Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants

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The development and utilization of a tissue culture system for the analysis of quiescent, nonreplicating herpes simplex virus type 1 (HSV-1) genomes is described. It was demonstrated previously that the HSV-1 Vmw65 mutant in1814, which is impaired for immediate early (IE) transcription, was retained for many days in human fetal lung (HFL) fibroblasts in a quiescent 'latent' state. Molecular analysis of the viral genome was not possible, however, due to residual expression of IE proteins and consequent cytotoxicity at high m.o.i. In the study reported here, IE transcription was reduced further by pretreatment of cells with interferon-α (IFN-α) and by the use of mutant in1820, a derivative of in1814 in which the Vmw110 promoter was replaced by the Moloney murine leukaemia virus (MomaLV) enhancer. The MomaLV enhancer was not expressed under IE conditions; thus in1820 was more impaired for replication than in1814 and behaved as if deficient for both Vmw65 and Vmw110. In cells pretreated with IFN-α and subsequently infected with in1820 cytotoxicity was overcome, enabling a tissue culture system to be developed in which all cells stably retained at least one quiescent viral genome. To assist the analysis of gene expression, in1820 was further modified by insertion of the Escherichia coli lacZ gene controlled by the human cytomegalovirus enhancer (mutant in1883) or the HSV-1 immediate early Vmw110 promoter (in1884). Expression of β-galactosidase was not detected after infection of IFN-α-pretreated cells with in1883 or in1884 but could be induced in almost all cells containing a viral genome, by superinfection of cultures. In1820-derived viruses were retained for at least 9 days and were not reactivated by subculture of cells. A regular arrangement of nucleosomes, as found in cellular chromatin, was not detected on the viral genome at the thymidine kinase locus. The non-linear genome was a template for reactivation with no requirement for prior conversion to a linear form. A small number of remaining linear genomes resulted from incomplete uncoating of input virus.

Introduction

Transcription of herpes simplex virus type 1 (HSV-1) immediate early (IE) genes is stimulated by the virus structural protein Vmw65 (also known as VP16 or α-TIF), resulting in efficient production of five IE proteins shortly after infection (Post et al., 1981; Campbell et al., 1984). Vmw65 forms a complex at the IE-specific sequence TAATGARAT (R is a purine) with at least two cellular proteins, Oct-1 and HCF, thereby bringing the C-terminal activating region of Vmw65 into proximity with cellular factors required for initiation of transcription (McKnight et al., 1987; O'Hare & Goding, 1988; Preston et al., 1988; Triezenberg et al., 1988; Sadowski et al., 1988; Stern et al., 1989; Katan et al., 1990; Kristie & Sharp, 1990; Wilson et al., 1993). Three of the IE proteins, Vmw175 (ICP4), Vmw110 (ICP0) and Vmw63 (ICP27), are known to be important regulators of viral gene expression. Vmw175 and Vmw63 are essential for virus replication, exerting effects at the transcriptional and post-transcriptional levels, respectively (Preston, 1979b; Watson & Clements, 1980; Dixon & Schaffer, 1980; Sacks et al., 1985; Everett, 1987; Sandri-Goldin & Mendoza, 1992), whereas Vmw110 is not essential but enables virus replication to be initiated efficiently, especially after infection at low m.o.i. (Stow & Stow, 1986; Sacks & Schaffer, 1987; Everett, 1989). Vmw110 is a potent activator of gene expression which acts on a wide variety of virus- or plasmid-borne promoters in a manner that is apparently not sequence-specific (Everett, 1984; O'Hare & Hayward, 1985; Cai & Schaffer, 1992; Chen & Silverstein, 1992).

At present, three properties of Vmw65 have been...
documented. The protein is a major structural component of the virus tegument and is therefore required for the formation of mature virions (Heine et al., 1974; Ace et al., 1988; Weinheimer et al., 1992). More recently, Vmw65 has been shown to associate with the virion host shutoff (vhs) protein (Smibert et al., 1994). The third function, activation of IE transcription, has been studied in great detail at the molecular level but only one virus mutant defective in this process, in1814, has been described to date (Ace et al., 1989). The Vmw65 protein specified by in1814 is deficient in transactivation of IE transcription due to the presence of a four-amino-acid insertion within a domain required for binding to Oct-1 and HCF (Ace et al., 1988; Greaves & O’Hare, 1990; Hayes & O’Hare, 1993). As a consequence of reduced IE transcription, in1814 is unable to initiate productive infection efficiently and thus, at low m.o.i., only a small proportion (0.1–1 %, depending on the cell type) of virus–cell interactions result in virus replication (Ace et al., 1989; Daksis & Preston, 1992). The vast majority of infected cells harbour the in1814 genome in a stable quiescent state that has been termed ‘in vitro latency’ (Harris & Preston, 1991). By 1 day after infection, in1814 genomes become insensitive to activation by Vmw65 or hexamethylene bisacetamide (HMBA), even though these agents effectively complement the defect in virus replication if applied at the time of infection (Ace et al., 1989; Harris & Preston, 1991; McFarlane et al., 1992). It is possible, however, to recover in1814 from cultures several days after initial infection by superinfection with HSV-1, provided the superinfecting virus expresses functional Vmw110 (Harris & Preston, 1991). Entry of in1814 into the quiescent state is probably due mainly to reduced synthesis of Vmw110, since mutants deleted for the Vmw110 gene have a phenotype similar to that of in1814 (Stow & Stow, 1986, 1989; Everett, 1989).

HSV latency in the peripheral nervous system of animals and humans is characterized by repression of all regions of the genome except for that encoding the latency-associated transcripts (LATs) (Croen et al., 1987; Spivack & Fraser, 1987; Stevens et al., 1987; Fraser et al., 1992). It has been suggested that a block to IE transcription, possibly imposed by inhibitory TAATGARAT-binding proteins, is primarily responsible for the abortion of lytic replication and hence the establishment of latency (Roizman & Sears, 1987; Kristie & Roizman, 1988; Kemp et al., 1990; Lillycrop et al., 1991, 1994; Sears et al., 1991). In support of this hypothesis, in1814 establishes latency after inoculation into mice even though productive infection in neurons is impaired, suggesting that IE gene expression is not required for latency (Steiner et al., 1990; Valyi-Nagy et al., 1991; Ecob-Prince et al., 1993). The phenotype of in1814 in tissue culture cells is therefore apparently similar to that observed in mice: productive replication is reduced and virus retention in a latent state is favoured. In1814 is the only virus mutant currently available to investigate the effects of reducing IE gene expression in tissue culture cells; therefore studies on the structure and function of the quiescent in1814 genomes are relevant to an understanding of the suppression of gene expression that occurs during latency.

