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Neurovirulent factor ICP34.5 uniquely expressed in the herpes simplex virus type 1 $\Delta\gamma_1 34.5$ mutant 1716

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The herpes simplex virus type 1 (HSV-1) diploid gene $\gamma_1 34.5$ encodes a neurovirulent factor, infected cell protein 34.5 (ICP34.5). The promoter to $\gamma_1 34.5$ is located within the HSV-1 genome where there are repeated sequences. This region of the genome also contains important overlapping transcripts involved with the virus's ability to establish lytic and latent infections and reactivation. These transcripts include the latency-associated transcripts and regulator proteins ICP0 and ICP4. This study aimed to separate ICP34.5 from these overlapping transcripts and test if its expression from a single gene could restore wild-type HSV-1 strain 17+ virulence. To address these aims, different recombinant viruses were constructed using the $\Delta\gamma_1 34.5$ mutant 1716. Immunoblots probed with different ICP34.5 antisera demonstrated that one of the newly generated recombinant viruses, 1622, overexpresses ICP34.5 relative to a panel of wild-type viruses. Interestingly, the overexpression of ICP34.5 does not yield a more virulent virus. The onset of ICP34.5 expression from 1622-infected cells *in vitro* matched that of 17+, and its expression restored the function of maintaining protein synthesis in human neuroblastoma cells. Replication of 1622, however, was only partially restored to 17+ levels *in vivo*. Additionally, plaque morphology from 1622-infected cells indicates there is an additional defect. The authors report that the mutant virus 1622 can express ICP34.5 from a single $\gamma_1 34.5$ gene and restore most (but not all) wild-type function. These findings are discussed with respect to the use of the $\gamma_1 34.5$ deleted mutant, 1716, in oncolytic viral vector therapies and future studies for ICP34.5. *Journal of NeuroVirology* (2008) **14,** 28–40.

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Introduction

The majority of herpes simplex virus type 1 (HSV-1) infections cause an as yet incurable latent neurotropic infection for the lifetime of the host. One of the neurovirulence factors of HSV-1 has been shown to be concentrated to a locus within the inverted repeat sequences and is due, in part, to the infected cell protein 34.5 (ICP34.5) (Thompson *et al*, 1983; Chou *et al*, 1990; MacLean *et al*, 1991a). The gene encoding ICP34.5, $\gamma_1 34.5$, has part of its promoter and a transcription initiation site within the unique repeat junction called the "a" sequence (Chou and Roizman, 1986; Figure 1A). Two commonly studied HSV-1 strains, F and 17+, show sequence homology within ICP34.5, with the exception of a peptide repeat (ProAlaThr) that is present 10 times in strain F
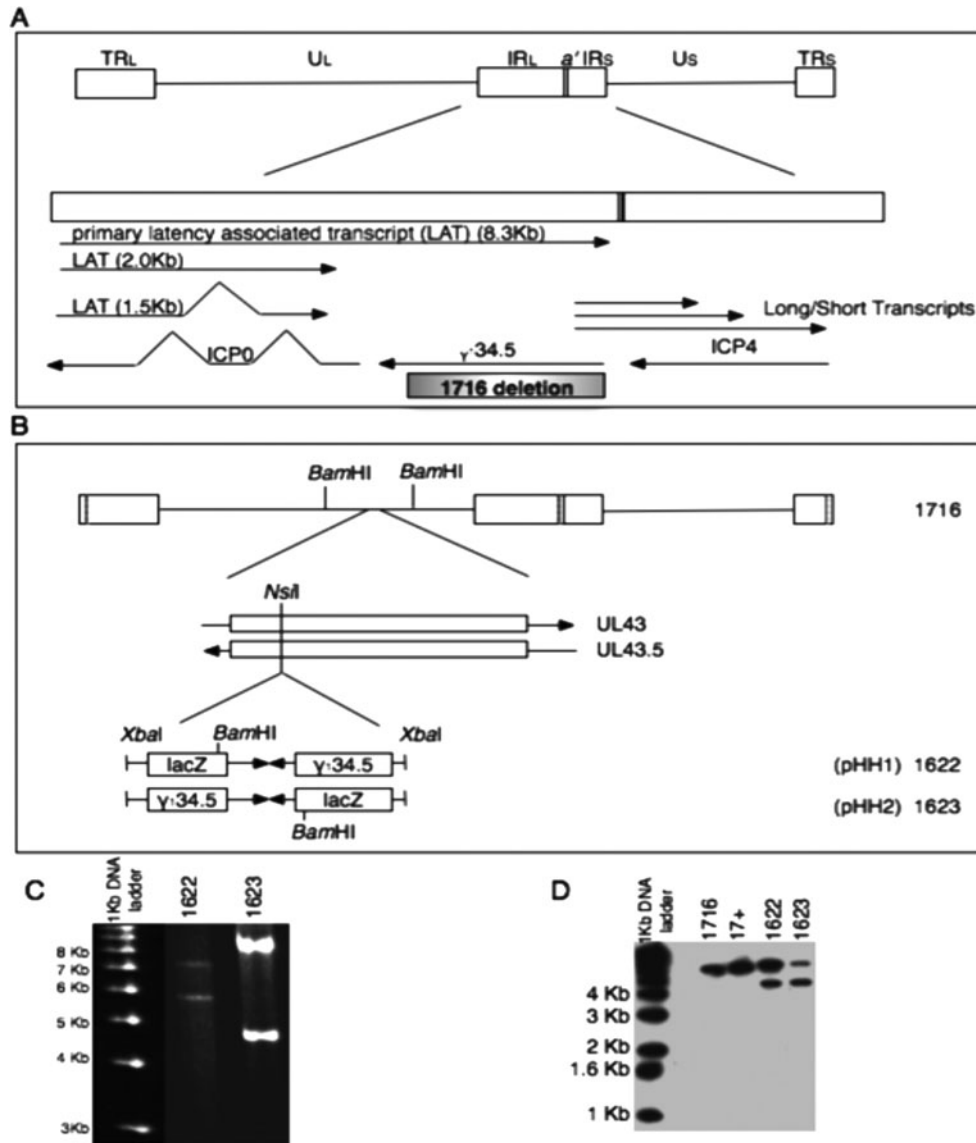
Figure 1 (**A**) A diagram of the HSV-1 genome containing the unique long (U$_L$) and short (U$_S$), regions, that are flanked by terminal and internal repeat sequences (TR$_L$, IR$_L$, and TR$_S$, IR$_S$). The junction where the TR$_L$ and TR$_S$ join is the *a* sequence (not shown), and where IR$_L$ and IR$_S$ meet is the *a′* sequence. The transcripts within the IR$_L$, IR$_S$, and *a′* sequence are shown with their relative orientation in more detail. (**B**) The site of the *UL43/UL43.5* locus and the ectopic locus of $\gamma_1 34.5$ within the 1716 recombinant viruses. (**C**) Agarose gel of pHH1 and pHH2 DNA digested with *Bam*HI, determining the orientation of the $\gamma_1 34.5$ and *lac*Z cassettes within the *UL43/UL43.5* locus. (**D**) A Southern blot of DNA prepared from virus stocks of 1716, 17+, 1622, and 1623. The blot was probed with p35(PacI) DNA spanning *UL41* to *UL44*. 17+ and 1716 have the naturally occurring 6.6-kb *Bam*HI fragment, whereas the recombinant viruses 1622 and 1623 have the additional $\gamma_1 34.5$ and *lac*Z cassette, introducing a new *Bam*HI site that cuts within the insert and thus generating two bands of 7.8 and 4.5 kb. A 1-kb DNA ladder is shown for size comparison.

but only 5 times in strain 17+. A recent study demonstrated that this difference in number of repeats does not play a role in neurovirulence (Jing and He, 2005).

