Herpes simplex virus type 1 mutants containing the KOS strain ICP34.5 gene in place of the McKrae ICP34.5 gene have McKrae-like spontaneous reactivation but non-McKrae-like virulence

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Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic double stranded DNA virus. Following peripheral infection, usually at a mucosal surface, HSV-1 establishes a lifelong latent infection in ganglionic sensory neurons. When HSV-1 infects the eye, latency occurs in the trigeminal ganglia (TG). The latent virus can reactivate sporadically throughout the life of the infected individual. The virus then travels down the nerve and returns to the original peripheral site of infection where it may produce recurrent disease.

The HSV-1 genome encodes more than 80 genes. However, the only gene abundantly transcribed during neuronal latency is the latency-associated transcript (LAT) (Rock et al., 1987; Stevens et al., 1987). The primary LAT is 8-3 kb long (Dobson et al., 1989; Zwaagstra et al., 1990) and overlaps the ICP0 gene in an antisense direction (Rock et al., 1987; Stevens et al., 1987). The primary LAT is highly unstable and difficult to detect, but a highly stable 2 kb LAT, an intron derived from the 8-3 kb LAT (Farrell et al., 1991), is present in readily detectable amounts during neuronal latency. The LAT also overlaps the ICP34.5 gene in an antisense direction. The LAT and ICP34.5 gene are located in the viral long repeats and are therefore diploid genes.

Differences in the virulence of HSV-1 have been observed between different strains (isolates) (Hill et al., 1987). Following ocular infection of rabbits with \(2 \times 10^5\) p.f.u. per eye of the McKrae strain of HSV-1, approximately 50% of the animals die (Perng et al., 1994, 1995, 1999). In contrast, ocular infection with the KOS(M) strain of HSV-1 does not result in the death of any rabbits (Hill et al., 1987; S. L. Wechsler, unpublished results). McKrae can therefore be considered a prototypic virulent HSV-1 strain, while KOS can be considered a prototypic avirulent strain.

Several viral genes have been linked to neurovirulence in...
infected animals. These genes include ICP34.5 (Perng et al., 1995; Thompson & Wagner, 1988), thymidine kinase (Gordon et al., 1984), ribonucleotide reductase (Cameron et al., 1988) and the US3 protein kinase (Kurachi et al., 1993). ICP34.5 has a major impact on neurovirulence, with some ICP34.5 gene mutants reported to reduce neurovirulence by a factor of 100000 or more (Perng et al., 1995; Thompson et al., 1983). ICP34.5 is a low-abundance protein that is essential for efficient replication of the virus in neurons in vivo. Unfortunately, there is no effective antibody currently available for studying ICP34.5. Detection and analysis of the ICP34.5 mRNA has also proven extremely difficult. Thus, our knowledge of ICP34.5 function comes almost exclusively from viral mutants. The requirement for ICP34.5 in HSV-1 replication is cell type- and cell state-dependent (Chou & Roizman, 1992; Chou et al., 1994; Perng et al., 1995). We have shown that spontaneous reactivation of d34.5, a McKrae strain ICP34.5 null mutant, is dose-dependent (Perng et al., 1996b). No spontaneous reactivation could be detected following ocular infection with the standard dose of 2 × 10^6 p.f.u. per eye. However, wild-type spontaneous reactivation levels were seen following infection with 1000-fold more virus. As with other ICP34.5 mutants, d34.5 dramatically reduced neurovirulence and did not result in the death of any rabbits, even at the 1000-fold higher infectious dose. Interestingly, since spontaneous reactivation was at wild-type levels at this infectious dose, these results showed that the phenotypes of spontaneous reactivation and neurovirulence are separable.

To determine whether the reduced virulence phenotype of KOS compared with McKrae might be partially or completely due to differences in the ICP34.5 genes of these two HSV-1 strains, in this report we constructed and studied two chimeric viruses. The first virus, designated 34.5KA, contained one copy of the KOS ICP34.5 gene inserted into an ectopic location in the viral unique long region (between UL37 and UL38) of the McKrae-based mutant d34.5, which has both copies of the ICP34.5 gene deleted (Perng et al., 1995). The second virus, designated d34.5KR, contained the ICP34.5 gene of KOS in place of both copies of the McKrae ICP34.5 gene on an otherwise wild-type McKrae genomic background. We also sequenced the ICP34.5 genes of McKrae and KOS to look for potential differences.