At present, the structural basis for the inactivity, and insensitivity to inducers, of quiescent in1814 genomes retained in fibroblasts is unclear. It is known that viral DNA is sequestered in a non-linear, probably circular, form analogous to that found during latency in vivo (Rock & Fraser, 1983; Efstathiou et al., 1986; Harris & Preston, 1991), but information is lacking on the nature of viral nucleoprotein complexes, either at specific promoters or the entire genome. Analysis of this problem presents technical difficulties because although the predominant outcome of infection is failure of in1814 to replicate, the small number of productive interactions that occur can form a significant background, especially if viral DNA replication occurs, thereby obscuring detection of the quiescent genomes. Furthermore, at high m.o.i. in1814 is cytopathic due to expression of IE proteins (Johnson et al., 1994). We describe here methods which reduce IE transcription from viruses containing the in1814 mutation, enabling the establishment of viable human fibroblast cultures containing at least one viral genome per cell. The methodology has been used to obtain novel information on the structure and expression of the quiescent genome.

**Methods**

**Cells.** Flow 2002 human fetal lung (HFL) fibroblasts (Flow Laboratories) were propagated in Eagle’s medium supplemented with fetal calf serum at 10% (EF10) or 5% (EF5) or with 5% human serum (EHu5). BHK-21 (clone 13) cells were grown in Eagle’s medium supplemented with 10% new born calf serum and 10% tryptophan phosphate. Human 143 thymidine kinase deficient (TK-) cells, transformed to a TK* phenotype by transfection of plasmid pTK1 (encoding the HSV-1 TK gene; Wilkie et al., 1979), were kindly provided by M. McFarlane (University of Glasgow, Molecular Biology, UK) and grown in EF10 containing 0.1 mM-hypoxanthine, 16 μM-thymidine, 0.4 μM-aminopterin and 3 μM-glycine. All cell culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

**Plasmids.** To prepare HSV recombinants containing the Escherichia coli lacZ gene inserted within the TK gene, plasmid pMJ27 was first constructed. The HindIII site within the vector sequences of pGX166 (pTK1 modified by the insertion of an Xhol linker at the SacI site within the TK coding sequences; kindly provided by V. G. Preston) was destroyed by cleavage, end-filling with Klenow enzyme and religation, to yield pOX166ΔH3. The E.coli lacZ gene plus simian virus 40 (SV40) promoter and enhancer was excised from plasmid pF33 (Rixon & McLauchlan, 1990) as a 4073 bp BamHI–XhoI fragment and cloned between the BamHI and XhoI sites of pUC18 (previously modified by insertion of an Xhol linker into the Smal site) to yield pUC18lacZ. The lacZ gene, together with the SV40 promoter and
polyadenylation signals, was excised from pUC18lacZ as a SalI–XhoI fragment and cloned into the XhoI site of pGX166ΔH3. A plasmid in which the direction of lacZ transcription was opposite to that of TK was selected and designated pMJ27. Plasmid pMJ27 contains unique XhoI and HindIII sites flanking the SV40 promoter plus enhancer, and has a unique XhoI site downstream of the SV40 polyadenylation signal.

The human cytomegalovirus (HCMV) Towne strain enhancer was cloned as a 760 bp Sau3AI fragment (Stinski & Roehr, 1985) from plasmid pHD101-4 (kindly provided by E. Blair, Wellcome Research Laboratories, Beckenham, UK) into the BamHI site of pUC18 (from which the SpII site had been removed by treatment with Klenow enzyme and religation), excised as an EcoRI (end-filled)–HindIII fragment and cloned between the XhoI (end-filled) and HindIII sites of pMJ27 to replace the SV40 enhancer, yielding plasmid pMJ101. The HSV-1 Vmw110 promoter was excised as an 836 bp BglII (end-filled)–SacI fragment from pJR3 (Everett, 1984) and cloned between the SpII (Klenow-treated) and SacI sites of pUC18. The promoter was then removed as an EcoRI (end-filled)–HindIII fragment and cloned between the XhoI (end-filled) and HindIII sites of pMJ27 to yield plasmid pMJ102. The structures of pMJ27, pMJ101 and pMJ102 are shown in Fig. 1.

Viruses. The HSV-1 strain 17 mutant in1814 contains a 12 bp insertion in the coding sequences for Vmw65 (Ace et al., 1989). Mutant in1820 was derived from in1814 essentially by replacing both copies of a 971 bp Ncol–SacI region containing the promoter for Vmw110 [nucleotide positions 124105–125076 in the inverted long repeat (IR-) copy; Perry & McGeoch, 1988] with a 760 bp DNA fragment containing the Moloney murine leukaemia virus (Molmuv) enhancer and promoter (Lang et al., 1983). A detailed description of the construction of in1820 will be presented elsewhere. 1814R was constructed by rescue of the Vmw65 mutation of in1814 (Ace et al., 1989) and in1825 was similarly produced by rescue of the Vmw65 mutation of in1820.

To construct mutants in1883 and in1884, in1820 DNA was cotransfected into BHK cells with SacI-cleaved pMJ101 and pMJ102, respectively. Progeny viruses expressing β-galactosidase (β-gal) were identified by the development of blue plaques in the presence of X-Gal (end-filled) and purified by three rounds of enrichment for lacZ-containing viruses. Final plaque isolates were grown as small scale cultures and DNA was purified from infected cells, cleaved with EcoRI and analysed by Southern transfer and hybridization, using 32P random primer extension (Feinberg & Vogelstein, 1983), in hybridization solution. The probes used were: purified HSV-1 EcoRI n, spanning the TK gene; BamHI k, spanning the joint; or a 500 bp BglI–SalI fragment from the Molmuv enhancer (Lang et al., 1983). Hybridization was continued at 70 °C for 20–24 h and membranes were washed once at 70 °C with hybridization solution followed by two washes at 70 °C with 0.2× SSC/0.1% SDS (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), each wash taking 45 min. Membranes were washed briefly with deionized water, dried and exposed to X-omat S film (Kodak) for autoradiography using an intensifying screen. Standard DNA for hybridization was extracted from partially purified in1820 virus particles released into culture medium. A portion was digested with BamHI and ribonuclease A, extracted with phenol–chloroform, ethanol precipitated and dissolved in deionized water. The DNA concentration was determined spectrophotometrically, and the measured value confirmed by electrophoresis of uncloned in1820 DNA with linearized plasmids of known concentrations.

Quantification of hybridization was achieved by phosphorimaging analysis, using Molecular Dynamics ImageQuant software. To calculate genome copy numbers and the relative proportions of linear and non-linear DNA, measured values of band peak areas were first corrected for variations in transfer and hybridization efficiencies of joint and terminal fragments by comparison with in1820 DNA standards. If x and y are the corrected values for joint and terminal fragments, respectively, the relative number of linear genomes is y and non-linear genomes 0.5(x–y). The total number of genomes was given by the sum of linear and non-linear molecules.