Deletion of the two $\gamma_1 34.5$ genes in HSV-1 strain F (mutant R3616) causes the HSV-1 virus to be non-neurovirulent by blocking host protein synthesis and viral replication in neuronal cells (Chou and Roizman, 1992). Several viruses have evolved similar mechanisms in specific cell types via the protein kinase R (PKR) pathway; these include polio virus, influenza virus, adenovirus, vaccinia virus, and herpes simplex virus (Black *et al*, 1993; Carroll *et al*, 1993; Davies *et al*, 1993; Katze, 1995; Lee *et al*, 1994; Lu *et al*, 1995; Mathews and Shenk, 1991). HSV-1 infections normally activate the PKR pathway, but without ICP34.5 binding to protein phosphatase 1α(PP1α) the translational initiation factor eIF2α is phosphorylated and the PKR pathway is not activated. Studies have shown that the carboxy terminus of ICP34.5 is responsible for binding to PP1αs which is highly homologous to the cellular

protein GADD34 (Chou and Roizman, 1992; He *et al*, 1996; Brown *et al*, 1997).

The HSV-1 strain 17+ mutant virus, 1716, has a 759-bp deletion in the two repeat regions corresponding to the $\gamma_1 34.5$ genes (MacLean *et al*, 1991; Figure 1A). Initial studies of 1716 by immunohistopathology demonstrated that by 28 days post injection into the central nervous system (CNS), there was neither evidence of virus antigen—nor a host-mounted immune response (McKie *et al*, 1998). Further characterization of 1716 tested its application against certain brain tumors by *in vitro* replication experiments in established human glioma cell lines and in primary cell cultures derived from human tumor biopsies. These studies demonstrated were good replication kinetics and eventual cell death from 1716 infections (McKie *et al*, 1998). However, replication of 1716 in various other non-neuronal cell lines showed restricted growth, for example, in murine 10T1/2 cells, stationary-phase primary mouse embryo cells (Bolovan *et al*, 1994), and mouse fibroblast (3T6) cells (Brown *et al*, 1994). We hypothesized that the phenotype and replication kinetics of the mutant virus 1716 was solely due to the lack of ICP34.5 and that expression of ICP34.5 from a single $\gamma_1 34.5$ gene would rescue the 17+ wild-type phenotype.

Here we report a new recombinant virus, 1622, containing a single copy of the $\gamma_1 34.5$ gene inserted into the 1716 genome. The $\gamma_1 34.5$ open reading frame (ORF) was placed under the control of the heterologous HSV-1 glycoprotein gD promoter to preclude rearrangements due to repeat sequences within the endogenous promoter. We demonstrate that the natural carboxy terminus of $\gamma_1 34.5$ maintained its function and allowed the virus to restore host cell protein synthesis. By immunoblotting, we found that 1622 expressed more ICP34.5 than wild-type 17+. The increased expression was due to the insertional orientation of the gene into the unique long 43 gene (*UL43)* and not the promoter sequence. This was determined by generating a second recombinant virus, 1623, where the $\gamma_1 34.5$ gene was inserted in the opposite orientation. 1623 yielded a marked reduction in ICP34.5 expression from 1622- and wild-type 17+-infected cells.

Furthermore the expression of ICP34.5 from HSV-1 viruses was identified as early as 2 h post infection *in vitro*. By indirect immunofluorescence ICP34.5 had similar punctate nuclear staining from wild-type 17+ and 1622-infected cells. However, 1622 maintained some characteristics of 1716, such as its small plaque morphology, and decreased replication kinetics in certain cell types *in vitro* and *in vivo.* Immunohistochemical experiments showed a reduction in positively stained neurons in trigeminal ganglia following 1622 infections compared to 17+ infections. The importance of these findings are evaluated and discussed with respect future directions for ICP34.5 studies.

Results

Construction of recombinant viruses
The $\gamma_1 34.5$ gene was originally derived from the HSV-1 *Bam*HI "k" fragment pBAMk/pAT153 (McGeoch and Barnett, 1991), the $\gamma_1 34.5$ ORF was cloned into plasmid pGEM34.5 (McKie *et al*, 1994), and this was finally subcloned into plasmid pFJ14H, positioning the $\gamma_1 34.5$ ORF immediately downstream of the HSV-1 gD promoter. The gD promoter was used instead of the $\gamma_1 34.5$ gene's own promoter sequence due to its location within the repeat sequences, which could potentially result in undesired rearrangements or repeats. The $\gamma_1 34.5$ ORF was cloned directly upstream of the HSV-2 immediate-early (IE5) polyadenylation sequence in pJF14H. Plasmid pFJ14H also contains the *lacZ* gene under the control of the SV40 promoter with its own SV40 polyadenylated sequence in the opposite orientation to $\gamma_1 34.5$.

The $\gamma_1 34.5$ and *lacZ* genes were digested from plasmid pJ14H as a single fragment, with *Xba*I and ligated into the HSV-1 *UL43* gene in plasmid p35(PacI) (MacLean *et al*, 1991b; McGregor *et al*, 1999). Two plasmids, pHH1 and pHH2, contained $\gamma_1 34.5$ but in opposite orientations (Figure 1B). *Bam*HI restriction enzyme digestions were carried out for orientation confirmation. pHH1 contains two DNA fragments of sizes 7.4 and 5.8 kb, indicating that the $\gamma_1 34.5$ gene is in the same direction as the *UL43.5* gene, and was used to generate the recombinant virus 1622. pHH2 contains two bands of sizes 8.5 and 4.7 kb (Figure 1C), demonstrating that $\gamma_1 34.5$ is in the same orientation as the *UL43* gene, and was used to generate the recombinant virus, 1623.

Plasmids pHH1 and pHH2 were linearized by digesting with *Xho*I for increased transfection efficiency and were individually co-transfected with 1716 DNA by calcium phosphatein BHK cells. Infected cells were allowed to display complete cytoplasmic effect and then were harvested into the media. Virus particles were released by sonication and titrated on BHK cell monolayers. Two days after titration, the supernatant was removed and an X-gal overlay was added. Detection by positive blue staining allowed individual recombinant viral plaques to be isolated and taken through plaque purification.

A Southern blot verified the recombinant virus DNA integrity after the growth of viral stocks. The membrane was probed with DNA that spans the *UL41* to *UL44* HSV-1 genome. Wild-type 17+ and the $\Delta\gamma_1 34.5$ mutant 1716 have one *Bam*HI fragment of 6.6 kb, whereas the recombinant viruses contain an extra *Bam*HI site plus DNA from pFJ14H generating fragments of 7.8 kb and 4.5 kb (Figure 1D).

Ectopic expression of ICP34.5 in 1716
Following confirmation of the correct recombinant viral DNA, ICP34.5 expression was examined.

Western blots were carried out using polyclonal anti-serum, 137, raised against a glutathione *S*-transferase (GST)-ICP34.5 fusion protein (Brown *et al*, 1997). Published data suggested that ICP34.5 is expressed late during infection (Chou and Roizman, 1986); however, we detected ICP34.5 as early as 2 h post infection (p.i.) from 17+- and 1622-infected BHK cells (Figure 2A). Expression of ICP34.5 from 17+- and 1622-infected cells steadily increased over time. The whole blot is shown to illustrate equivalent loading.