We report here that, compared with the McKrae ICP34.5 gene, the KOS ICP34.5 gene supported virus virulence very poorly. In contrast, the KOS ICP34.5 gene supported high levels of spontaneous reactivation of the McKrae strain, even though the KOS strain of HSV-1 does not re activate in the rabbit model. Thus, the reduced neurovirulence of KOS compared with McKrae appeared to be at least partially due to one or more of the sequence differences that we found in the KOS ICP34.5 gene compared with the McKrae ICP34.5 gene, while the reduced reactivation phenotype of KOS compared with McKrae could not be accounted for by differences in their ICP34.5 genes.

**Methods**

- **Cells and viruses.** Rabbit skin (RS) cells were grown in Eagles’s minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). CV-1 cells were grown in MEM supplemented with 10% FCS. CV-1 cells were used for growth kinetic studies. RS cells were used for all other tissue culture procedures, including preparation of virus stocks. All mutants were derived from HSV-1 strain McKrae. The parental McKrae virus and all mutants were triple plaque-purified and passaged only one or two times prior to use. The properties of wild-type McKrae and the properties and construction of 34.5A and d34.5 have been previously described (Perng et al., 1995, 1996c).

- **Construction of the KOS ICP34.5 chimeric viruses.** The parental virus for construction of 34.5KA and d34.5KR was d34.5, a McKrae-derived mutant in which both copies of the ICP34.5 gene (one in each long repeat) are deleted. The construction of 34.5KA, containing a single copy of the KOS ICP34.5 gene (a DraI–SphI restriction fragment corresponding to HSV-1 genomic nt 125900–124480) in an ectopic location between the UL37 and UL38 genes was identical to that of 34.5A (Perng et al., 1996a), except that the ICP34.5 gene fragment was from KOS instead of McKrae. Construction of d34.5KR was identical to that of the marker-rescued virus 34.5KR (Perng et al., 1995), except that the ICP34.5 gene fragment (a SmaI–SphI restriction fragment corresponding to HSV-1 genomic nt 126770–124480) used to restore both deleted copies of the ICP34.5 gene was from KOS instead of McKrae. This fragment contains 582 nucleotides of the KOS sequence downstream of the 3’ end of the d34.5 deletion and 791 nucleotides of the KOS sequence upstream of the 5’ end of the d34.5 deletion. This resulted in sufficient overlapping DNA at both ends of the deletion for homologous recombination to occur. All mutant viruses were triple plaque-purified and their structures were confirmed by extensive restriction enzyme digestion and Southern blot analysis.

- **Replication in tissue culture.** CV-1 cell monolayers at 70–80% confluency were infected with virus at 0.01 p.f.u. per cell and all monolayers were refed with exactly the same amount of MEM containing 10% FCS. Virus was harvested at various times by two cycles of freeze-thawing of the monolayers and medium (from −80 °C to room temperature). The amount of virus in each sample was then determined by standard plaque assay on RS cells.

- **Animals.** Rabbits were 8- to 10-week-old male New Zealand Whites from Irish Farms. Rabbits were treated in accordance with the Association for Research in Vision and Ophthalmology, the American Association for Laboratory Animal Care and the National Institutes of Health guidelines.

- **Rabbit model of ocular HSV-1 infection, latency and spontaneous reactivation.** As previously described (Perng et al., 1994, 1995), rabbits were bilaterally infected by placing 2 × 10^5 p.f.u. of virus per eye into the conjunctival cul-de-sac, closing the eye and rubbing the lid gently against the eye for 30 s. To examine acute replication of virus in rabbit eyes, tear films were collected from five eyes per group, each eye from a different rabbit, on days 3, 5, 7 and 10 post-infection (p.i.). The amount of virus was determined by standard plaque assay. Virulence (or neurovirulence) was defined as death due to viral encephalitis within the first 21 days p.i. Beginning on day 31 p.i., tear films were collected daily from each eye for 26 days using a nylon-tipped swab. The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect RS cell monolayers. These monolayers were observed in a masked fashion by phase light microscopy for up to 5 days for HSV-1 cytopathic effects (CPE). All positive monolayers were blind-passaged on to fresh cells to confirm the presence of virus. DNA was purified from positive cultures and analysed by restriction enzyme digestion and Southern analysis to confirm that the
CPE was due to reactivated HSV-1 and that the reactivated virus was identical to the input virus (data not shown).