Nucleic acid digestion of nuclei. Nuclei prepared as described above were resuspended in 10 mM-Tris–HCl pH 7.5, 2 mM-MgCl2, 10% (w/v) sucrose, and micrococcal nuclease (MN; Pharmacia P-L) or DNase I (DN-EP; Sigma) added at various concentrations. MN digestion was carried out in 20 mM-PiPES pH 7.0, 0.25 mM sucrose, 10 mM-NaCl, 1 mM-MgCl2, 1 mM-CaCl2, 5 mM-mercaptoethanol, 0.1 mM-PMSF. DNase I digestion was carried out in 10 mM-PiPES pH 7.0, 0.25 mM sucrose, 10 mM-NaCl, 3 mM-MgCl2, 5 mM-mercaptoethanol, 0.1 mM-PMSF. After incubation at 37 °C for fixed times of 2–30 min, reactions were stopped by addition of ice-cold EDTA to a final concentration of 12 mM. Samples were digested by addition of 0.4 mg/ml trypsin–HCl pH 7.5, 25 mM-EDTA, 0.5% SDS and 100 μg/ml proteinase K and
incubation at 37°C for 5–16 h. DNA was extracted and either electrophoresed directly on 2% agarose gels or, after cleavage with BamHI, on 0.6% agarose gels. Southern transfer and hybridization was carried out as described above. DNA analysed on 2% gels was partially depurinated by incubation of the gel in 0.25 M-HCl for 15 min prior to transfer.

Analysis of IE RNA. Monolayers of HFL cells were infected with 10 p.f.u. of virus per cell in the presence of 50 μg of cycloheximide/ml. After incubation at 37°C for 5 h in the continuous presence of cycloheximide, RNA was extracted and analysed by dot-blot hybridization using radiolabelled probes specific for the IE genes or β-actin, as described previously (Daksis & Preston, 1992).

Histochemical staining for β-galactosidase. Culture medium was removed and monolayers fixed by addition of 1% glutaraldehyde, dissolved in PBS, for 1 h at room temperature. After removal of glutaraldehyde, monolayers were washed twice with PBS, and reaction mixture (5 mM-potassium ferricyanide, 5 mM-potassium ferrocyanide, 2 mM-MgCl₂, 0.01% NP40, 1 mg/ml X-Gal, all dissolved in PBS) was added. After incubation for an appropriate time (usually 3–5 h) at 37°C, monolayers were washed with water. Monolayers were counterstained by addition of Carmaun stain (25 g/l carnine; 25 g/l aluminium potassium sulphate; 2.5% (v/v) glacial acetic acid) and incubation at 4°C for 1–2 days, washed with water, dried and mounted.

Results

Properties of mutants in1820, in1883 and in1884

As described in the Introduction, we wished to examine the quiescent viral genomes which are retained in HFL cells after infection with in1814. We considered that it was essential to meet two criteria: first, for structural studies the viral genome must, realistically, be present at one or more copies per cell; and, second, evidence must be provided that the genomes detected were biologically relevant. In practice, the latter proposition demanded the demonstration that most of the quiescent genomes detected were capable of resuming gene expression in response to an appropriate stimulus. Since there was no model in which these objectives had been attempted or achieved, it was necessary to develop a new cell culture system for the study of quiescent genomes. Extending our unpublished observations, which were in agreement with those reported by Johnson et al. (1992, 1994), that production of IE proteins resulted in cell degeneration, we attempted further to reduce IE transcription after infection of HFL cells with in1814. The first step involved specifically reducing Vmw110 synthesis, since mutants deleted only for this gene can also persist in a quiescent state (Stow & Stow, 1989). Mutant in1820 was derived from in1814 by replacement of the Vmw110 gene promoter with the Momulv promoter and enhancer. Additional insertion, at the TK locus, of the E. coli lacZ gene controlled by either the HCMV enhancer or the HSV-1 Vmw110 promoter yielded in1883 and in1884, respectively. When titrated on HFL cells, in1820 and its derivatives were more impaired for replication than in1814, with virus stocks exhibiting an approximately 10-fold higher particle:p.f.u. ratio (results not shown). This observation was extended by analysis of IE RNA accumulation after infection of HFL cells in the presence of cycloheximide (Fig. 2). As shown previously (Ace et al., 1989; Daksis & Preston, 1992), levels of IE RNAs were lower in in1814-infected cells compared with 1814R-infected cells. The amounts of IE RNAs encoding Vmw175, Vmw68 and Vmw63 were similar in in1814- and in1820-infected cells, but IE RNA encoding Vmw110

<table>
<thead>
<tr>
<th>Probe</th>
<th>110</th>
<th>63</th>
<th>175</th>
<th>68</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1814R</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>in1814</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>in1820</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>in1825</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 2. IE RNA synthesis. HFL cells were infected with 10 p.f.u. of virus/cell or mock-infected, and incubated at 37°C for 5 h in the presence of 50 μg of cycloheximide/ml. RNA was extracted, 1 μg was applied to nitrocellulose membranes and hybridization with gene-specific probes was carried out.
was not detectable in \textit{in}1820-infected cells. HFL cells infected with \textit{in}1825, the resurgent of \textit{in}1820 at the Vmw65 locus, also showed no detectable production of Vmw110 RNA. Thus, the Momul enhancer was not recognized as an IE promoter, with the consequence that \textit{in}1820 behaved as a Vmw65/Vmw110 double mutant in HFL cells. A detailed description of the properties of \textit{in}1820 and \textit{in}1825 will be published elsewhere.

Viruses with mutations in Vmw110 or Vmw65 exhibit low titres in plaque assays because initiation of productive replication is extremely inefficient at low m.o.i. (Stow \& Stow, 1986; Sacks \& Schaffer, 1987; Ace \textit{et al.}, 1989; Everett, 1989). This causes difficulties in accurately assessing the effective input m.o.i., as discussed by Cai \& Schaffer (1992). To overcome this problem, we standardized virus preparations by titration on BHK cells in the presence of 3 mM-HMBA, which allows \textit{in}1814 and \textit{in}1820 to be assayed essentially as wild-type viruses (McFarlane \textit{et al.}, 1992). This procedure provided a sound basis for comparison of virus stocks but, as shown below, a value approximately three times higher was obtained by co-infection with 1 p.f.u. of the HSV-1 mutant \textit{tsK} per cell and subsequent incubation at 37 °C. Co-infection with \textit{tsK} provides both Vmw65 and Vmw110, thereby fully complementing the defects of \textit{in}1820. When ‘p.f.u.’ was measured by complementation with \textit{tsK}, particle:p.f.u. ratios of \textit{in}1814- and \textit{in}1820-based mutants were equivalent to those of wild-type HSV-1 stocks.