To determine if one ICP34.5 antiserum had a higher affinity for ICP34.5 in 1622, different ICP34.5 antisera were tested. An antibody raised against a synthetic peptide of the $(PAT)_{10}$ repeat from ICP34.5 was used in immunoblots (McKay *et al*, 1993; Figure 2B, *top*). This antiserum consistently and preferentially recognizes ICP34.5 in strain F, which also has 10 PAT repeats. Anti-$(PAT)_{10}$ sera does not strongly recognize ICP34.5 in 17+ (which has only 5 repeats), it shows a faint level; however, this antiserum further supports that 1622 overexpresses ICP34.5 relative to strains F and 17+. To confirm equal loading, the blot was incubated with an antiserum raised against the HSV protein UL42 (Figure 2B, *bottom*). The level of UL42 expression was similar among 17+, 1716, and 1622, but it was less well recognized in strain F. Finally, another generated polyclonal antiserum raised against GST-$\gamma_1$34.5 protein, R1, also showed an
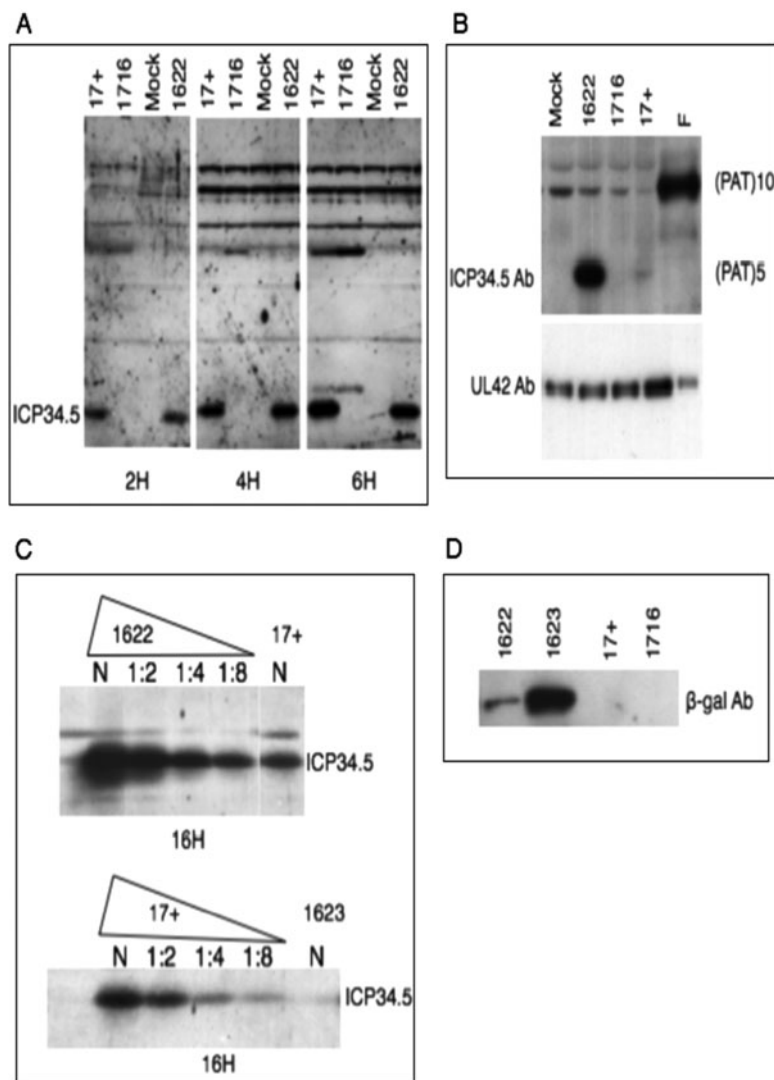

Figure 2 Expression of ICP34.5 in infected BHK cells. (**A**) BHK cells were either infected with 17+, 1716, 1622, or mock. Both 17+ and 1622 show the same levels of ICP34.5 expression at 2 h p.i. and increasing levels at 4 and 6 h p.i. (**B**) Western blot using a monoclonal antibody generated against a synthetic peptide of the ten PAT repeats in strain F ICP34.5. A monoclonal antibody against the *UL42*, 65-kDa DNA-binding protein was also used to confirm equal loading. (**C**) Comparison of ICP34.5 levels from 1622- and 17+-infected BHK cells 16 h p.i. Serial twofold dilutions of lysates were made in SDS lysis buffer starting with undiluted [N], 1:2, 1:4, and 1:8 dilutions of either 1622 or 17+ ICP34.5 expression shown on a Western blotting using antiserum against ICP34.5. (**D**) A Western blot reacted with *β*-galactosidase antiserum showing *β*-galactosidase expression levels from recombinant viruses 1622 and 1623.

increased level of expression of ICP34.5 from 1622- relative to 17+- or strain F-infected cell extracts (data not shown).

At later times after infection when ICP34.5 was previously known to be expressed, 1622 demonstrated a dramatic overexpression or abundance of ICP34.5 in BHK cells by Western blot (Figure 2C). However, the mutant virus 1623 had little detectable ICP34.5 by immunoblotting. In order to qualitatively assess the differences in ICP34.5 expression, a series of twofold dilutions were made of 1622- (top) or 17+- (bottom) infected BHK cells This demonstrates that 1622 expresses at least eight times more ICP34.5 than 17+ at late times post infection. Serial dilutions of 17+ were also carried out to compare the level of ICP34.5 expression from 1623; there was approximately 8 times less ICP34.5 from 1623-infected cells.

Due to the effect on expression of placing $\gamma_1 34.5$ either with *UL43* or *UL43.5*, we examined the expression levels of β-galactosidase in these recombinant viruses. A Western blot using antiserum against β-galactosidase showed an increased expression level of the β-galactosidase from 1623-infected cells over that from 1622-infected cells (Figure 2D), which is consistant with the ICP34.5 orientations in these viruses. Infections of 17+ and 1716 were used as negative controls.

The detection of a more native form of ICP34.5 expression was examined by immunoprecipitations from cells infected with 17+, 1622, or 1716. Equal quantities of the whole-cell extract (WCE) and immunoprecipitants (IPs) were analyzed by Western blot using an antiserum against ICP34.5 (Figure 3A). The WCE of 17+- and 1622-infected cells recognized ICP34.5 expression; however, the IP showed ICP34.5 from 1622, whereas ICP34.5 was not detected in the 17+ immunoprecipitation.

Host protein synthesis from viral infections in vitro
These experiments were carried out to determine whether ICP34.5 from strain 17+ and 1622 infections could rescue the ICP34.5 function of maintaining host protein synthesis similar to that reported in HSV-1 strain F. BHK and SK-N-SH cells were infected at a multiplicity of infection (MOI) of 10 with either 17+, 1716, 1622, or mock, and then pulse-labeled with[^{35}S]methionine for 16 h p.i. In BHK cells, all viruses gave the same protein synthesis profile (Figure 3B). The overall cell density was lower in SK-N-SH cells due to the slow growth properties of the cells. However, in these cells little protein synthesis occurred from 1716 infections. The host protein synthesis profile from 1622-infected cells was identical to that from 17+-infected SK-N-SH cells.

