Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2

S. Moira Brown,* June Harland, Alasdair R. MacLean, Jürgen Podlecht† and J. Barklie Clements

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, U.K.

The herpes simplex virus (HSV) gene RL1 encodes the protein ICP34.5, which is a specific neurovirulence factor. Null mutants in RL1 fail to replicate in the central nervous system of mice and are therefore totally non-neurovirulent. Additionally, they fail to replicate in neurons of the peripheral nervous system, although they are capable of establishing and reactivating from a latent infection. As the precise function of ICP34.5 in HSV-neuronal interactions is unknown, we have studied the role of ICP34.5 in vitro by examining in detail the phenotypes of RL1-negative viruses in two defined tissue culture systems. The first was mouse embryo fibroblast 3T6 cells, in which RL1-negative mutants are impaired and the in vivo phenotype is mimicked. This impairment is amplified when the cells are in the stationary state. The second was mouse embryo testicular carcinoma F9 cells which, in the undifferentiated state, provide a reversal of phenotype; wild-type virus fails to grow but RL1-negative virus replicates efficiently. Differentiation results in the ability to support wild-type virus growth. The stage at which the replication cycle is blocked plus the role of cellular factors is addressed in both tissue culture systems. Evidence is provided that cell type and cell state are crucial to ICP34.5–cellular interaction and hence, based on these parameters, ICP34.5 can be defined as a host-range determinant. Identification of cellular proteins that specifically interact with or are homologues of ICP34.5 may lead to the identification of neuron-specific proteins that have a similar role.

Introduction

Understanding the pathogenesis of herpesvirus infections, especially in neonates and immunocompromised individuals, is becoming increasingly important. Crucial to the development of effective antiviral drugs and vaccines is the identification of genes and their products that determine particular features of the viral life cycle, such as latency and virulence, in vivo. To this end, we have constructed and isolated mutants with deletions in the long repeat regions of the herpes simplex virus (HSV) genome which, on intracerebral inoculation of mice, have LD50 levels as much as 10^4-fold higher than their parental counterparts (Taha et al., 1989a, b; MacLean et al., 1991a, b). The deletions were mapped to a region of the genome subsequently shown to encode a gene (RL1) both in HSV-1 strain F (Chou & Roizman, 1990) and strain 17+ (Dolan et al., 1992) and in HSV-2 strain HG52 (McGeoch et al., 1991). RL1 encodes the protein ICP34.5 (Ackermann et al., 1986; McKay et al., 1993) which is the only identified, specific HSV neurovirulence factor. As well as being unable to replicate productively and hence destroy the central nervous system (CNS), mutants in RL1 are also incapable of replication in neurons of the peripheral nervous system (PNS), although they are able to establish and reactivate from latency (Robertson et al., 1992).

The precise virus–host interactions involving ICP34.5 that confer neurovirulence are unknown. The in vivo experimental techniques that are available to elucidate the function of ICP34.5 are restricted and information gained from animal models is limited. We have therefore chosen to complement our in vivo studies by developing tissue culture systems in which the virus phenotype in vivo is mimicked in vitro. There is no evidence to date to indicate that growth impairment of ICP34.5 null mutants in vivo is restricted to neurons; other cell types may well be involved. We therefore decided not to restrict our studies to neuronal lines but to include a number of cell systems that could potentially provide information on cellular factors interacting specifically with ICP34.5 to allow or preclude HSV replication. This paper describes our findings on the identification of one cell line that is non-permissive for RL1-negative virus, and another in which growth of wild-type HSV is significantly impaired but virus lacking ICP34.5 replicates efficiently.