Use of IFN-α pretreatment to reduce IE gene expression

Although the use of \textit{in}1820, instead of \textit{in}1814, increased the number of virus particles that could be added to monolayers, cell degeneration still occurred after infection with \textit{10}^6 p.f.u. per plate (approximately 1 p.f.u. per cell). Further reduction of IE gene expression was achieved by pretreatment of cells with IFN-α, a procedure known to inhibit IE transcription and reduce cell killing (Mittnach \textit{et al.}, 1988; Oberman \& Panet, 1989; Johnson \textit{et al.}, 1992).

Monolayers were pretreated with \textit{10}^6 units of IFN-α/ml for 16–20 h prior to infection with \textit{1814R}, \textit{1814}, \textit{in}1814, \textit{in}1820 or \textit{in}1883 (Table 1). The titre of \textit{1814R} was reduced by approximately 7-fold after IFN-α pretreatment. The titre of \textit{in}1814 was reduced to an even greater extent, although application of \textit{10}^6 p.f.u. resulted in cell destruction irrespective of IFN-α pretreatment. When infected with \textit{10}^6 p.f.u. of \textit{in}1820, however, IFN-α-pretreated monolayers withstood infection and produced only five plaques per plate, whereas at lower m.o.i. no plaques were observed. The use of \textit{in}1883, which permitted subsequent staining for β-gal expression, provided a more sensitive means of detecting viral replication or gene expression but even in this case only 16 ‘plaques’, some of which were single cells, were observed on plates infected with \textit{3 × 10}^4 p.f.u. of \textit{in}1883.

Pretreatment with IFN-α thus permits the survival of cultures after infection with at least 1 p.f.u. of \textit{in}1820 per cell. Since the titre of \textit{in}1820 after complementation with \textit{tsK} was approximately 3-fold higher than the value measured on BHK cells in the presence of HMBA, it follows that an input m.o.i. of \textit{3 × 10}^6 p.f.u. per monolayer of \textit{8 × 10}^5 cells should deliver an average of at least one genome per cell. Under these circumstances, molecular analysis of the genome should be possible, provided the small number of potentially active genomes (16 after infection with \textit{3 × 10}^6 p.f.u. per plate; Table 1) was not amplified by replication. An inhibitor of DNA replication, AraC, was therefore added after infection of IFN-α-pretreated monolayers.

To determine whether IFN-α pretreatment or the presence of AraC exerted significant influence on the retention of virus, HFL cells were infected with \textit{30} p.f.u. of \textit{in}1883, incubated with or without AraC for 2 days, then superinfected with \textit{tsK} (Table 2). Co-infection with \textit{tsK} resulted in 85 plaques on untreated cultures or 65 on IFN-α-pretreated monolayers. \textit{In}1883 was recovered by superinfection after 2 days, as expected from previous studies (Harris \& Preston, 1991); a process referred to as ‘reactivation’ hereafter. Treatment with AraC did not reduce the reactivation of \textit{in}1883 and IFN-α pretreatment had only a small effect. Crucially, reactivation of \textit{in}1883 from cells treated with both agents (140 plaques) was close to that observed in untreated cells (178 plaques), demonstrating that the combination of IFN-α pretreatment and AraC addition did not significantly affect retention and reactivation of the \textit{in}1883.

**Table 1. Inhibition of HSV replication by IFN-α pretreatment**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inoculum (p.f.u. per plate)</th>
<th>−IFN-α</th>
<th>+IFN-α</th>
</tr>
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<tbody>
<tr>
<td>1814R</td>
<td>\textit{1 × 10}^8</td>
<td>CPE†</td>
<td>196</td>
</tr>
<tr>
<td>1814R</td>
<td>\textit{1 × 10}^6</td>
<td>98</td>
<td>14</td>
</tr>
<tr>
<td>\textit{in}1814</td>
<td>\textit{1 × 10}^6</td>
<td>CPE</td>
<td>CPE</td>
</tr>
<tr>
<td>\textit{in}1814</td>
<td>\textit{1 × 10}^6</td>
<td>146</td>
<td>1</td>
</tr>
<tr>
<td>\textit{in}1820</td>
<td>\textit{1 × 10}^4</td>
<td>CPE</td>
<td>5</td>
</tr>
<tr>
<td>\textit{in}1820</td>
<td>\textit{3 × 10}^4</td>
<td>CPE</td>
<td>0</td>
</tr>
<tr>
<td>\textit{in}1820</td>
<td>\textit{1 × 10}^4</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>\textit{in}1883‡</td>
<td>\textit{3 × 10}^6</td>
<td>CPE</td>
<td>16</td>
</tr>
<tr>
<td>\textit{in}1883</td>
<td>\textit{1 × 10}^8</td>
<td>72</td>
<td>3</td>
</tr>
<tr>
<td>\textit{in}1883</td>
<td>\textit{3 × 10}^4</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values represent the means of duplicate or triplicate determinations.
† CPE: extensive cytopathic effect prevented estimation of plaque numbers.
‡ Plaques, or single cells, were counted after reaction of \textit{in}1883-infected monolayers with X-Gal.
Table 2. Complementation of in 1883 by tsK *

<table>
<thead>
<tr>
<th>Time of superinfection</th>
<th>Plaques on monolayers†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AraC</td>
</tr>
<tr>
<td>0 h‡</td>
<td>85</td>
</tr>
<tr>
<td>2 days</td>
<td>178</td>
</tr>
<tr>
<td>2 days</td>
<td>174</td>
</tr>
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</table>

* Monolayers of HFL cells, either pretreated with INF-α or mock-pretreated, were infected with 30 p.f.u. of in1883 and incubated at 37 °C with or without AraC. After 2 days, monolayers were washed and superinfected with 8 x 10⁶ p.f.u. of tsK per plate, overlaid with EHu5 and incubated at 37 °C for 24 h. Plaques were identified by expression of β-gal. No plaques were detected on monolayers infected with in1883 alone.
† Values represent the means of triplicate determinations.
‡ Monolayers were mock-superinfected with 30 p.f.u. of in1883 and 8 x 10⁶ p.f.u. of tsK.

Table 3. Stability of the virus–cell interaction*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plaques per monolayer†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mock si</td>
</tr>
<tr>
<td>Superinfect day 3</td>
<td>0</td>
</tr>
<tr>
<td>Subculture 1:3 on day 3, superinfect on day 6‡</td>
<td>0</td>
</tr>
<tr>
<td>Subculture 1:3 on day 6, superinfect on day 9§</td>
<td>0</td>
</tr>
</tbody>
</table>

* IFN-α-pretreated HFL cultures were infected with 40 p.f.u. of in1883 and maintained at 37 °C for 3 days in the presence of AraC. Monolayers were washed and incubation continued at 37 °C for a further 3 days. Cultures were mock-superinfected (mock si) or superinfected with 8 x 10⁶ p.f.u. of tsK per plate (si tsK) and maintained at 37 °C for 24 h prior to staining for β-gal.
† Values represent the means of between three and nine determinations.
‡ After 3 days in the presence of AraC, monolayers were washed, trypsinized and replated at one third of the original density. On day 6, cultures were mock-superinfected or superinfected with 8 x 10⁶ p.f.u. of tsK per plate and maintained at 37 °C for 24 h prior to staining for β-gal.
§ Monolayers subcultured on day 3 were again subcultured 1:3 on day 6 and superinfected on day 9.