ICP34.5 immunofluorescence from 17+, 1716, and 1622 infections
Localization of ICP34.5 from 1622-infected cells was analyzed by indirect immunofluorescence (Figure 4).
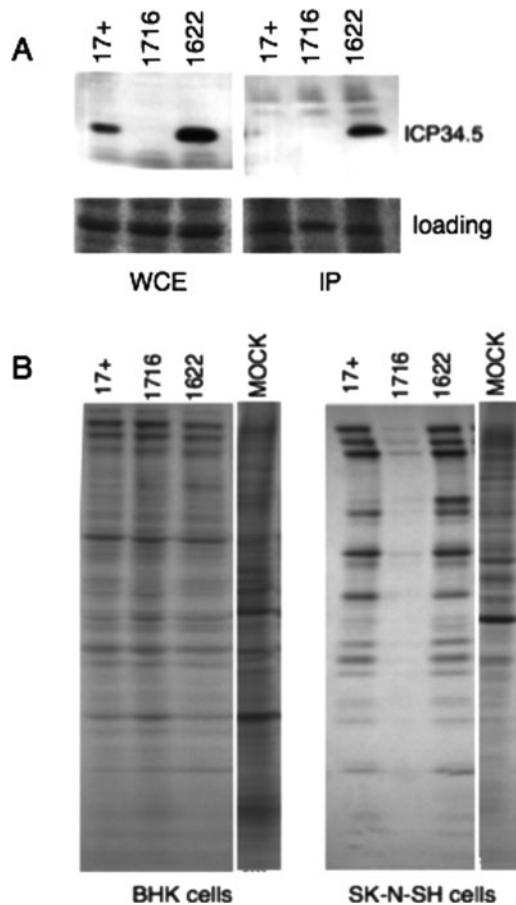

Figure 3 Immunoprecipitation of ICP34.5 from different virus-infected BHK cells. (**A**) Western blot of ICP34.5 (*top*) and radiolabeled for loading control (*bottom*). (**B**) BHK and SK-N-SH cells were infected at an MOI of 10 were pulse-labeled with [^{35}S]methionine from 14 to 16 h p.i.

BHK cells were either infected with 17+, 1716, or 1622 at a MOI of 10 for 5 h, fixed on coverslips, and incubated with the ICP34.5 antiserum, 137. The ICP34.5 staining produced similar punctate nuclear staining in 1622- and 17+-infected cells, whereas 1716-infected cells had diffuse background staining.

Plaque phenotype
When cells were infected at a low MOI (0.01) and allowed to form plaques on a monolayer of BHK cells, there was a specific phenotype from 1716 infection that was mimicked in the 1622 and 1623 infections (Figure 5A). Plaque sizes were semiquantitated by taking photographs, cutting around the plaque, and weighing. Averages from the different virally infected cells are given in Table 1.

To determine if the small plaque morphology from 1716- and 1622-infected cells was a direct result of either deleting the diploid gene $\gamma_1 34.5$, its ectopic expression, or the mutation of another region, rescue experiments of 1716 were carried out using cosmids spanning the entire HSV-1 17+ genome
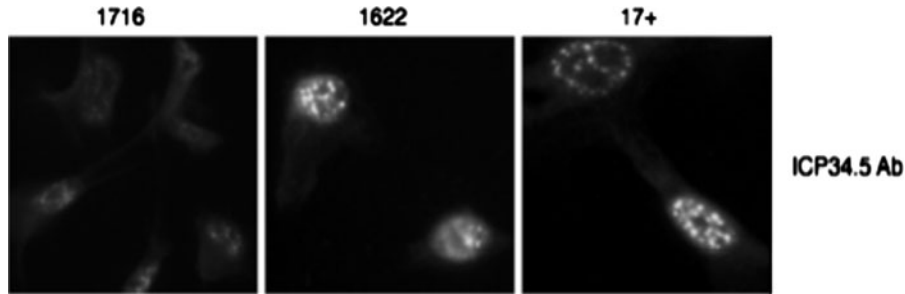
Figure 4 Nuclear localization of ICP34.5 following infection of BHK cells. Immunofluorescence of ICP34.5 in BHK cells 5 h p.i. from 1622 and 17+ showing punctate nuclear staining.

by cotransfecting with 1716 (Figure 5B). Two cosmids rescued wild-type 17+ plaque morphology in 1716, cos48 and cos6 (Figure 5C); these cosmids span the internal and terminal repeat sequences, respectively.

In vitro viral replication kinetics

This laboratory and others have shown that mutating or deleting the $\gamma_1 34.5$ genes dramatically reduces the virus's ability to replicate in some, but not all, cell lines. In a single cycle of viral replication using a high

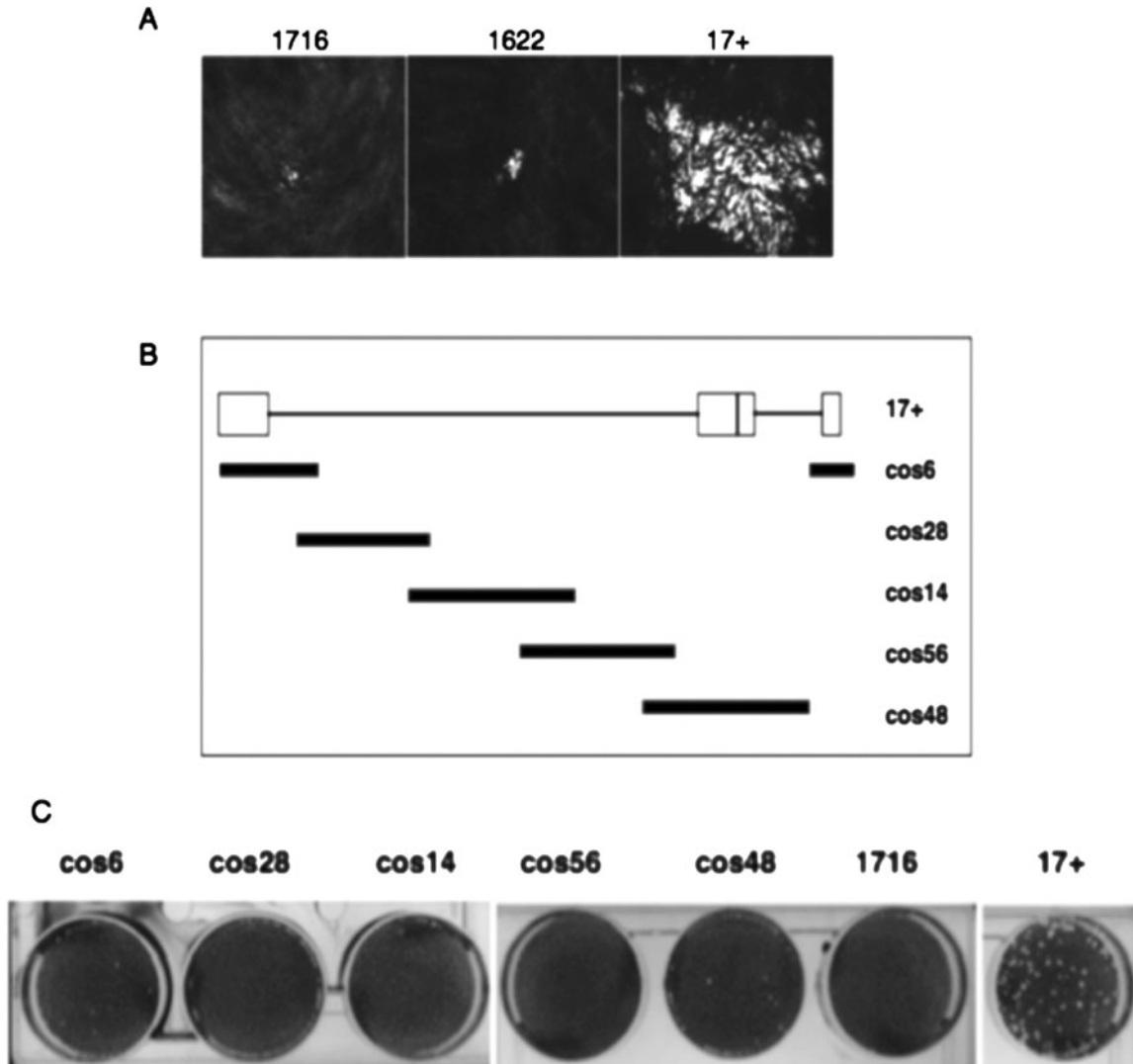**Figure 5** Cosmid rescue experiment of wild-type 17+ virus plaque morphology. (**A**) Plaque morphology of 1716, 1622, and 17+ in a BHK cell monolayer by crystal violet staining shows differences in plaque size. (**B**) Schematic representation of cosmids spanning the HSV-1 17+ genome. (**C**) Crystal violet–stained BHK cell monolayers of 1622-infected cells cotransfected with the different cosmids.