† Present address: Institut für Virologie, Universitätsklinik, Mainz, Germany.
Methods

Cells. Baby hamster kidney clone 13 cells [BHK-21(C13); MacPherson & Stoker, 1962] were propagated in Eagle's medium containing twice the normal concentration of vitamins and amino acids, 5% (v/v) tryptose phosphate broth and 10% (v/v) calf serum. MRC-5 human fetal lung cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% non-essential amino acids and 10% fetal bovine serum (FBS). Mouse embryo fibroblasts (3T6) were grown in DMEM containing 10% FBS. To cause 3T6 cells to enter their stationary state the serum concentration was reduced to 0.5% and cells were left undisturbed for 5 to 6 days before infection. PC12 rat adrenal pheochromocytoma cells were grown in DMEM containing 10% horse serum (HS) and 5% FBS, and were induced to differentiate to the neuronal phenotype by the addition of nerve growth factor (NGF) at a concentration of 100 ng/ml. ND7 cells (Wood et al., 1990) had been initially prepared by fusing rat dorsal root ganglia cells with the mouse N18 neuroblastoma cell line and selecting for HAT resistance of the ganglion cells. These cells were grown in Leibovitz's L-15 medium with 10% FBS and induced to differentiate to the neuronal phenotype in serum-free medium (Wood et al., 1990). F9 mouse testicular carcinoma cells were grown in DMEM containing 5% FBS on gelatin-coated plastic surfaces. Differentiation to parietal endoderm was induced in the presence of retinoic acid and dibutyryl cAMP (Strickland et al., 1980). Differentiation was confirmed by the change in morphology from a flat cell appearance to a rounded refractile phenotype with processes extending several cell diameters. TM4 mouse testis Sertoli cells were grown in DMEM with 5% HS and 2.5% FBS, mixed in a ratio of 1:1 with Ham's F12 medium.

Viruses. Virus stocks were grown and titrated in BHK-21(C13) cells as previously described (Brown et al., 1973). The parental HSV-1 strain was Glasgow strain 17+ (Brown et al., 1973) and the RL1 mutants were 1716 (MacLean et al., 1994a) and 17171 (E. McKay et al., 1994). The parental HSV-2 strain was HG52 (Timbury, 1971) and the RL1 mutant was JH2604 (Harland & Brown, 1985).

Virus growth properties in vitro. Cells were infected either at an m.o.i. of 5 p.f.u./cell (single cycle) or 0.01 p.f.u./cell (multiple cycles). After adsorption for 45 min at 37 °C, the monolayers were washed, overlaid with the appropriate medium and incubated at 37 °C. At intervals up to 48 h (single cycle) or 72 h (multiple cycles) post-infection samples were harvested and virus, released by sonication, was titrated on BHK-21(C13) cells. Growth experiments with HSV-1 were carried out in all the cell types listed above, and in BHK-21(C13), 3T6 and F9 cells with HSV-2.

Western blotting. Infected cell extracts were prepared as described previously (McKay et al., 1993). After transfer, the nitrocellulose was blocked for 1 h in PBS containing 5% dried milk and incubated with the various antisera, diluted as described below in PBS containing 1% BSA. Detection was carried out using Protein A peroxidase (Sigma) and chemiluminescence reagents (ECL; Amersham). The following antisera were used for Western blotting and/or immunofluorescence: antipeptide sera against Vmw110 (number 95) at a dilution of 1:50, against Vmw63 (number 43) at a dilution of 1:100 and against Vmw21 (number 14473/4) at a dilution of 1:20 (MacLean et al., 1987), monoclonal ascites sera against Vmw65 (number Z1F11) at a dilution of 1:1000 and against glycoprotein C (gC) at a dilution of 1:200. The sera were kindly supplied by Drs Howard Marsden and Anne Cross.

Immunofluorescence. Plastic Petri dishes (35 mm) containing two coverslips were seeded with approximately 1 x 10^5 cells/plate and incubated at 37 °C overnight. Cells were infected at an m.o.i. of 0.1 p.f.u./cell with either strain 17+ or 1716 and incubated at 37 °C. At selected time points post-infection, cells were fixed with 90% methanol and stored in PBS at 4 °C until immunofluorescence staining was carried out. Cells were initially stained with one of the antibodies listed above, diluted appropriately in PBS. The second antibody, either goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Sigma) or goat anti-rabbit IgG conjugated to FITC (Sigma), was diluted 1:80 in PBS. Coverslips were mounted onto slides using Citifluor AF1. To minimize non-specific staining, antibodies were pre-absorbed onto uninfected monolayers for 30 min.