The information from the previous sections was used to develop a system in which each HFL cell should contain at least one quiescent viral genome. Infection of IFN-α-pretreated monolayers with 30 p.f.u. of in1883 yielded 140 plaques after superinfection with tsK (Table 2); thus addition of 3 x 10⁴ p.f.u. of in1883 should result in almost all cells in a monolayer of 8 x 10⁵ cells receiving, on average, one reactive genome. To demonstrate directly the validity of this extrapolation, IFN-α-pretreated monolayers were infected with 3 x 10⁴ p.f.u. or greater of in1883 per plate, maintained in the presence of AraC for 2 days and superinfected with tsK. To prevent intercellular spread of virus, Brefeldin A was present during superinfection (Cheung et al., 1991). Control experiments showed that in cells treated with Brefeldin A, β-gal and its reaction product were restricted to individual cells (results not shown). After superinfection, monolayers were stained for the presence of β-gal (Fig. 3).

Retrieval of the viral genome after infection at high m.o.i.

The stability of retention of in1820-based viruses was investigated (Table 3). IFN-α-pretreated monolayers were infected with 40 p.f.u. of in1883 per plate and, after 3 days in the presence of AraC, monolayers were superinfected with tsK or mock-superinfected. No virus replication or expression of β-gal was detected on untreated plates, but superinfection with tsK yielded an average of 169 plaques per plate. Subculture and reseeding at 3-fold dilution after 3 days in the presence of AraC did not result in reactivation of in1883. Once monolayers had become confluent, superinfection with tsK yielded approximately one-third of the initial number of plaques per plate. A further cycle of subculture again failed to reactivate virus, and the number of plaques per plate reactivated by superinfection with tsK was again reduced by a factor of approximately three. Therefore, trypsinization, subculture and regrowth of HFL cells did not reactivate quiescent in1883, emphasizing that repression of gene expression could not be overcome by the changes in cell metabolism which occur during subculture. There was also no evidence for co-replication with the host cell, since reactivation competent cells were diluted in approximately the same ratio as the total cell population. The in1883 genome remained within cells and could be reactivated for at least 9 days after infection.
was therefore verified directly. It was possible to produce cultures with each cell containing at least one reactivatable genome and with the background of cells harbouring potentially replicative genomes contributing less than 0.01% of the population. The results also demonstrate that the HCMV enhancer and Vmw110 promoter were not detectably active, except in a small number of cells.

\[ \text{Table 4. Relationship between genome copy number and reactivatable virus} \]

<table>
<thead>
<tr>
<th>in1883 (p.f.u. per plate)</th>
<th>Genomes retained after 3 days*</th>
<th>( \beta )-Galactosidase-expressing cells†</th>
<th>Genomes per reactivating cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 \times 10^4</td>
<td>3.7 \times 10^6 (2)§</td>
<td>0.6 (6)§</td>
<td>2.7 \times 10^6</td>
</tr>
<tr>
<td>1 \times 10^5</td>
<td>1.0 \times 10^6 (4)</td>
<td>5.6 (6)</td>
<td>7.6 \times 10^5</td>
</tr>
<tr>
<td>3 \times 10^5</td>
<td>2.3 \times 10^6 (2)</td>
<td>32.2 (6)</td>
<td>8.0 \times 10^6</td>
</tr>
</tbody>
</table>

* Data from Southern blots, as shown in Fig. 4, were used to calculate genome copy number in IFN-\( \alpha \)-pretreated HFL cell cultures infected with in1883. The results were correlated with analysis of \( \beta \)-gal-expressing cells, as shown in Fig. 3.
† Monolayers infected with in1883 were stained for \( \beta \)-gal after mock-superinfection (mock si) or superinfection with 8 \times 10^5 p.f.u. of tsK per plate (si tsK) in the presence of Brefeldin A.
‡ Calculated from the percentage of cells staining for \( \beta \)-gal and the measured average cell density of 8 \times 10^5 cells per monolayer.
§ Mean value, number of determinations in brackets.

In addition, superinfection with in1814R (essentially wild-type HSV-1) and incubation for 7 h in the presence of Brefeldin A, or AraC, reactivated in1883 or in1884 as efficiently as superinfection with tsK (results not shown).

The extrapolation from results obtained at low m.o.i. was therefore verified directly. It was possible to produce cultures with each cell containing at least one reactivatable genome and with the background of cells harbouring potentially replicative genomes contributing less than 0.01% of the population. The results also demonstrate that the HCMV enhancer and Vmw110 promoter were not detectably active, except in a small number of cells.

Structure and copy number of DNA

Previous experiments showed that viral DNA was sequestered as a non-linear molecule when HFL cells were infected with in1814 at an m.o.i. of approximately 0.1 p.f.u. per cell and subsequently maintained in the presence of AraC or aphidicolin (Harris & Preston, 1991). The configuration of viral DNA was therefore investigated after infection of IFN-\( \alpha \)-pretreated HFL cells with 10^6 p.f.u. of in1820 per plate and maintenance in the presence of AraC. DNA was extracted from cell nuclei, cleaved with BamHI and enriched for viral sequences by equilibrium buoyant density gradient centrifugation. Hybridization with the joint-spanning BamHI k fragment from HSV-1 revealed a preponderance of joint fragment after 1 day in culture (Fig. 4a, lane 1) and an almost complete absence of termini after 2 days (lane 2), demonstrating conversion to a non-linear form.

To obtain an estimate of viral genome copy number, DNA was extracted from nuclei of cells pretreated with IFN-\( \alpha \), infected with 3 \times 10^5 or 1 \times 10^6 p.f.u. of in1883 and maintained in the presence of AraC for 7 h or 3 days. Hybridization was carried out on unfractionated DNA using a radiolabelled DNA fragment from the Momulv enhancer, thereby avoiding problems of cross-hybridization with cellular sequences which can occur with HSV joint-spanning fragments (Fig. 4b). This probe detected the joint-spanning fragment, the L terminus and minor species containing additional ‘a’ sequences, and a minor band of unknown origin smaller than the L terminus. The results again demonstrated that the DNA was predominantly non-linear (Fig. 4b, lanes 6 and 7). Quantification was carried out on the data shown in Fig. 4(b) and on four independent experiments, and the total amount of viral DNA was compared with the number of reactivation competent cells as estimated from proportions of \( \beta \)-gal-containing cells after superinfection with tsK (Table 4). After infection with 3 \times 10^4 and 1 \times 10^6 p.f.u. of in1883 per plate, the situations in which \( \beta \)-gal expression is proportional to input m.o.i., cultures retained, respectively, 3.7 \times 10^5 and 1.0 \times 10^6 in1883 DNA molecules per monolayer of 8 \times 10^5 cells. The estimated numbers of \( \beta \)-gal-expressing cells upon superinfection with tsK were 2.7 \times 10^6 (33%) and 7.6 \times 10^5 (95%), as derived from experiments represented in Fig. 3; thus cultures contained 1:3–1:4 genomes per reactivated cell. Most genomes detectable at 3 days after infection were therefore competent for reactivation, as measured by expression of \( \beta \)-gal after superinfection.