Table 1 Viral plaque size determined by photography and weight

Virus	Number of plaques counted	Average weight (g)	Percentage of 17+ plaque weight
1622	26	0.3101	19%
1716	26	0.0926	6%
17+	22	1.5975	100%

MOI of either 17+, 1716, or 1622, replication kinetics were analyzed in BHK or 3T6 cells (Figure 6A). All viruses showed similar replication kinetics when infecting BHK cells; however, in 3T6 cells, 1716 did

not replicate as well as 17+ and 1622. 1716 yielded a maximum titer at 36 h that was at least 1 log less than 1622 or 17+.

Multicycle viral replication kinetics were also tested from 17+, 1716, and 1622 with the additional $\gamma_1 34.5$ mutant virus, 17termA (Bolovan *et al*, 1994), and its corresponding rescuant, 17termAR. BHK and RS cells were infected at a low MOI of 0.01, and at time points 0, 15, 50, and 96 h p.i.; samples were titrated in triplicate onto RS cells. The 1622 recombinant virus started to show a slight defect in replicating by 10 h in BHK cells (Figure 6B). In RS cells, 1716, 1622, and 17+ all had similar replication
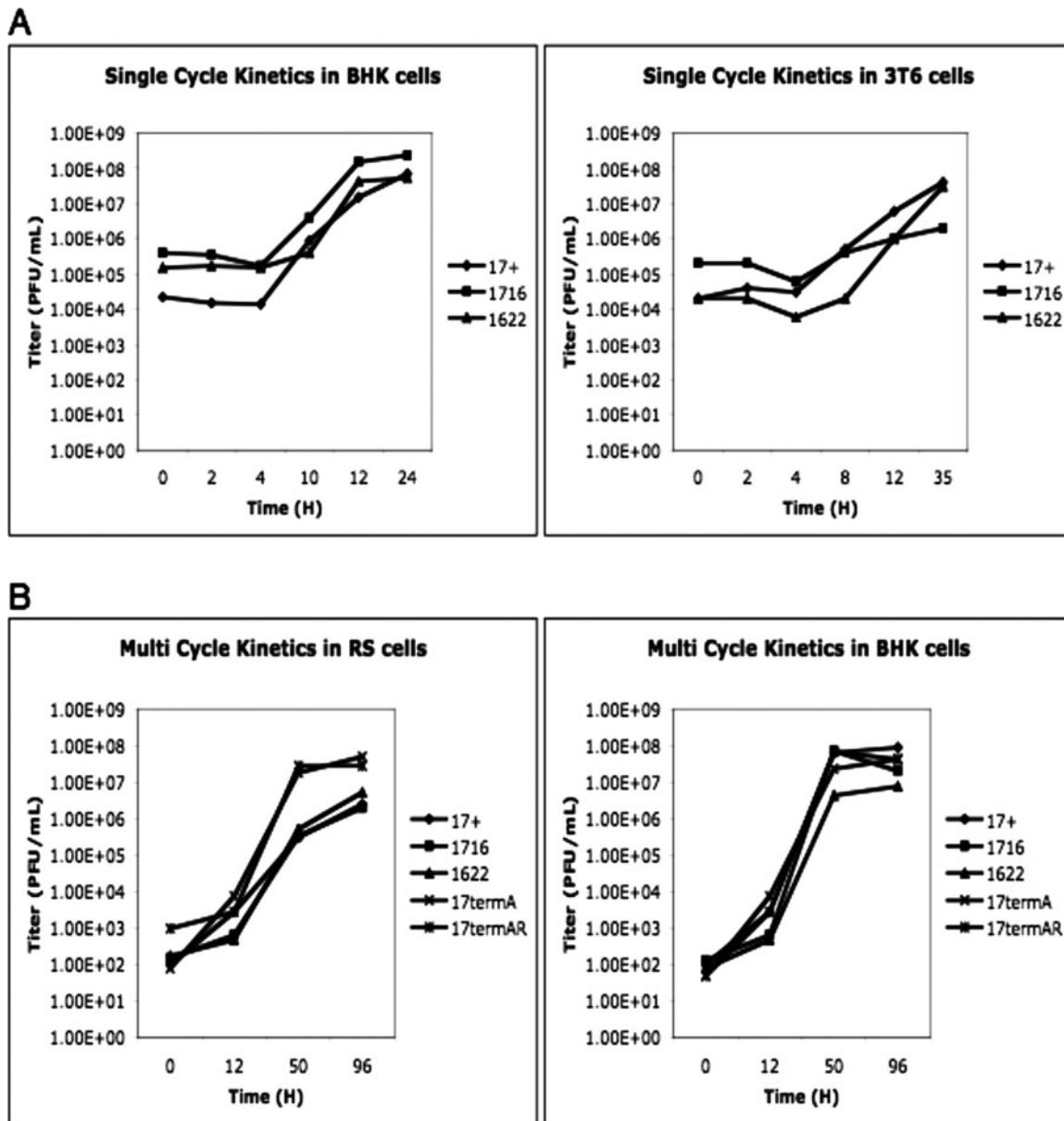
Figure 6 Replication kinetics in different cell lines. BHK and 3T6 cells (**A**) were infected with either 1716, 1622, or 17+ at a MOI of 10 to study single-cycle growth kinetics. At times 0, 2, 4, 10, 12, and 24 or 36 h p.i., cells were scraped into the medium, sonicated, and titrated onto BHK cells. In 3T6 cells, 1716 did not replicate as well as 17+ and 1622, the latter reaching titers of 7×10^7 and 8×10^7 PFU/ml, respectively, at 36 h p.i. (**B**) Multicycle replication kinetics in RS cells and in BHK cells.

Table 2 The LD_{50} values following intracranial inoculations of different viruses

	1×10^1	1×10^2	1×10^3	1×10^4	1×10^5	1×10^6	LD_{50}
17+	4/4	4/4	ND	ND	ND	ND	$<3.2^*$
1716R	4/4	4/4	ND	ND	ND	ND	<3.2
1716	ND	ND	ND	ND	0/4	1/4	1.8×10^6
1622	0/4	1/4	4/4	4/4	ND	ND	1.8×10^3

ND = Not Done.
*PFU/mouse.

kinetics as one group, whereas 17termA abd 17termAR grew similarly in as another group. The two groups of viruses had an approximately 2-log difference at 50 h p.i. and 1-log difference at 96 h p.i. The overall grouping of 17+, 1716, and 1622, versus 17termA and 17termAR, was due to the growth of viral stocks in either BHK cells or RS cells, respectively.