DNA sequencing. Purified HSV-1 DNA was used as a template for PCR. Due to the high GC content of the sequence, PCR was performed using a series of primers (MWG Biotech Inc.), which generated 15 overlapping products encompassing the entire ICP34.5 gene. A Clontech Advantage-GC2 polymerase Mix kit (Clontech) was used according to the manufacturer’s protocol. The resultant PCR products were sequenced using the SequiTherm EXCEL II DNA Sequencing Kit (Epicenter Technologies).

Results

Structure of chimeric viruses containing the KOS ICP34.5 gene on a McKrae background

To determine whether the KOS ICP34.5 gene could efficiently replace the McKrae ICP34.5 gene and produce the McKrae phenotypes for spontaneous reactivation and neurovirulence, we constructed two different chimeric viruses. A schematic representation of these virus is shown in Fig. 1. The details of the construction of the mutants is given in Methods. The parental virus for both chimeric viruses was d34.5 (Perng et al., 1995), an ICP34.5 null mutant of McKrae containing a 917-nucleotide deletion that removes the entire ICP34.5 open reading frame. The first mutant, 34.5KA, contained one copy of the KOS ICP34.5 gene inserted into an ectopic location in the unique long region of d34.5 between the genes for UL37 and UL38. This virus was identical to our previously published mutant, 34.5A (Perng et al., 1996a), except that in 34.5KA the inserted ICP34.5 gene was from KOS instead of from McKrae. The second mutant, d34.5KR, was a ‘marker-rescued’ virus of the d34.5 mutant in which both deleted copies of the ICP34.5 gene (one in each long repeat of the virus) were restored using

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![Diagram of HSV-1 mutants](https://via.placeholder.com/150)

**Fig. 1.** Genomic structure of the HSV-1 mutants. (A) Schematic representation of the wild-type HSV-1 genome from strain McKrae. TRL and IRL indicate the terminal and inverted long repeats. IRS and TRS indicate the inverted and terminal short repeats. UL and US indicate the unique long and unique short regions. The TRL and IRL are expanded (dashed lines) to show the LAT region. Details of the long repeats are shown below. The primary LAT transcript is indicated by the large arrow. The solid rectangle represents the stable 2 kb LAT intron. The relative locations of the ICP0 and ICP34.5 transcripts are shown for reference. (B) The d34.5 mutant has a complete deletion of both copies of the ICP34.5 gene in HSV-1 McKrae. (C) The 34.5A mutant contains one copy of the complete ICP34.5 gene of McKrae inserted into the UL region between genes UL37 and UL38 of d34.5. (D) The 34.5KA mutant is identical to 34.5A except that the ICP34.5 gene inserted between UL37 and UL38 is from HSV-1 strain KOS. (E) The d34.5KR mutant is the marker rescuant of d34.5 in which both copies of the ICP34.5 gene have been restored using the ICP34.5 gene of HSV-1 McKrae. (F) The d34.5KR mutant is identical to d34.5R, except that both copies of ICP34.5 were rescued with the KOS ICP34.5 gene.
the KOS ICP34.5 gene instead of the McKrae ICP34.5 gene. This mutant corresponded to our previously published marker-
rescued virus d34.5R (Perng et al., 1995). Thus, 34.5KA contained one copy of the KOS ICP34.5 gene located in an
ectopic location in the otherwise ICP34.5 McKrae deletion
mutant d34.5, while in d34.5KR, both copies of the McKrae
ICP34.5 gene were replaced by the KOS ICP34.5 gene.