Results

Growth characteristics of HSV-1 strain 17+ and the mutant 1716 in a range of tissue culture cell lines

Non-neurovirulent deletion mutants (RL1-negative) of HSV-1 strain 17+ and HSV-2 strain HG52 are unable to grow in mouse brain following intracerebral inoculation, whereas their parental wild-type viruses grow, resulting in destruction of neurons and supporting cells and encephalitis (Taha et al., 1990; Robertson et al., 1992). In BHK-21(C13) cells (the cell line in which the virus is normally grown and titrated) these mutants show no impairment in growth. In an attempt to identify a tissue culture system that reflects the in vivo phenotype of RL1-negative virus, a number of cell lines have been studied as substrates for single cycle and multiple cycle growth experiments. The experiments were originally performed with the HSV-1 RL1-negative mutant 1716 and its parental wild-type strain 17+; and the HSV-2 RL1-negative mutant 2604 and its parental wild-type strain HG52. However, HSV-2 strain HG52, which in BHK-21(C13) gives an average burst size of 10 p.f.u./cell compared to 100 p.f.u./cell for HSV-1 strain 17+, was found to be restricted in most cell lines studied. Hence, any significant differences between the wild-type and 2604 were difficult to evaluate. The majority of experiments were therefore confined to HSV-1 strain 17+ and the mutant 1716.

Fig. 1 shows the results of single cycle growth experiments with an initial infecting multiplicity of 5 p.f.u./cell. The cell lines used were BHK-21(C13), MRC-5, 3T6, ND7, PC12, F9 and TM4 (as described in Methods), infected with the viruses HSV-1 strain 17+ and 1716. In BHK-21(C13), MRC-5 and ND7 cells, the growth patterns of 17+ and 1716 were similar, with a 10^2- to 10^3-fold increase in the exponential phase of growth. In 3T6 and PC12 cells, the wild-type virus grew normally but the yield of the mutant 1716 was impaired, indicating that lack of ICP34.5 was detrimental to virus replication. Strikingly, in the mouse teratocarcinoma line TM4 neither 17+ nor 1716 demonstrated any replication and these cells were totally non-permissive for HSV. The mouse testicular carcinoma cell line F9 gave an unexpected result. With wild-type 17+ there was less than a 10-fold increase in growth between 8 h and 24 h post-infection. In contrast, with the mutant 1716 over the same time scale, the virus titre rose by 100-fold. It
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Fig. 1. Growth kinetics of HSV-1 strain 17+ (■) and the RL1-negative mutant 1716 (○). BHK-21(C13), MRC-5, 3T6, ND7, PC12, F9 and TM4 cells were infected at an m.o.i. of 5 p.f.u./cell at 37 °C and at various times post-infection, the infected cells were harvested. The virus was released by sonication and titrated on BHK-21(C13) cells at 37 °C.

appears therefore that expression of ICP34.5 in undifferentiated F9 cells has a negative effect on virus replication.

To determine the ability of virus to be released from infected cells and undergo further rounds of replication, various cell lines were infected at an m.o.i. of 0.01 p.f.u./cell with 17+ and 1716. Progeny virus were harvested up to 72 h post-infection (multiple cycle growth). As expected (Fig. 2a), the phenotypes of 17+ and 1716 were virtually identical in BHK-21(C13) cells. In 3T6 cells there was a very large difference in growth between 17+ and 1716, with a $5 \times 10^4$-fold increase in titre between 6 h and 48 h for 17+, compared to a 50-fold increase for 1716 over the same time period. Another HSV-1 RL1-negative mutant, 1771, with a stop codon 9 bp downstream of the initiating methionine and which
Fig. 2. (a) Growth kinetics of HSV-1 strain 17\textsuperscript{+} (●) and mutant 1716 (○) following infection at 37 °C of different cell types at an m.o.i. of 0.01 p.f.u./cell. Cultures were harvested at intervals up to 72 h post-infection, sonicated and progeny virus was titrated on BHK-21(C13) cells at 37 °C. (b) Growth kinetics of HSV-2 strain HG52 (▲) and the RL1-negative mutant 2694 (△) following infection at 37 °C of BHK-21(C13) cells and 3T6 cells at an m.o.i. of 0.01 p.f.u./cell. Cultures were harvested at intervals post-infection and progeny virus titrated on BHK-21(C13) cells at 37 °C.
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Fig. 3. Growth kinetics of HSV-1 strain 17\(^{+}\) (●) and the mutant 1716 (○) in BHK-21(C13) and 3T6 cells. Cells were infected at an m.o.i. of 0.01 p.f.u./cell when the monolayers were totally confluent and following serum starvation for 5 days (resting). Cultures incubated at 37 °C were harvested at the times indicated post-infection and progeny virus titrated on BHK-21(C13) cells at 37 °C.

