. In

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**Fig. 2. Latent \textit{in1814} DNA.** HFL monolayers were mock-infected or infected with five particles of \textit{in1814} per cell and incubated at 42 °C (lanes 4 to 7), or at 37 °C with AraC (lanes 11 to 14) or aphidicolin (lanes 18 to 21). Mock-infected cells were harvested after 4 days (lanes 4, 11 and 18). Infected cells were harvested after 5 h (lanes 5, 12 and 19), 2 days (lanes 6, 13 and 20) or 4 days (lanes 7, 14 and 21). Density gradient-enriched DNA was cleaved with \textit{Bam}HI and probed with radiolabelled \textit{Bam}HI \textit{k}. Reconstructions consisting of 5, 1.5 and 0.5 ng of HSV-1 DNA are shown in lanes 1 to 3, 8 to 10 and 15 to 17. \textit{Bam}HI \textit{k}, the joint-spanning fragment, and \textit{Bam}HI \textit{q} and \textit{s}, the termini, are indicated.
initial experiments, it was found that incubation of HFL cells at 42 °C for 2 days immediately after infection reduced the titre of 1814R by approximately 1000-fold (similar to the effect of the in1814 mutation), whereas the drop in in1814 titre was only fourfold (R. A. Harris, unpublished results). An important effect of incubation at 42 °C may, therefore, involve disruption to the activity of Vmw65.

The observation that in1814 cannot be complemented by Vmw65 once latency has been established suggests that IE promoters rapidly become insensitive to transactivation. This may reflect an overall ‘silencing’ of the genome, perhaps by conversion to a chromatin-like structure (Deshmanc & Fraser, 1989), the presence of a specific inhibitor of IE transcription, as exists in mouse C1300 neuroblastoma cells (Kemp et al., 1990), or a combination of the two. Whatever the cause of silencing, gene expression remains sensitive to trans-activation by Vmw110. We have been unable to detect gene expression from the in1814 genome during latency at 42 °C or 37 °C, using the polymerase chain reaction with primers specific for thymidine kinase or LATs (R. A. Harris, unpublished observations). Thus, in HFL cells, expression was not detected from any HSV genes, including LAT, in contrast to the situation in vivo. This finding may reflect the neuron-specific nature of the LAT promoter (Batchelor & O’Hare, 1990).

The known particle: p.f.u. ratios of in1814 stocks predict that addition of five virus particles per HFL cell would result in the formation of between 100 and 500 plaques on a monolayer of 10^6 cells (Ace et al., 1989). To prevent these interactions from supporting productive infection and forming a significant background, it was necessary to prevent virus replication by addition of an inhibitor. This procedure reduces the number of cells containing potentially ‘non-latent’ viruses to 100 to 500 in a population of 1.3 × 10^5 to 2.1 × 10^5 cells harbouring latent virus. Thus the background contributed by ‘non-latent’ in1814 is not more than 0·05% to 0·4%. From the data in Table 2 and Fig. 1 and 2, it is clear that AraC does not fundamentally alter the in vitro latency system.

Conversion of the genome to a non-linear form can be detected as soon as 5 h after infection, and is complete by 2 days. The amount of DNA recovered from cultures remained constant or decreased slightly during this period, suggesting that DNA replication did not occur during latency. The calculated low genome copy number supports this conclusion. In a related study, analysis of the HSV-2 genome during latency at 42 °C also showed the retention of non-linear DNA at low copy number (C. M. Preston & J. Russell, unpublished results). The HSV-2 joint fragment was present at approximately double the molarity of a unique fragment, suggesting that the genome was present in a circular form (C. M. Preston & J. Russell, unpublished results). By analogy, it is likely that in1814 is present as a circular episome during latency at 42 °C or 37 °C. It is not clear why our observations differ from those of Wigdahl et al. (1984a); although the procedures used in their system are similar to ours, the differences may be crucial.

Latency with in1814 in vitro reflects the in vivo phenomenon in three ways. As found in the mouse eye model (Steiner et al., 1990), the mutant is impaired in its ability to undergo productive infection but is predisposed to latency. The genome is non-linear, and gene expression is severely depressed. These findings support the view that a failure of Vmw65 to activate IE transcription may be the normal route to establishment of latency. The use of in1814 at relatively high m.o.i. will enable further molecular studies to be performed with the system, thereby providing information that should be relevant to latency in vivo.

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References


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