Replication of 34.5KA and d34.5KR in tissue culture

We have previously reported that the McKrae ICP34.5 null mutant d34.5 has greatly restricted replication in CV-1 cells
(Perng et al., 1995). To determine whether the KOS ICP34.5
gene could restore normal replication to d34.5 in CV-1 cells,
subconfluent monolayers of CV-1 cells were infected with 0.01
p.f.u. per cell of 34.5KA, 34.5A, d34.5 or wild-type McKrae
(Fig. 2A), or with 0.01 p.f.u. per cell of d34.5KR, d34.5R, d34.5
or wild-type McKrae (Fig. 2B). The d34.5 mutant replicated
poorly in CV-1 cells, reaching a maximum titre of only about
1 x 10^3 p.f.u./ml, compared with over 1 x 10^8 p.f.u./ml for
34.5A, d34.5R and wild-type McKrae. Replication of both
chimeric viruses (34.5KA, d34.5KR) was indistinguishable
from their non-chimeric counterparts, 34.5A and d34.5R, and
from wild-type McKrae. Thus, the KOS ICP34.5 gene was
capable of restoring wild-type replication in CV-1 cells to
d34.5 in both chimeric viruses. This indicated that, for the
phenotype of replication in CV-1 cells, the KOS ICP34.5 gene
was comparable with the McKrae ICP34.5 gene. This was
expected, since KOS replicates efficiently in CV-1 cells.

Replication of 34.5KA and d34.5KR in rabbit eyes

We have previously reported that d34.5 does not replicate
well in rabbit eyes (Perng et al., 1995). To determine whether
34.5KA and 34.5KR could rescue this defect, rabbits were infected with $2 \times 10^6$ p.f.u. per eye of either 34.5KA, 34.5A or wild-type McKrae (Fig. 3A), or with d34.5KR, d34.5 or wild-type McKrae (Fig. 3B) as described in Methods. Tears were collected at the times indicated, and the amount of virus in each of five eyes per group (one eye per rabbit) was determined by standard plaque assay as described in Methods. As expected from our previous studies, d34.5 replicated poorly (Fig. 3B, open circles). In contrast, peak titres of 34.5KA and d34.5KR in rabbit eyes were similar to those of 34.5A and wild-type McKrae. Thus, as in CV-1 cells, the KOS ICP34.5 gene appeared to restore fully wild-type levels of virus replication in rabbit eyes. This suggests that, for the phenotype of replication in rabbit eyes, the KOS ICP34.5 gene is comparable with the McKrae ICP34.5 gene.

These results also provided information regarding the requirement of corneal scarification (i.e. scratching the cornea with a needle just prior to ocular infection) for efficient ocular infection. Because McKrae does not require corneal scarification, corneal scarification was not employed in these studies. Unlike McKrae, KOS does require corneal scarification for efficient replication in rabbit eyes (Hill et al., 1987). The efficient replication of 34.5KA and d34.5KR without corneal scarification therefore revealed that a ‘defect’ in the KOS ICP34.5 gene is not responsible for the requirement of the KOS strain for corneal scarification.

Survival of rabbits ocularly infected with 34.5KA and d34.5KR

We have previously reported that d34.5 does not result in the death of any rabbits following ocular infection, even at doses as high as $1 \times 10^8$ p.f.u. per eye (Perng et al., 1996b). To determine whether virulence (as defined by death following ocular infection) was restored in 34.5KA or d34.5KR, rabbits were ocularly infected with $2 \times 10^5$ p.f.u. of virus per eye, as described above. As expected, about 60% of rabbits infected with wild-type McKrae survived in these two experiments. We have previously shown that 34.5A, containing one copy of the McKrae ICP34.5 gene in an ectopic location, only partially restored virulence. The results shown here (78% survival; Fig. 4A) are consistent with this. In contrast, 34.5KA did not result in the death of any rabbits (Fig. 4A). However, because of the intermediate phenotype of 34.5A, it was not clear from the 34.5KA results whether the KOS ICP34.5 gene can substitute for the McKrae ICP34.5 gene as regards the McKrae virulence phenotype.

We have previously shown that marker-rescued virus (d34.5R) fully restores the wild-type McKrae virulence phenotype (Perng et al., 1995). In contrast, marker rescue of d34.5 with the KOS ICP34.5 gene (d34.5KR) did not appear to rescue completely the McKrae virulence phenotype. Ninety-four per cent of the rabbits infected with d34.5KR survived (Fig. 4B). This was significantly different from wild-type McKrae ($P = 0.02$) and not significantly different from d34.5 ($P = 1.0$). The results with d34.5KR strongly suggest that the KOS ICP34.5 gene cannot support wild-type McKrae virulence in this rabbit system.