Fig. 4. Growth kinetics of HSV-1 strain 17\(^{+}\) (●) and the mutant 1716 (○) in differentiated PC12 and ND7 cells. PC12 cells were induced to differentiate to the neuronal phenotype by the addition of NGF at a concentration of 100 ng/ml. ND7 cells were induced to differentiate to the neuronal phenotype in serum-free medium. Cells were infected at an m.o.i. of 0.01 p.f.u./cell (a) or 5 p.f.u./cell (b) and cultures incubated at 37 °C were harvested at intervals up to 72 h. Progeny virus was titrated on BHK-21(C13) cells at 37 °C.
therefore fails to synthesize ICP34.5 (McKie et al., 1994), was also used in low multiplicity infections of BHK-21(C13), 3T6 and F9 cells. The growth patterns of 171 were indistinguishable from those of 1716. In confluent 3T6 cells at 48 h post-infection, the titre of 1771 was $1.2 \times 10^8$ and that of $17^+$ was $3 \times 10^7$ p.f.u./10^6 cells. A similar effect was observed in the growth patterns of HSV-2 strain HG52 and its RL1-negative mutant JH2604, in confluent 3T6 cells after infection at 0.01 p.f.u./cell (Fig. 2b). By 48 h post-infection there was a $10^2$-fold differential between the wild-type and mutant viruses.

In ND7 cells (Fig. 2a) the difference in growth between $17^+$ and 1716 was apparent following a low multiplicity infection. Strain $17^+$ exhibited a 100-fold increase in growth by 72 h, with the rate of increase still increasing, whereas with 1716 the titre between 6 and 72 h was virtually unaltered. ICP34.5 expression is thus required in ND7 cells for low multiplicity infection to proceed. In contrast, in PC12 cells, the 1716 defect seen at high m.o.i. (Fig. 1) was not amplified. It is apparent that lack of ICP34.5 expression in these cells does not prohibit virus replication. At the lower multiplicity, the phenotype of 1716 and $17^+$ in F9 cells was as expected; $17^+$ demonstrated no growth whereas 1716 had increased in titre by more than 100-fold by 72 h. Expression of ICP34.5 caused an inability of HSV-1 to replicate efficiently in F9 cells.

The marked difference between $17^+$ and 1716 in multicycle growth experiments in 3T6 cells could imply that only a proportion of the cells are capable of infection by the RL1-negative mutants or that the capability of released virus to infect surrounding cells is impaired. To determine whether the poor yield of 1716 in 3T6 cells was due to inefficient virus release from infected cells, progeny virus from $17^+$ and 1716 infections was separated into cell-associated and cell-released fractions prior to titration.

In BHK-21(C13) cells the titres of cell-released virus, although lagging behind up to 24 h, paralleled that of cell-associated virus both in $17^+$ and 1716 infections, with very similar yields at both 48 h and 72 h. In 3T6 cells the ratio of $17^+$ virus in the supernatant to cell-associated virus was similar to that obtained with BHK-21(C13) cells, but the yield of 1716 cell-associated virus was very low (less than 9 p.f.u./10^6 cells at both 48 h and 72 h). The supernatant virus titres were even lower, never rising above the titre at infection (data not shown) and so it was not possible to draw definitive conclusions on the efficiency of 1716 release.