This conclusion is crucial because it can be stated with certainty that the genomes detected biochemically were templates for reactivation. As a consequence, studies on the structure of the quiescent genome in HFL cells and the changes that occur upon reactivation will be meaningful since they pertain to biologically relevant material. It is important, therefore, to define the limits within which the conclusion is based. The values presented rely on calculations that are individually subject to error, but over a number of experiments using 1 \times 10^5 or fewer p.f.u. of in1883 and, to a lesser extent, in1884, the genome copy per reactivating cell varied only between 1:0 and 2:0. The proportion of non-linear genomes at 3 days post-infection ranged between 70%
Fig. 3. Expression of β-gal after superinfection. Cultures were pretreated with IFN-α and infected with in1883 or in1884 for 48 h in the presence of AraC. Monolayers were stained for β-gal 24 h after mock-superinfection (a, e) or superinfection with 8 × 10^5 p.f.u. of tsK per plate (b, c, d, f, g, h) in the presence of 2.5 μg/ml Brefeldin A. Cultures were initially infected with in1883 at 3 × 10^5 (a, b), 1 × 10^5 (c) or 3 × 10^4 (d) p.f.u. per plate, or in1884 at 3 × 10^5 (e, f), 1 × 10^5 (g) or 3 × 10^4 (h) p.f.u. per plate. Cells expressing β-gal on mock-superinfected plates (a, e) are labelled with arrows. Bar represents 120 μm.

and > 95 %, thus the number of non-linear genomes per reactivating cell was, in some cases, lower than presented in Table 4 and closer to one.

Comparison of β-gal-expressing cell numbers (Table 4) with values for plaque formation (Table 2) revealed a discrepancy: after superinfection with tsK, cultures infected with 3 × 10^4 p.f.u. of in1883 yielded approximately 2.7 × 10^5 β-gal-expressing cells yet an input of
Fig. 4. Analysis of viral DNA. (a) IFN-α-pretreated cultures were infected with \(1 \times 10^6\) p.f.u. of \(\text{in}1820\) per plate. AraC was added, and DNA was extracted after 1 (lane 1) or 2 (lane 2) days. Viral DNA was cleaved with \(\text{BamHI}\) and partially purified by equilibrium buoyant density gradient centrifugation prior to Southern transfer and hybridization, using radiolabelled HSV-1 \(\text{BamHI} k\) fragment as probe. Mock-infected cell DNA of equivalent density (lane 3), and 1.5 ng of \(\text{in}1820\) DNA (lane 4) were also analysed. (b) IFN-α-pretreated cultures were infected with \(3 \times 10^4\) (lanes 6 and 8) or \(1 \times 10^5\) (lanes 7 and 9) p.f.u. of \(\text{in}1883\) per plate and nuclear DNA was extracted at 7 h (lanes 8 and 9) or 3 days (lanes 6 and 7) post-infection. Standards consisted of \(3 \times 10^7\) (4.5 ng, lane 2), \(1 \times 10^7\) (lane 3), \(3 \times 10^6\) (lane 4) or \(1 \times 10^6\) (lane 5) copies of \(\text{in}1820\) DNA. DNA extracted from \(1 \times 10^5\) p.f.u. of \(\text{in}1820\) was applied to lane 1. The probe was a Momulv-specific fragment.

Fig. 5. Fate of \(\text{in}1883\) DNA. (a) IFN-α-pretreated cultures were infected with \(3 \times 10^5\) p.f.u. of \(\text{in}1883\) per plate and DNA was extracted from the nuclear fraction (lanes 1–6), NP40 supernatant plus sucrose wash (lanes 7–12) or culture medium (lanes 13–17) at 2 h (lanes 1, 7 and 13), 7 h (lanes 2, 8 and 14), 1 day (lanes 3, 9 and 15), 2 days (lanes 4, 10 and 16), 3 days (lanes 5, 11 and 17) or 4 days (lanes 6 and 12) after infection. The probe was a Momulv-specific fragment. (b) The signals from lanes 16 were quantified by phosphorimage analysis, and the amounts of total (○), linear (□) and non-linear (●) \(\text{in}1883\) DNA plotted.

30 p.f.u. per plate resulted in approximately 100 plaques (140 in the experiment described in Table 2), instead of the 270 expected. This observation was found to be due to a suppressive effect of the human serum used to prevent virus spread during virus titration. Cultures were co-infected with 30 p.f.u. of \(\text{in}1883\) and \(8 \times 10^5\) p.f.u. of \(\text{tsK}\), overlaid with various combinations of serum and Brefeldin A, and incubated at 37°C for 24 h prior to staining for β-gal. Monolayers overlaid with EHu5 alone yielded 73 plaques (mean of triplicate determinations), whereas those overlaid with EF5 containing 2.5 μg/ml Brefeldin A resulted in 151 stained cells. EHu5 containing Brefeldin A gave 57 stained cells. The presence of human serum lowered the 'titre' by a factor of more than two, by an unknown mechanism, thereby accounting for the differences between the reactivation efficiencies derived
from plaque numbers or stained cell counts. 'Reactivation', as used in Table 4, therefore strictly applies to activation of gene expression rather than the production of infectious virus.

Fate of input DNA

The experiment shown in Fig. 4(b) also includes hybridization to DNA from an input inoculum of $1 \times 10^5$ p.f.u. of in1883 (lane 1) and DNA present at 7 h after infection of IFN-α-pretreated cells with $3 \times 10^4$ and $1 \times 10^5$ p.f.u. in the presence of AraC (lanes 8 and 9). Over five determinations, $1 \times 10^5$ p.f.u. corresponded to $1.07 \times 10^7$ input DNA molecules, of which $2.9 \times 10^6$ were detected in the nuclear fraction at 7 h post-infection. The 7 h value thus exceeded the number of genomes retained after 3 days ($1 \times 10^6$), and an explanation was sought for this unexpected finding.