In vivo *viral replication and immunohistochemistry of HSV-1*

Using a murine model, we tested for 50% of the lethal dose (LD_{50}) of 17+, 1622, 1716, and its rescuant, 1716R. Three-week-old BALB/c mice were administered different concentrations of viruses by intracranial inoculations. Both 17+ and 1716R had LD_{50} values of less than 3.2 PFU/mouse (Table 2); whereas 1622 had LD_{50} value of at least 2 logs less and the LD_{50} value for 1716 is greater than 1.8×10^6 plaque forming units (PFU)/mouse.

In vivo lytic infections with different viruses were carried out to study viral replication at the site of infection (snouts) and in trigeminal ganglia (the normal end point of lytic infections). The replication of lytic infection in snouts from 17+ showed an efficient replication between days 2 (S2) and 4 (S4) post infection (Figure 7A). Four days post infection (S4), 17+ titers decreased and continued to decrease for the rest of the experiment. The replication kinetics of 1716 and 1622 in snouts were similar. Both 1716 and 1622 maintained a constant replication until 4 days post infection (S4), but their titers did not increase like 17+. By 6 days post infection in snouts (S6), there was no detectable 1716 or 1622 virus. In trigeminal ganglia (TG), 17+ and 1716 initially had a titer of 1×10^3 PFU/ml at 2 days post infection (TG2), but the titer of 1716 dropped at 4 days post infection (TG4), and its titer continued to drop to undetectable levels by 6 days post infection (TG6), whereas 17+ had an increased titer between days 2 (TG2) and 4 (TG4) post infection. Although 1622 showed an initial slower growth than 17+ and 1716 in TG, it demonstrated an ability to replicate efficiently between day 2 (TG2) and day 4 (TG4) post infection (Figure 7B).

Immunohistochemistry experiments were carried out to further examine the differences these viruses in the trigeminal ganglia of mice, which were infected for four days (Figure 7C). Typical images are shown that depict the lack of any HSV antigen staining from 1716 infection. Positive neurons for HSV antigen (as indicated by arrows) were present from 1622 infections as well as many positively stained neurons from 17+-infected TG.

Discussion

Due to the chimeric nature of the HSV-1 genome, sequences spanning the junctions have presented complex overlapping, antiparallel, and diploid transcripts. One such open reading frame is responsible for the neurovirulence factor, ICP34.5. It was hypothesized that generating a recombinant virus containing a single copy of the $\gamma_1 34.5$ gene would provide a better understanding of this transcript. Two such $\gamma_1 34.5$ recombinant viruses, 1622 and 1623, both express ICP34.5 ectopically from a single copy of the gene in the $\Delta\gamma_1 34.5$ mutant 1716. The strategy of cloning $\gamma_1 34.5$ into the *UL43/UL43.5* locus was based on earlier studies by MacLean *et al*, who demonstrated that by inserting the *lacZ* gene into the *UL43* ORF, expression of UL43 was precluded and viral replication kinetics were indistinguishable from that of its wild-type parental strain 17+ (MacLean *et al*, 1991b). Another mutant virus where most of the *UL43* gene was deleted did not impair the ability of the mutant to replicate *in vivo* at the periphery, spread, or replicate within the nervous system in a mouse ear model. Therefore, the *UL43* gene was considered to be nonessential. Subsequent studies by others discovered that there was a transcript antisense to *UL43*, named *UL43.5*, which is expressed at late times post infection (Ward *et al*, 2003). In the same study, Ward *et al* determined that *UL43.5* was also dispensable in cell cultures.

The initial experiments of this studies examined the orientation of the $\gamma_1 34.5/lacZ$ cassette cloned within the *UL43/UL43.5* locus. It was determined that when $\gamma_1 34.5$ is inserted in the same direction as *UL43.5,* there is a significant overexpression of ICP34.5. A previously published study showed that when the *lacZ* gene under the control of the glial fibrillary acidic protein promoter was cloned in the *UL43* locus and in the same direction as the UL43 transcript in 1716, it selectively replicated in astrocytes in the CNS using a brain tumor model (McKie *et al*, 1998b). However they did not establish if there was an oritentation effect on expression in this locus. With the findings reported here, it may be worthwhile to further test the potential use of the *UL43.5* promoter and orientation for gene therapies.

Detection methods used for examining the expression of ICP34.5 were previously based on Western blotting. Here we report the visualization of ICP34.5 intracellulary by indirect immunofluorescence and showed punctate nuclear staining in fibroblasts *in vitro*. Although this could be of interest for future *in vivo* studies in different tissues and cell types, it would also be beneficial to look for ICP34.5
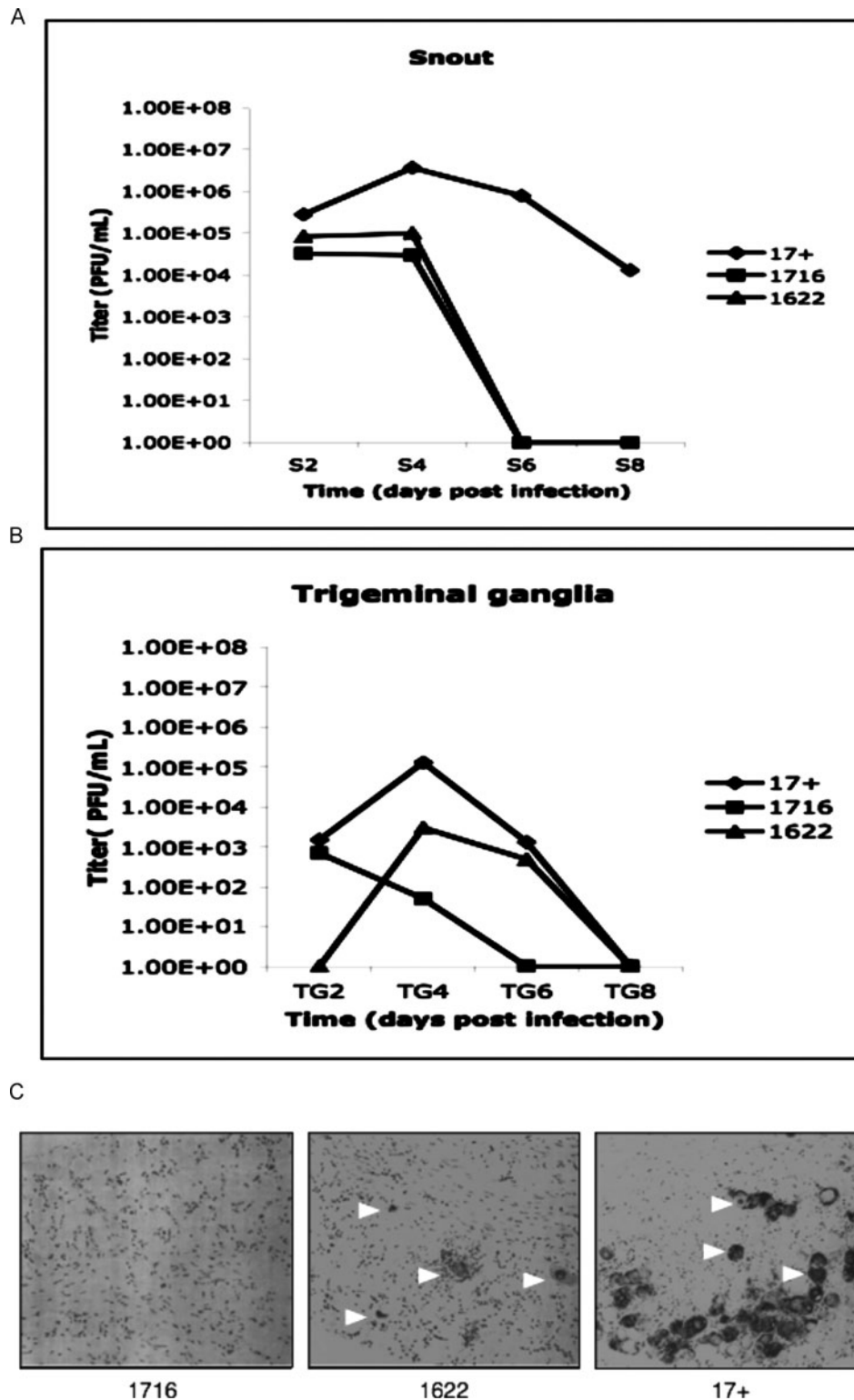
Figure 7 *In vivo* replication kinetics and immunohistochemistry following viral infections. (**A**, **B**) Viral titers are shown graphically from plaque growth in RS cell monolayers. (**A**) Replication of 17+, 1716, and 1622 viruses in the snouts was monitored at days 2 (S2), 4 (S4), and 6 (S6) post infection. (**B**) In trigeminal ganglia, 1716 did not appear to replicate over time as seen at 2 (TG2), 4 (TG4), and 6 (TG6) days post infection. (**C**) Immunohistochemistry of trigeminal ganglia 4 days post infection of 17+, 1716m, or 1622. Shown are microscopic sections from trigeminal ganglia. The darker positive HSV staining of neurons in sections of TG were infected with 1622 or 17+. TG from mice infected with 1716 had a lack of any positive HSV staining. Photomicrographs were taken at 20× magnification.