It was apparent by microscopic analysis that once 3T6 monolayers became confluent there was very little additional cell division. The cells were strongly contact-inhibited and did not become overgrown following several days incubation at 37 °C. The relative growth patterns of $17^+$ and 1716 in exponentially growing 3T6 cells compared to those in confluent cells were determined and it was clear that growth of the mutant was more impaired in contact-inhibited 3T6 cells. This indicated that the cell state was playing a role in the ability to support growth of the RL1-negative virus. This was investigated further by synchronizing cell cultures by serum starvation for 5 days preinfection. Confluent and resting BHK-21(C13) cells gave essentially the same pattern of virus growth following $17^+$ and 1716 infection. In 3T6 cells, 1716 was impaired in confluent cells but in resting cells the virus yield at 72 h post-infection was only marginally above the titre of the input virus immediately post-absorption. Wild-type virus growth in the synchronized 3T6 cells was also impaired, with a $10^8$-fold increase between 8 and 48 h, compared to a $10^2$-fold increase in confluent cells (Fig. 3). The yield of 1716 in dividing 3T6 cells was 6% of the $17^+$ yield, in confluent cells it was 0.7% and in stationary cultures it was 0.1%. These results were confirmed in cells that had been synchronized in the G1-S phase of division following an aphidicolin–thymidine block, and demonstrated that cell state is important for HSV growth and the absence of ICP34.5 accentuates its importance.

A further way to assess the role of cell state in determining permissivity is to induce differentiation, thereby altering the cellular phenotype. This was carried out for PC12, ND7 and F9 cells. In the presence of NGF, PC12 cells differentiate to a neuronal phenotype, a neuronal phenotype is induced in ND7 cells by growth in defined serum-free medium, and F9 embryonic ectoderm cells differentiate to become parietal endoderm in the presence of retinoic acid and cAMP. Growth of $17^+$ and 1716 following a low multiplicity infection in differentiated PC12 and ND7 cells is shown in Fig. 4. In differentiated PC12 cells the growth differential between $17^+$ and 1716 is more marked than in undifferentiated cells (Fig. 2) but 1716 is still capable of several rounds of replication. Differentiation of ND7 cells improves the ability to support strain 1716 replication. Thus, the cell state in PC12 and ND7 cells is important for virus growth but is not crucial for replication of virus lacking the RL1 gene.

Differentiation of F9 cells resulted in increased permissivity for 1716 replication at both high and low multiplicities. Additionally, differentiation rendered F9 cells permissive for $17^+$ virus replication at both multiplicities, although 1716 still grew faster and with a higher burst size/cell (Fig. 5). Transformation from the stem cell state to the committed state of the parietal endoderm phenotype resulted in an increased capability to support HSV replication per se, and the expression of ICP34.5 was of less significance, although not irrelevant.
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Fig. 5. Growth kinetics of HSV-1 strain 17 + (●) and the mutant 1716 (○) in differentiated and undifferentiated F9 cells following infections at multiplicities of 0.01 (a) and 5 (b) p.f.u./cell. F9 cells were induced to differentiate to parietal endoderm in the presence of retinoic acid and dibutyryl cAMP. Cultures were incubated at 37 °C and harvested at the indicated times post-infection. Progeny virus was titrated on BHK-21(C13) cells at 37 °C.

The growth experiments in BHK-21(C13) and 3T6 cells were carried out 15 to 20 times and those in F9 cells 10 times. The average titres were calculated and the results are shown in Table 1.

On the basis of the growth experiments, stationary state 3T6 cells were chosen as a system in which the in vivo phenotype of RL1-negative mutants was mimicked. Strain 1704 (Maclean et al., 1987), a null mutant in the latency-associated transcripts grew as well as wild-type virus in stationary 3T6 cells, and 1716R, a recombinant in which the RL1 deletion is corrected, also gave the wild-type phenotype (data not shown). The non-permissivity of 3T6 cells was specific for virus lacking RL1. By contrast, F9 cells provided a system to investigate the role of cell state in HSV permissivity and ultimately the specific interaction between ICP34.5 and cellular proteins. All further experiments were therefore restricted to BHK-21(C13), 3T6 and F9 cells.