Spontaneous reactivation

We have previously shown that the ICP34.5 deletion mutant d34.5 has significantly reduced spontaneous reactivation following ocular infection of rabbits with $2 \times 10^5$ p.f.u. of virus per eye (Perng et al., 1995). We have also shown that 34.5A and d34.5R both have a wild-type McKrae spontaneous reactivation phenotype (Perng et al., 1995, 1996a). To examine spontaneous reactivation of 34.5KA and d34.5KR (the corresponding chimeric viruses containing the KOS ICP34.5 gene in place of the McKrae ICP34.5 gene), all eyes from the surviving rabbits in the experiments shown in Fig. 4(A) and (B) were swabbed daily to collect tear films for analysis of spontaneously reactivated virus as described in Methods. Eye-swab collection began 31 days p.i., at which time latency had already been established (Perng et al., 1994). The cumulative
Fig. 5. Spontaneous reactivation of 34.5KA and d34.5KR. Spontaneous reactivation was determined as described in Methods using the rabbits shown in Fig. 4. Briefly, eye swabs were performed daily and the presence of spontaneously reactivated virus in each eye was determined by plating the swabs on indicator cells. The y-axis shows the cumulative number of eye swab cultures per eye that contained reactivated virus.

Table 1. Spontaneous reactivation of 34.5KA and d34.5KR

Results taken from the experiments shown in Fig. 5.

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of positive cultures/total (%)</th>
<th>Probability vs chimera*</th>
<th>No. of positive eyes/total (%)</th>
<th>Probability vs chimera*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.5KA</td>
<td>50/468 (11%)</td>
<td></td>
<td>15/18 (83%)</td>
<td></td>
</tr>
<tr>
<td>34.5A</td>
<td>39/364 (11%)</td>
<td>(P = 1.0)</td>
<td>11/14 (79%)</td>
<td>(P = 1.0)</td>
</tr>
<tr>
<td>McKrae</td>
<td>75/572 (13%)</td>
<td>(P = 0.25)</td>
<td>15/22 (68%)</td>
<td>(P = 0.46)</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d34.5KR</td>
<td>77/850 (9%)</td>
<td></td>
<td>22/34 (65%)</td>
<td></td>
</tr>
<tr>
<td>d34.5</td>
<td>5/500 (1%)</td>
<td>(P &lt; 0.0001)</td>
<td>4/20 (20%)</td>
<td>(P = 0.002)</td>
</tr>
<tr>
<td>McKrae</td>
<td>57/500 (11%)</td>
<td>(P = 0.19)</td>
<td>12/20 (60%)</td>
<td>(P = 0.78)</td>
</tr>
</tbody>
</table>

* Fisher’s exact test (GraphPad Instat software version 2.02).

number of virus-positive tear film cultures (indicative of spontaneously reactivated virus) during the 26 day study period are shown in Fig. 5. As expected, d34.5 appeared to have a dramatically reduced spontaneous reactivation rate compared with wild-type McKrae (Fig. 5B), consistent with our previous findings. In contrast, 34.5KA and 34.5A (Fig. 5A) and d34.5KR (Fig. 5B) all appeared to have a wild-type McKrae spontaneous reactivation phenotype.

Statistical analyses of these experiments are shown in Table 1. In experiment A, approximately 11% of cultures from rabbits latently infected with 34.5KA contained spontaneously reactivated virus. This was similar to that for 34.5A (11%) and wild-type McKrae (13%) (\(P = 1.0\) and 0.25, respectively). The percentage of eyes in which spontaneously reactivated virus was detected at least once showed the same pattern. Again, spontaneous reactivation of d34.5KR (65% of eyes) was significantly greater than that of d34.5 (20%; \(P = 0.002\)) and similar to that of wild-type McKrae (60%; \(P = 0.78\)). These analyses all suggested that both of the chimeric viruses containing the KOS ICP34.5 gene in place of the McKrae ICP34.5 gene (34.5KA and d34.5KR) had spontaneous reactivation phenotypes indistinguishable from that of wild-type McKrae. This strongly suggests that the ICP34.5 function(s) required for the high spontaneous reactivation phenotype is similar in KOS and McKrae.