IFN-α-pretreated cells were infected with $3 \times 10^6$ p.f.u. of in1883, washed twice with EF5 after 1 h at $37^\circ C$, and overlaid with EF5 containing AraC. At various times after addition of virus to cells, monolayers were harvested and nucleic acids extracted from the nuclear fraction, the NP40 supernatant plus 10% sucrose wash of crude nuclei (cytoplasmic fraction), and from the culture medium. Nucleic acids were cleaved with BamHI plus ribonuclease A and analysed by Southern hybridization (Fig. 5a). Viral DNA was predominantly in the nuclear fraction at all times, although some linear molecules were found in the cytoplasmic fraction up to 1 day after infection. No viral DNA was detected in the culture medium at any time. When the data from Fig. 5(a) were expressed graphically (Fig. 5b), it was found that the total amount of in1883 DNA fell by approximately 3-fold during the first 2 days of infection, but that the number of non-linear genomes present at 2 days remained stable. Input DNA not converted to the non-linear form during the course of the experiment was presumably degraded, since it could not be recovered in the cytoplasmic fraction or growth medium.

Regular nucleosome arrangement cannot be detected at the TK locus

Cellular DNA is organized into chromatin, of which the nucleosome is the basic unit. It has been shown that HSV DNA forms a chromatin-like structure when sequestered in mouse brain stem (Deshmukh & Fraser, 1989). To investigate whether in1820 DNA was organized similarly in HFL cells, nuclei from IFN-α-pretreated cells infected with in1820 and maintained in the presence of AraC were partially digested with MN, the fragments separated by electrophoresis, and hybridization carried out using radiolabelled EcoRI n from the TK gene (Fig. 6). As a control, nuclei from human 143 TK- cells stably transformed to a TK+ phenotype by transfection of the HSV-1 TK gene were analysed in parallel.

Examination of ethidium-bromide-stained gels demonstrated that cellular chromatin was digested to the characteristic 'ladder' of DNA fragments. When viral
Retention of HSV DNA

DNA was examined by hybridization, however, material of heterogeneous sizes was observed, with no evidence for the presence of regularly spaced nucleosomes (Fig. 6, lanes 5–8). The HSV-1 TK gene, when present in 143 TK- cells, was organized into chromatin (Fig. 6, lanes 9–12), demonstrating that there are no intrinsic features of the TK gene which preclude the formation of regularly packaged nucleosomes.

Maintenance of cells in the presence of AraC might have reduced cellular histone pools due to inhibition of DNA synthesis, thereby preventing the formation of properly spaced nucleosomes on viral DNA. IFN-α-pretreated, in1820-infected HFL cells were therefore treated with 5 μM-acyclovir, which specifically inhibits HSV DNA synthesis. Nuclear DNA was analysed after digestion with MN (Fig. 6, lanes 13–18), but again no evidence was obtained for a chromatin-like structure of the in1820 genome. Similar results were obtained when phosphonoacetic acid was used to prevent HSV DNA replication (results not shown).

These experiments show that the in1820 genome, at the TK locus, was not arranged into the regular structure characteristic of cellular chromatin.

Non-linear DNA is a template for reactivation

Superinfection of cultures resulted in activation of expression from the HCMV enhancer (in in1883) and the Vmw110 promoter (in in1884) (Fig. 3). To investigate whether reactivation was accompanied by alteration of the non-linear genome configuration, IFN-α-pretreated monolayers were infected with in1820, maintained for 2 days in the presence of AraC and superinfected with 1814R in the continuous presence of AraC. At 8 h after infection, nuclear DNA was prepared, cleaved with BamHI and analysed by Southern hybridization, using the probe specific for the Momulv enhancer (Fig. 7). Reactivation did not result in a change in the relative proportions of non-linear and linear molecules (lanes 1 and 2), even though this process was known to activate gene expression. The non-linear genome was therefore the template for reactivation, and conversion to a linear form was not a prerequisite for this event to occur. In addition, the residual linear DNA remained linear throughout the 8 h period after infection.

Linear DNA represents non-uncoated genomes

Even after 2–3 days in the presence of AraC a small amount of in1820 DNA was linear (Figs 4, 5 and 7). To determine the nature of this material, nuclear DNA was prepared from HFL monolayers pretreated with IFN-α, infected with in1820 and incubated for 2 days in the presence of AraC. Nuclei were incubated with MN or DNase I, and DNA was extracted, cleaved with BamHI and electrophoresed. The probe was a Momulv-specific fragment. DNA from mock-infected cells is shown in lanes 1 and 7. DNA from latently infected cells was digested with no enzymes (lanes 2 and 8), MN at 1 unit for 3 min (lane 3) or 3 units for 5 min (lane 4), or DNase I at 1 unit for 3 min (lane 9), 2 units for 5 min (lane 10) or 5 units for 20 min (lane 11). Lanes 5 and 6 show hybridization to 3 x 10⁷ and 1 x 10⁷ copies of in1820 DNA, respectively.
proceeded, hybridization to the joint-spanning fragment decreased more sharply than to the terminal fragment until the residual, essentially nuclease resistant, viral DNA (lanes 4 and 11) was composed only of the linear form with joint and terminal fragments essentially equimolar. This result demonstrates that the small amount of linear DNA remaining in cells at 2–3 days after infection presumably originated from particles that had not undergone full uncoating and therefore protected the DNA from the action of nuclease.

Discussion

We describe the development of a cell culture system in which the HSV genome was stably retained in a quiescent state in human fibroblasts at high efficiency without cell destruction. The crucial feature is the incorporation of steps designed to reduce IE gene expression, namely the use of viruses with mutations in Vmw65 and the Vmw110 promoter together with pretreatment with IFN-α. It appears that cytotoxicity during nonpermissive infection with HSV-1 (strain 17) was due mainly to expression of IE proteins and that virus structural components, including the vhs factor, were not deleterious at the multiplicities used. This conclusion agrees with that reached in studies by Johnson et al. (1992, 1994), who also reported that pretreatment with IFN-α reduced cytotoxicity by HSV-1. Reactivation by superinfection was almost as efficient in IFN-α-pretreated cultures as in untreated cultures (Table 2). For investigation of genome structure, which realistically demands the presence of at least one genome per cell, it was necessary to include an inhibitor of DNA synthesis to prevent replication, and hence amplification, of the low background of potentially replicative virus. The origin of this virus is unclear: a small population of cells may be inherently more permissive for in1814, or a proportion of cells may receive a larger number of virus particles (possibly due to aggregation of virions) and thus overcome the block to replication. It is important to emphasize that the use of IFN-α pretreatment and AraC did not fundamentally affect the interaction of in1820 with the host cell; these agents merely enabled the system to be adapted for use at an m.o.i. of 1 p.f.u. or greater per cell and thus for analysis of the viral genome.