Virus-infected cell polypeptide expression

To determine whether there is a specific stage at which virus replication is blocked in 3T6 and F9 cells, the expression of viral proteins was studied by both Western blotting and immunofluorescence. HSV protein expression is temporally regulated, resulting in three broad classes of proteins, immediate early (α), early (β) and late (γ). The expression of proteins in each category was determined using monoclonal antibodies or antipeptide sera to specific proteins in each class. The proteins were Vmw110 and Vmw63 (α), Vmw65 (β) and Vmw21 and gC (γ). In all infections used to determine polypeptide expression, cell numbers were equalized.

Using Western blotting and the antipeptide serum directed against Vmw110, the amount of this protein expressed by 17 + and 1716 in BHK-21(C13) cells was found to be virtually equivalent at both 8 and 24 h post-infection (Fig. 6a). In both 17+- and 1716-infected confluent 3T6 cells, by 8 h there were comparable amounts of the protein, whereas by 24 h it was apparent that the amount of Vmw110 expressed by 1716 was at least twofold less than that expressed by 17+. In 17+-infected 3T6 resting cells, the protein was just apparent by 8 h and had increased in amount by 24 h post-infection. In 1716-infected 3T6 resting cells there was little Vmw110 detectable at either 8 or 24 h.

By contrast, in F9 cells (Fig. 6b) the amount of Vmw110 synthesized in 1716-infected cells was markedly

Table 1. Virus titres per 10⁶ cells of various cell types at different times post-infection

<table>
<thead>
<tr>
<th>Cells</th>
<th>M.o.i. p.f.u./cell</th>
<th>Strain 17+ titre (± s.d.)</th>
<th>Strain 1716 (± s.d.)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>48 h 72 h</td>
<td>48 h 72 h</td>
</tr>
<tr>
<td>BHK-21(C13)</td>
<td>0.01</td>
<td>2.0 x 10⁶ (± 3.1)</td>
<td>2.2 x 10⁶ (± 2.6)</td>
</tr>
<tr>
<td>3T6 (confluent)</td>
<td>0.01</td>
<td>6.2 x 10⁵ (± 2.3)</td>
<td>1.0 x 10⁶ (± 2.5)</td>
</tr>
<tr>
<td>24 h 48 h</td>
<td>24 h 48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9 (undifferentiated)</td>
<td>5</td>
<td>1.6 x 10⁶ (± 1.8)</td>
<td>1.5 x 10⁶ (± 1.7)</td>
</tr>
<tr>
<td>BHK-21(C13)</td>
<td>5</td>
<td>1.2 x 10⁶ (± 2.0)</td>
<td>1.3 x 10⁶ (± 2.0)</td>
</tr>
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greater at both 8 and 24 h compared to 17⁺-infected cells. The amount of Vmw110 synthesized by 24 h in 17⁺-infected cells was similar to that expressed in 1716-infected F9 cells following differentiation. At 8 h the large disparity in synthesis of Vmw110 was even more marked than in undifferentiated cells.

Similar results were obtained in both 3T6 and F9 cells with the antibody to the other immediate early protein Vmw63.

Using a monoclonal antibody directed against the early protein Vmw65, the pattern of synthesis paralleled that of Vmw110 synthesis in 3T6 and F9 cells (data not shown). Using antipeptide serum directed against the late Vmw21 protein, the amount of synthesis in BHK-21(C13) cells by 1716 and 17⁺ was virtually the same at each time post-infection, with the expected increase in the level at 24 h. In 1716-infected 3T6 confluent cells, the Vmw21 protein was only detected at 24 h post-infection and at a level markedly reduced compared to 17⁺-infected cells. In 3T6 resting cells no protein was detected with 1716 and was only present by 24 h with 17⁺ (Fig. 7a). In 1716-infected F9 cells, Vmw21 was detectable by 8 h and increased by 24 h, whereas 17⁺ infection only
growth experiments, a lower overall level of viral synthesis in terms of infectious virus. A similar conclusion can be drawn for strain 17\(^+\) infection of F9 cells.