transcripts at early times to elucidate potential differences between 1622 and 17+ or in fact among other HSV-1 strains. It is probable that there is a low level of ICP34.5 expression from 17+ before 2 h p.i. that may play a critical role in the virus's ability to lytically infect TG.

Time-course experiments showed that the onset and duration of ICP34.5 expression in 17+, 1622, and 1623 infections were similar. The overexpression of ICP34.5 in 1622 led us to inquire if the replication kinetics of 1716 *in vitro* would be restored to wild-type levels or if 1622 would be even more virulent. The reduced replication kinetics of 1716 *in vitro* in certain cell lines (3T6) was supported by previously published data of HSV-1 strain F and its $\gamma_1 34.5$ negative mutant, R3616 (He *et al*, 1997).

The $\Delta\gamma_1 34.5$ mutant 1716 from strain 17+ is unable to maintain host protein synthesis as reported on the $\Delta\gamma_1 34.5$ mutant R3616 from HSV-1 strain F. Additionally, a single copy of $\gamma_1 34.5$ in 1622 restored protein synthesis. Another group published a similar finding using the recombinant virus Myb34.5 from HSV-1 strain F, where they used the *UL39* loci to insert a single copy of the $\gamma_1 34.5$ under the control of the E2F-responsive cellular B-*myb* promoter (Chung *et al*, 1999).

Plaque morphology data showing differences between wild-type HSV-1 strains and $\Delta\gamma_1 34.5$ mutant viruses has been previously reported by Mao and Rosenthal (2003). The data presented here indicate that the smaller plaque phenotype may correlate with less virulence. The underlying cause of this smaller plaque was determined by cosmid rescue experiments. The plaque size of 1716 and 1622 returned to normal when cosmids containing the terminal and inverted repeat sequences were cotransfected. However, further investigation with smaller sequences to rescue the wild-type plaque phenotype could be conducive to understanding what other factors may be involved in the 1622 phenotype, for example, reduced replication *in vivo* and lower number of infected neurons in TG. Three leading explanations remain for the reduction in replication kinetics *in vivo* and plaque morphology from 1622. One reason could be due to the ICP34.5 expression from a single $\gamma_1 34.5$ gene. Although this is not a favored explanation due to the rescued phenotype of 1622 maintaining host protein synthesis *in vitro*, it should not be ruled out. One approach to address this could be to add a second copy of $\gamma_1 34.5$ in another nonessential locus such as *UL39*. A second and more attractive hypothesis for why 1622 does not completely rescue wild-type kinetics *in vivo* may be due to the partially overlapping transcription factors, ICP4 and ICP0. Expression of ICP0 and ICP4 were examined by Western blot and did not show any differences between 1716, 1622, and wild-type expression levels (data not shown). Although this result reflects an *in vitro* infection and it might be different *in vivo*, this would not be expected. A third hypothesis is that there is

another mutation elsewhere in the 1716 and/or 1622 genome. This is not likely because the cosmid experiments rescued the aberrant plaque morphology of 1716 and 1622.

The importance of this work relates to several clinical studies in which HSV-1 vectors are being used for potential therapies. A phase I clinical trial reported that there can be a relapse in malignant glioma's using 1716 with no adverse effects, including no encephalitis, and no reactivation of the mutant virus (Rampling *et al*, 2000). Another report using a combination of $\Delta\gamma_1 34.5$ and disabled *UL39* gene in the parental HSV-1 strain F, G207, also in phase I clinical trials showed that there was no development of HSV encephalitis, toxicity, or serious adverse effects (Markert *et al*, 2000). The findings described here provide a good rationale for future studies of ICP34.5 and this region in HSV viral vectors for gene delivery.

Materials and methods

Cells and viruses
Baby hamster kidney (BHK) cells were grown in Glasgow modified Eagle's medium supplemented with tryptose phosphate broth and 10% newborn calf serum. Rabbit skin (RS) cells and mouse embryonic fibroblast (3T6) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Human neuroblastoma (SK-N-SH) cells, a generous gift of B. Roizman, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. All cells were grown in an atmosphere of 5% CO_2 and 37°C. The HSV-1 17+, 1716, 1622, and 1623 viruses were plaque-purified, grown, and titred on BHK cell monolayers as described previously (MacLean *et al*, 1991a).

Plasmids and recombinant viruses
The DNA fragment containing $\gamma_1 34.5$ was initially isolated from the viral genome by restriction enzyme digestion using *Rsa*I (126530) and *Alu*I (1250741) and was then cloned into the plasmid pGEM, producing pGEM34.5. The pGEM34.5 plasmid was digested with *Nco*I and *Bam*HI to isolate the $\gamma_1 34.5$ open reading frame (ORF).

A multi-cloning site was synthesized consisting of a top linker, AGCTTAGATCTCCATGGCCCGGGA, and a bottom linker, ATCTAGAGGTACCGGGCC-CTTCGA, which were ligated into the *Hin*dIII site of plasmid pFJ14, generating plasmid pFJ14H. The $\gamma_1 34.5$ and *lacZ* genes were isolated in one fragment from pFJ14H using *Xba*I and cloned into the nonessential *UL43/UL43.5* loci in plasmid p35(PacI) (A. McGregor, unpublished data), generating plasmids pHH1 ($\gamma_1 34.5$ in the direction of *UL43.5*), and pHH2 ($\gamma_1 34.5$ in the direction of *UL43*).

Plasmids pHH1 and pHH2 were individually cotransfected with the variant 1716 strain using calcium phosphate and salmon sperm DNA to generate the recombinant viruses 1622 and 1623, respectively.

Three rounds of plaque purification were carried out and recombinant virus was isolated by blue plaque morphology in the presence of X-gal, as described previously (MacLean *et al*, 1991a). Southern blot analysis was performed as previously described, to verify the integrity of recombinant virus DNA. Briefly, a *Bam*HI fragment from p35(PacI) corresponding to *UL41* to *UL44* genes was labeled with [^{32}P]dCTP by random priming High Prime (Roche Applied Science).