Sequence of the McKrae and KOS ICP34.5 gene

The region of the ICP34.5 gene containing the ICP34.5 open reading frame corresponding to genomic nt 124710–125950 was sequenced from HSV-1 strains McKrae and KOS(M) as described in Methods (Fig. 6). The corresponding
Role of ICP34.5 gene in HSV-1 virulence

Fig. 6. Sequence of the ICP34.5 gene. Genomic DNA from strains McKrae and KOS was sequenced as described in Methods. The sequence of strain 17syn+ is reproduced from GenBank accession no. X14112. Dashes indicate the same nucleotide as McKrae. ∆ indicates a missing nucleotide compared with McKrae.

Role of ICP34.5 gene in HSV-1 virulence

Fig. 7. Predicted amino acid sequences of ICP34.5. The predicted amino acid sequences based on the DNA sequences in Fig. 6 are shown.
sequence for 17syn*, the only strain of HSV-1 to be completely sequenced, was included for comparison (McGeoch, 1987; Perry & McGeoch, 1988). HSV-1 17syn* has a virus virulence phenotype similar to that of McKrae.

The predicted amino acid sequences for the ICP34.5 protein of these three HSV-1 strains are shown in Fig. 7. The N-terminal region of ICP34.5 contains an arginine (R)-rich cluster (Mao & Rosenthal, 2002), with KOS containing one fewer arginine than McKrae and two fewer arginines than 17syn*. The central region of ICP34.5 contains a proline–alanine–threonine (PAT) repeat region (Mao & Rosenthal, 2002). KOS contains only five PAT repeats compared with six for 17syn* and nine for McKrae. At amino acid 188 of McKrae, KOS has a threonine instead of the alanine found in both McKrae and 17syn*. KOS also has a C nucleotide inserted after McKrae nucleotide 692 (Fig. 6) and a C deleted at McKrae nucleotide 754. This shifts the reading frame of KOS relative to McKrae and 17syn* (from McKrae aa 232–251), resulting in 19 of 20 amino acids being mismatched compared with McKrae and 17syn*.

Discussion

Mutants of KOS and McKrae, along with 17syn*, have been commonly used to study HSV-1 reactivation and virulence (defined in this study as death from encephalitis following ocular infection). In the rabbit eye model, McKrae and 17syn* have similar high spontaneous reactivation rates (approximately 5–10% of eyes containing spontaneously reactivated virus at any given time) and similar high virulence (resulting in the death of approximately 30–50% of rabbits from encephalitis). In contrast, in the same model, KOS has a completely negative spontaneous reactivation phenotype and is completely avirulent.

Mutations that delete or significantly alter the ICP34.5 gene greatly reduce virus virulence as measured by both peripheral and intracranial challenge (Perng et al., 1995; Thompson et al., 1983). Reactivation is also greatly reduced in these mutants following infection with the doses of virus typically used (i.e. approximately 2 × 10^6 to 2 × 10^8 p.f.u.) (Perng et al., 1995). To determine whether a ‘defect(s)’ in the KOS ICP34.5 gene might account for either the inability of KOS to reactivate spontaneously in the rabbit eye model or for avirulence of KOS in this model, we constructed and reported here on two different chimeric viruses. Both chimeric viruses were constructed on the genomic background of the McKrae ICP34.5 null mutant d34.5. Both copies of the ICP34.5 gene (one in each long repeat) are deleted and, like KOS, d34.5 is avirulent and has a negative spontaneous reactivation phenotype following infection of rabbits with 2 × 10^8 p.f.u. of virus per eye. To construct the first chimeric virus, 34.5KA, one copy of the KOS ICP34.5 gene was inserted into an ectopic location in the unique long region of d34.5. This chimeric virus was identical to our previous mutant 34.5A, except that in 34.5A the ectopic ICP34.5 gene was from McKrae rather than KOS. The second chimeric virus, d34.5KR, was constructed by marker rescue of both ICP34.5 gene deletions in d34.5 using a small (2.3 kb) piece of KOS DNA. d34.5KR was identical to d34.5R (marker-rescued d34.5 using McKrae DNA) and also to wild-type McKrae, except that the ICP34.5 gene (and an indeterminate amount of flanking DNA, but less than 1 kb on each side of the gene) was from KOS.