**Discussion**

It is now accepted that both HSV-1 and HSV-2 encode a gene, RL1, located within the terminal portion of the long repeat region of the genome and whose promoter is in the ‘a’ sequence (Chou & Roizman, 1986; McGeoch et al., 1991; Dolan et al., 1992). The protein ICP34.5 (Ackermann et al., 1986; McKay et al., 1993) encoded by RL1 is the only specific HSV neurovirulence factor. Mutants that fail to make ICP34.5 are totally non-neurovirulent in the CNS of mice (Taha et al., 1989a, b; Chou & Roizman, 1992; MacLean et al., 1991a, b) but are capable of establishing and reactivating from latent infections in the PNS following peripheral inoculation (MacLean et al., 1991b; Robertson et al., 1992). The only studies of the pathology caused by RL1-negative mutants have been on mouse brains infected with the HSV-2 strain HG52 RL1-negative mutant JH2604, which failed to produce necrotizing encephalitis. The only morphological change it induced was immune cell infiltration. Using a polyclonal antiserum, viral antigens were found to be mostly localized to astrocytes and ependymal cells, close to the site of inoculation (Taha et al., 1990). These results clearly showed that absence of ICP34.5 results in failure of HSV to replicate productively in neurons of the CNS and possibly also in some supporting cells within the brain. Absence of ICP34.5 expression also results in failure to replicate in peripheral neurons (Robertson et al., 1992), although it does not preclude limited replication in mouse footpad tissue. Using the mouse eye route of infection, it appears that ICP34.5 expression is necessary for replication in corneal cells (J. G. Spivack, personal communication). These results suggest that, at least in vivo, the block in replication of RL1-negative virus is not confined to neurons.

The ICP34.5 protein has a conserved region, which is highly similar to a mouse myeloid protein (McGeoch & Barnett, 1991) and to an African swine fever virus protein that is thought to have a role in host range determination (Sussman et al., 1992). However, the precise role of HSV ICP34.5 in virus–cell interactions is unknown.

As information to be gained from in vivo models is limited, it is important to develop tissue culture systems for the analysis of phenotype. Such in vitro analysis may provide clues to ICP34.5 function and allow in vivo experiments with more defined goals to be designed. Based on this rationale, we have carried out the series of experiments described here.
As ICP34.5 has been defined as a neurovirulence factor, it could be considered that, ideally, any tissue culture system should be neuronal in origin. This premise, however, may be naïve, as lack of productive replication of ICP34.5-negative virus in vivo does not appear to be confined to neurons. When neuroblastoma cell lines were tested for HSV permissivity, they proved to be unsatisfactory for a number of reasons (especially lack of virus growth) and it was therefore decided to test a range of cell lines that were diverse in origin and cell type. Following results from the initial growth experiments we decided to concentrate our studies on three lines: BHK-21(C13) cells, our normal laboratory host cells for growth and titration of HSV; 3T6 mouse embryo fibroblasts and F9 mouse teratocarcinoma stem cells. The BHK-21(C13) cells therefore provided a control, and the 3T6 and F9 cells provided environments in which RL1-negative HSV displayed different phenotypes. It was decided not to investigate ND7 and PC12 cells in depth as the difference in these cells varied, but the results nonetheless add weight to the conclusions on the role of cell type and cell state.