Immunoblotting

Mouse fibroblast cells either mock infected or infected with different viruses at a multiplicity of infection (MOI) of 10 PFU/cell. After different times post replication, infected cells were harvested into the media, centrifuged, and the cellular pellet was rinsed with phosphate-buffered saline (PBS). Samples were solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 2.75% sucrose. Samples were sonicated, boiled, and subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred from the gel to nitrocellulose membranes, blocked with 5% nonfat milk, and reacted with two different anti-GST/HSV-1 ICP34.5 rabbit polyclonal sera, namely R1 and 137 (Brown *et al*, 1997; McKay *et al*, 1993); anit-HSV-1 (PAT)$_{10}$ serum (Ackermann *et al*, 1986); anti-β-galactosidase serum; and anti-HSV-1 gC serum. The membranes were washed in PBS and reacted with either anti-rabbit or anti-mouse IgG–horseradish peroxidase conjugate (Sigma). Using an enhanced chemiluminescence (ECL) kit (Amersham), the blot was exposed to film.

Immunoprecipitation

BHK cells (5×10^6) were infected with either wild-type virus or recombinant virus at a MOI of 20. For equal loading, duplicate infected cells were labeled with 50 μCi/ml [^{35}S]methionine in Eagle's medium containing one-fifth the normal concentration of methionine and 2% calf serum (Emet/5C2 medium). At 16 h post infection, cells were harvested by washing with PBS and suspended in binding buffer (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% [*v/v*] NP40, 0.5% [*w/v*] sodium deoxycholate, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) at 4°C for 15 min. Cellular debris was removed by centrifugation at $13,000 \times g$ for 15 min. Immunoprecipitations were carried out by incubating cells with anti ICP34.5 serum overnight at 4°C. Immunoprecipitants were allowed to bind to Protein G for 1 h at 4°C and washed before separation of proteins by an SDS-polyacrylamide gel.

Host protein synthesis shutoff

Host protein synthesis shutoff experiments were carried out by infecting BHK and SK-N-SH cells at a MOI of 10 and radiolabeling with 100 μCi/ml of [^{35}S]methionine in Emet/5C2 medium between 14 and 16 h p.i. (labeled for 2 h). Cells were harvested into SDS loading buffer, boiled, and analyzed by electrophoresing through a 10% SDS-polyacrylamide gel, drying the gel, and exposing it to film (Chou and Roizman, 1992).

Immunofluorescence of ICP34.5 and plaque morphology

BHK cells were grown on 13-mm-diameter glass coverslips and were then infected with either 1716, 1622, or 17+ at a MOI of 5. At 5 h post infection, cells were washed twice with PBS containing 1% FCS and then fixed by incubation for 5 min at −20°C with precooled 95% ethanol 5% glacial acetic acid. The cells were then washed three times with PBS 1% FCS. Cells were permeabilized for 5 min in a PBS solution containing 0.5% NP-40 and 10% sucrose. The primary antisera raised against ICP34.5 was diluted 1:200 in PBS plus 1% FCS. After incubation at room temperature for 1 h, coverslips were washed three times with PBS and then treated with the secondary antibody (fluorescein isothiocyanate–conjugated donkey anti-rabbit immunoglobulin G) (Sigma) diluted 1:100 in PBS. After a further 30-min incubation, coverslips were washed three times with PBS and then washed once with water before being mounted on glass slides using Citifluor.

Plaque morphologies were observed by infecting cells at a MOI of 0.01. Plaque formation was allowed to develop for up to 16 h. Cells were fixed and stained with 1% crystal violet dissolved in a 10% formalin solution, washed with distilled water, and allowed to dry. Subsequent imaging was done using a 4× objective lens on inverted microscope.

In vitro *viral replication assays*

In vitro growth analyses of these viruses were carried out as previously described (Brown, 1994). One-step and multistep replication kinetic experiments were performed at 37°C on either confluent BHK cells, 3T6 cells, or rabbit skin cells, in one-step experiments using a MOI of between 10 and 50, and in multistep experiments using a MOI of 0.01. After 1 h of adsorption, the inoculum was removed and replaced with 1 ml of medium. Depending on the multiplicity of infection, at different times post infection cells were collected. Following disruption of cells by sonication, viral titres were determined on BHK cells or RS cells. Duplicate samples were analyzed in parallel at each time point, and the data shown are representative from three experiments.

LD$_{50}$

Three-week-old BALB/c mice were intracranially innoculated with individual virus stocks (MacLean *et al*, 1991). Briefly, mice were anaesthetized with ether and 0.025 ml of appropriate virus dilution in phosphate-buffered saline (PBS) and 5% calf serum

was inoculated into the central region of the left cerebral hemisphere. Four mice were inoculated with each virus dilution. The virus stocks were retitrated to confirm the inoculation dose on the day of infection. Daily observations of the mice were done and the LD_{50} calculated on the basis of number of deaths up to 21 days post inoculation, according to Reed and Muench (1938).

Inoculation of mice*

All procedures involving animals were approved by the Cincinnati Children's Hospital Institutional Animal Care and Use Committee and were in compliance with the *Guide for the Care and Use of Laboratory Animals.* Animals were housed in American Association for Laboratory Animal Care–approved quarters. Male, outbred, Swiss Webster mice (Harlan Laboratories) 4 to 5 weeks of age were used throughout these studies. Prior to inoculation, mice were anesthetized by intraperitoneal injection of sodium pentobarbitol (Nembutal; 50 mg/kg body weight). In preliminary studies it was determined that replication of $\gamma 34.5$ mutants was severely impaired on the corneal surface (data not shown) and (Sawtell, 2003). However, we found that replication on the surface of the snout was not reduced significantly from wild type. One goal of the *in vivo* studies was to determine the ability of these mutants to replicate in the nervous system. Impaired replication at the surface, i.e., corneal surface, would complicate the interpretation of downstream events. Therefore, the surface of the snout was utilized as the inoculation site. A 20-μl drop containing a titer of 4×10^5 PFU was placed onto each scarified whisker pad as described previously (Sawtell, 2003).

Viral replication in vivo*

Three mice from each inoculum group were sacrificed on days 2, 3, and 4 post infection. Infected tissues, whisker pads, and trigeminal ganglia were removed, homogenized, and assayed for infectious virus titer using a standard serial dilution plaque assay on RS cell monolayers (Sawtell, 2003).

Immunohistochemistry of HSV-1 on trigeminal ganglia*

Immunohistochemistry on trigeminal ganglia (TG) was carried out as previously described (Sawtell, 2003). On day 4 p.i., three to four mice from each group were sacrificed, the TG removed and fixed in 4% paraformaldehyde overnight at 4°C. Fixed tissues were rinsed in PBS, dehydrated in graded ethanols, cleared in xylene, and embedded in paraffin. Eight-micrometer sections were cut and placed on superfrost plus slides (Fisher). Following removal of paraffin and rehydration, endogenous peroxidase activity was blocked by incubating sections in a methanol bath containing 0.15% hydrogen peroxide for 15 min. Sections were then rinsed in PBS and incubated for 30 min in 5% nonfat dry milk, rinsed in PBS, and then incubated for 1 h in rabbit anti-HSV1/2 antibody (Accurate) used at 1:3000 dilution. Sections were again rinsed in PBS and incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (Vector), rinsed and then incubated for 1 h in avidin-HRP conjugate (Vector). Antigen-antibody complexes were detected by incubating tissue in a solution of 0.05% diaminobenzidine and 0.002% hydrogen peroxide in Tris, pH 7.6.

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