In the experiments presented here, the KOS ICP34.5 gene successfully replaced the McKrae ICP34.5 gene and produced McKrae-like phenotypes for replication in CV-1 cells, replication in rabbit eyes and spontaneous reactivation. These results demonstrate that the KOS ICP34.5 gene is capable of supporting the high-level spontaneous reactivation phenotype when placed in an appropriate virus genetic background. Thus, ‘defects’ in ICP34.5 cannot account for the negative spontaneous reactivation phenotype of KOS in rabbits. Differences in LAT also cannot account for the reduced spontaneous reactivation phenotype of KOS, since we have previously shown that a chimeric virus in which the functional portion of the McKrae LAT gene was replaced with the corresponding portion of the KOS LAT had a McKrae spontaneous reactivation phenotype (Drolet et al., 1998). Thus, both the KOS LAT and the KOS ICP34.5 genes are capable of supporting a McKrae-like spontaneous reactivation phenotype even though KOS itself does not reactivate spontaneously.

In contrast to the findings for replication in CV-1 cells and rabbit eyes and the spontaneous reactivation phenotype, the KOS ICP34.5 gene was not able to substitute efficiently for the McKrae ICP34.5 gene for the phenotype of virus virulence. This strongly suggests that differences between the KOS and McKrae ICP34.5 genes may play an important role in the low virus virulence of KOS compared with McKrae. However, they do not rule out the possibility that differences in additional KOS genes may also be significant. In fact, there are at least three reports indicating that a defect in gB significantly contributes to the reduced pathogenicity of KOS (Kosovsky et al., 2000; Kostal et al., 1994; Yuhasz & Stevens, 1993). These results also confirm our previous findings that the phenotype of virus virulence can be separated from both the spontaneous reactivation phenotype and the ability of the virus to replicate efficiently in rabbit eyes (Perng et al., 1996a, b).

To look for differences in the KOS ICP34.5 gene that might alter its ability to support the virus virulence phenotype, we sequenced the ICP34.5 gene from KOS and McKrae and compared these sequences to each other and to 17syn*. Since 17syn* and McKrae have similar virus virulence phenotypes, it seems logical to assume that if the KOS sequence differs from both McKrae and 17syn*, this difference may affect the virus virulence phenotype. Several differences were noted between the KOS and McKrae sequences for the ICP34.5 protein. The predicted ICP34.5 protein sequence contains an N-terminal arginine (R)-rich cluster and a more central proline–alanine–threonine (PAT) repeat region. It has been proposed...
that one or both of these regions is involved in cellular localization of the ICP34.5 protein and in its neurovirulence properties (Mao & Rosenthal, 2002). The arginine cluster of the McKrae, KOS and 17syn\(^+\) ICP34.5 proteins are similar, containing eight, seven and nine arginines, respectively. This small range of differences is not thought to be significant (Mao & Rosenthal, 2002). In contrast, McKrae contains nine PAT repeats while KOS contains only five PAT repeats, suggesting that the reduced number of PAT repeats in KOS may account for the decreased virulence properties of its ICP34.5 gene. However 17syn\(^+\), which has virulence and reactivation properties similar to those of McKrae, has six PAT repeats, only one more than KOS.

Compared with McKrae and 17syn\(^+\), the KOS ICP34.5 amino acid sequence differs in two additional locations. At the location corresponding to amino acid 188 of McKrae, KOS contains a threonine, while McKrae and 17syn\(^+\) both contain an alanine. Compared with McKrae and 17syn\(^+\), KOS has a C nucleotide inserted into the DNA sequence after the nucleotide corresponding to McKrae nt 692 and a C deleted from the DNA sequence at McKrae nt 754. This produces a frame shift at the location corresponding to aa 233–251 of McKrae. This results in a stretch of 19 out of 20 mismatched amino acids in the KOS sequence compared with both McKrae and 17syn\(^+\). At this time it is unknown which of the difference(s) in the KOS sequence results in the inability of the KOS ICP34.5 protein to support the high virus virulence phenotype.

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