From the virus growth experiments and the experiments monitoring virus antigen expression throughout the replicative cycle, in mouse fibroblast 3T6 cells the cell state in terms of metabolism and stage in the cell cycle was of vital importance in determining the permissivity for HSV that fails to express ICP34.5. This was shown by 1716, the RL1 deletion mutant, and 1771, the RL1 stop codon mutant, of HSV-1 strain 17 + and the RL1 deletion mutant JH2604 of HSV-2. Confluent 3T6 cells are in fact now used routinely in our laboratory as a selection system for isolating HSV mutants that fail to synthesize ICP34.5. Three fundamental conclusions can be drawn from the results obtained from the 3T6 infections. (i) Exponentially growing 3T6 cells express a cellular protein that can partially compensate for ICP34.5 to allow virus replication. (ii) In contact-inhibited and stationary cells this cellular protein is depleted to such an extent that virus lacking ICP34.5 is significantly disadvantaged. (iii) From the immunofluorescence experiments, a small proportion of the cells in a stationary culture support 1716 replication, whereas the majority appear to be uninfected. Thus, 3T6 cells produce a protein that, either alone or in a complex with other cellular and/or viral proteins, can replace ICP34.5 to allow virus replication. The protein could be homologous to, or totally unrelated to ICP34.5. From the immunoblotting results and more particularly from the fluorescence experiments, the significantly reduced polypeptide expression in RL1-negative virus-infected cells appears to result from the small proportion of cells demonstrating positive staining, rather than a block at a specific stage in the replication cycle. These permissive cells may not be in the stationary state and perhaps if 100 % of the cells were synchronized in the G1-S phase, the amount of virus replication would be even more limited. Alternatively the low number of positively staining cells could be due to the limiting of virus replication to cells that were initially infected and, owing to a defect in virus maturation within these cells, there is then a resultant depletion in virus capable of infecting surrounding cells.

There are two basic explanations for the differing phenotypes of wild-type and RL1-negative mutants in undifferentiated F9 cells. (i) Expression of ICP34.5 induces cell death and hence virus replication cannot take place; F9 cells infected with 17 + at high multiplicity take on a rounded appearance very soon after infection. This is not classic viral c.p.e. as it occurs too soon after infection, does not occur with 1716 and, in addition, virus replication is minimal. The rounded appearance could be due to cell death. An apoptosis assay (Henderson et al., 1991) in differentiated and undifferentiated F9 cells infected with 17 + and 1716 failed to show DNA degradation into nucleosomal lengths and acridine orange staining did not show condensation of chromatin. Apoptosis can therefore be ruled out but necrosis could possibly be taking place. (ii) There is a cellular protein that interacts antagonistically with ICP34.5 to stop virus replication or to block the ability of the cells to support virus replication. When the F9 cells are induced to differentiate, levels of the cellular protein could be reduced such that its interaction or competition with ICP34.5 is minimized, thereby allowing virus replication. The increased ability of RL1-negative mutants to grow in differentiated F9 cells could indicate that the cellular protein itself is deleterious but less so than when coupled with ICP34.5.

The 3T6 and F9 cell systems provide novel evidence that cell type and cell state are important in supporting replication of HSV that fails to express ICP34.5. The results in undifferentiated and differentiated ND7 and PC12 cells provide supporting evidence that cell state is important. Serum-starved BHK-21(C13) cells show no impairment in their ability to support 1716 replication. ICP34.5 thus determines host range in terms of cell type but not species (mouse 3T6 and rat PC12 are both non-permissive). For virus growth in vivo, cell type or metabolic state are also crucial for permissivity (Taha et al., 1989a,b; MacLean et al., 1991a; Robertson et al., 1992). The basis is therefore provided for investigation of cellular proteins that may interact with ICP34.5. We are currently trying to identify ICP34.5 cellular homologues in F9 and 3T6 cells by screening cDNA libraries. The recent availability of an expressed ICP34.5 fusion protein should allow experiments to identify specific protein–protein interactions with
ICP34.5. In addition the processing of viral RNA and proteins and the maturation of virus particles in 3T6 and F9 cells is under investigation.

While the role of ICP34.5 in vivo may be complex and more difficult to define, and 3T6 and F9 cells are not neurons, identification of proteins from these cells that specifically interact with ICP34.5 may lead to the identification of neuron-specific proteins that have a similar role. Neurons express a large number of genes coding for proteins specific for brain function and F9 cells have been shown to express the N component of the brain-specific small ribonucleoprotein U2, whose expression is dependent on the differentiated state of the cells (McAllister et al., 1988). In some ways they may thus mimic a neuronal phenotype.

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