Pretreatment of cells with IFN-α inhibited plaque formation by in1814 more severely, in percentage terms, than 1814R (Table 1). The blocks to IE transcription imposed by the Vmw65 mutation and IFN-α pretreatment were thus additive in their effects on virus replication, a result apparently at variance with the view that IFN-α pretreatment inhibits transactivation by Vmw65 (DeStasio & Taylor, 1990). If this were the case, it might be expected that in1814 and related viruses with impaired transactivation would be resistant to the action of IFN-α. Two possible explanations exist. The insertion mutation in the in1814 genome may not completely inactivate Vmw65, leaving a residual activity which is the target for IFN-α. Although we have failed to detect activity of the mutant Vmw65 in functional assays in vivo or in vitro (Ace et al., 1988, 1989), it remains possible that it can form an unstable complex with Oct-1 and HCF in the infected cell and hence activate IE transcription to some extent. Alternatively, IFN-α may reduce IE transcription in a manner that does not involve Vmw65. This possibility is implied by the observation that expression from the HCMV enhancer, which is active under IE conditions, is also inhibited by IFN-α pretreatment (Johnson et al., 1992; C. M. Preston, unpublished observations). Perhaps IFN-α prevents the onset of IE transcription in a manner analogous to the effect of the agent on SV40 early transcription (Brennan & Stark, 1983).

During the quiescent state, expression of β-gal from the HCMV enhancer and Vmw110 promoter could not be detected in the vast majority of cells, indicating that these elements were not active. In contrast, enzyme was readily detected in the few cells which presumably had produced sufficient amounts of IE proteins. The block to IE gene expression could easily account for the absence of transcription from the remainder of the viral genome since early and late genes are inactive without IE proteins. The HCMV enhancer (present in in1883) is, however, expressed in the absence of IE proteins and is not dependent upon functional Vmw65 (C. M. Preston, unpublished observations). The lack of detectable β-gal expression in in1883-infected cells implies that the block to gene expression also applies to the HCMV enhancer and thus may not be strictly promoter-specific. Once established, repression of gene expression is stable since genomes remain quiescent even during cell subculture. The cell-cycle related factor(s) which mimic Vmw110 action (Cai & Schaffer, 1991) or influence IE transcription (Daksis & Preston, 1992; Ralph et al., 1994) are thus unable to reactivate quiescent genomes from in1820-based viruses.

No similar studies on the fate and structure of HSV DNA in the absence of IE transcription have been carried out, thus it is difficult to make detailed comparisons with the work of others. B. L. Wigdahl and colleagues pretreated HFL cells with IFN-α and found that infection with HSV-1 at up to 2.5 p.f.u. per cell could be tolerated without cell degeneration. Following incubation for 7 days in the presence of an inhibitor of viral DNA synthesis, virus could not be detected provided cultures were maintained at a supraoptimal temperature of 39.5–40.5 °C (Wigdahl et al., 1982). Temperature downshift to 37 °C resulted in virus
replication. Linear HSV genomes were found in cultures (Wigdahl et al., 1984), in contrast to the findings reported here. From our studies, the presence of linear DNA implies that the genome was present in virus particles that had not been fully uncoated or had been packaged de novo into nucleocapsids. The problem in interpreting the data of Wigdahl et al. (1984) is that cultures contained more than 0.5 genomes per cell, but not more than 3% of cells produced virus, even when reactivation was induced by superinfection (Wigdahl et al., 1982, 1984). It was therefore not possible to state that the linear genomes were templates for reactivation since a small proportion of non-linear molecules would not have been detected. In our experiments, the close correlation between reactivation and copy number demonstrates that non-linear DNA is the relevant form.

During latency in animals and humans, HSV DNA in the peripheral and central nervous systems is non-linear (Rock & Fraser 1983; Efstratiou et al., 1986), and conversion of input genomes to a non-linear form seems to occur in the absence of viral DNA replication (Slobedman et al., 1994). In addition, genomes extracted from the brain-stem were found to be packaged into a chromatin-like structure (Deshmane & Fraser, 1989). While these data clearly describe the structure of the number of cells from which virus can be reactivated. In to occur in the absence of viral DNA replication (Leinbach & Summers, 1980; Muggeridge & Fraser, 1986), although the most abundant form of DNA was resistant to nuclease digestion. Nonetheless, the genome we detect is transcriptionally inactive, and the basis for the block is unclear. Non-linearity per se was not the primary reason for the absence of gene expression, since non-linear molecules were templates for superinfection-induced reactivation, but circularization may be required to initiate further structural changes involved in silencing the genome. Formation of a tight chromatin structure, at least at the TK locus, does not occur and we have found no CpG methylation of the CCGG sites in the β-gal gene of in1820 (C. M. Preston, unpublished observations). HSV can be recovered from the central nervous system only at very low efficiency (Roizman & Sears, 1987); thus it is not possible to be certain that the chromatin-like genomes are able to reactivate.

Surprisingly, full uncoating of the in1820 genome was slow, only reaching completion 2–3 days after infection. The existence of large numbers of non-uncoated genomes has been recognized previously (Jacob & Roizman, 1977), but long-term examination of the progression to uncoating of all genomes has not been possible due to viral replication and/or cell destruction. The early block imposed by inhibition of IE transcription has enabled us to demonstrate that almost all genomes that remain in cells eventually become uncoated. It should be noted that the rate of uncoating of strain 17-derived viruses in HFL cells was slower than found in other cell types (Poffenberger & Roizman, 1985; L. H. Robinson, unpublished observations), but that neither IFN-α pretreatment nor the insertion mutation in Vmw65 affected the process (Oberman & Panet, 1989; L. H. Robinson, unpublished observations). A large proportion (up to 80%) of genomes which become associated with cells are lost, presumably due to degradation since they could not be detected in cytoplasm or culture medium (Fig. 5a, b). The way in which viral DNA is degraded and the apparently selective resistance of non-linear molecules is unclear at present, and we are not aware of any comparable studies using different cell types and virus strains.

At the structural level, our results indicate that the in1820 genome undergoes some of the events that normally occur prior to DNA synthesis. Circularization of the genome occurs even in the absence of protein synthesis (Poffenberger & Roizman, 1985). The acquisition of sensitivity to MN and the existence of a structure unlike chromatin has been described previously during productive infection (Leinbach & Summers, 1980; Muggeridge & Fraser, 1986), although the most abundant form of DNA was resistant to nuclease digestion. Nonetheless, the genome we detect is transcriptionally inactive, and the basis for the block is unclear. Non-linearity per se was not the primary reason for the absence of gene expression, since non-linear molecules were templates for superinfection-induced reactivation, but circularization may be required to initiate further structural changes involved in silencing the genome. Formation of a tight chromatin structure, at least at the TK locus, does not occur and we have found no CpG methylation of the CCGG sites in the β-gal gene of in1820 (C. M. Preston, unpublished observations). Future studies will use the approaches described here to investigate the overall organization of the quiescent viral genome and to examine specific regions in detail.

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*Retention of HSV DNA* 1431

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