

The Region of the Herpes Simplex Virus Type 1 LAT Gene That Is Colinear with the ICP34.5 Gene Is Not Involved in Spontaneous Reactivation

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The goal of this report was to determine if the region of the LAT gene that is colinear with ICP34.5 (kb 6.2 to 7.1 of LAT) is involved in spontaneous reactivation of herpes simplex virus type 1. We inserted one copy of the ICP34.5 gene into the unique long region of a herpes simplex virus type 1 (strain McKrae) mutant lacking both copies of ICP34.5 (one in each viral long repeat) and the corresponding 917-nucleotide colinear portion of LAT (kb 6.2 to 7.1). Rabbits were ocularly infected with this mutant, and spontaneous reactivation relative to that for the wild-type virus and the original mutant was measured. As we previously reported, the original ICP34.5-deleted virus (*d34.5*) was significantly impaired for spontaneous reactivation and virulence (G. C. Perng, R. L. Thompson, N. M. Sawtell, W. E. Taylor, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler, *J. Virol.* 69:3033–3041, 1995). In contrast, we report here that restoration of one copy of ICP34.5 at a distant location completely restored the wild-type level of *in vivo* spontaneous reactivation, despite retention of the deletion in LAT (spontaneous reactivation rate = 0.3 to 1.4% for the ICP34.5 deletion mutant, 7.7 to 19.6% for the wild type, and 9 to 16.1% for virus with one copy of ICP34.5). Thus, the 917-nucleotide region of LAT from kb 6.2 to 7.1 was not involved in the LAT function required for wild-type spontaneous reactivation. We also found that restoration of a single ICP34.5 gene in a novel location did not restore wild-type virulence (rabbit death rate = 0% [0 of 15] for the original ICP34.5 deletion mutant, 8% [2 of 24] for the single-copy ICP34.5 virus, and 52% [14 of 27] for wild-type virus; $P < 0.001$ for one versus two copies of ICP34.5). It is likely that either two gene doses of ICP34.5 or its location in the long repeat is essential for full functionality of ICP34.5's virulence function. Furthermore, the ability of the single-copy ICP34.5 virus to reactivate at wild-type levels despite being significantly less virulent than wild-type virus separates the spontaneous reactivation phenotype from the virulence phenotype.

Following peripheral infection, herpes simplex virus (HSV) establishes a lifelong latent infection in host sensory neurons. At various times, the latent virus can reactivate, travel back down the nerve, and produce recurrent disease at the original peripheral site. Recurrent HSV type 1 (HSV-1) corneal infection, which can lead to blindness due to scarring of the cornea, is the most common cause of infectious blindness in the developed world (14).

During latency in sensory neurons, viral RNA transcription appears to be limited to the latency-associated transcript (LAT) RNAs (21, 28). LAT is located in the long repeat and is therefore present in two copies. The primary LAT transcript is 8.3 kb long and completely overlaps the ICP0 and ICP34.5 genes in an antisense direction. In addition to the primary LAT, which is unstable and difficult to detect, two very stable LATs of 2 and 1.5 kb are present in readily detectable amounts during neuronal latency (4, 7, 21, 25, 27, 28, 30–34, 36) (see Fig. 1). The 2-kb LAT appears to be an intron derived from the 8.3-kb LAT (36). The 1.5-kb LAT appears to be derived from the 2-kb LAT (32).

Since its discovery as the only viral gene abundantly tran-

scribed during latency, LAT has been assumed to be an important factor in some aspect of the latency-reactivation cycle. LAT promoter deletion mutants appear to reduce explant or induced reactivation (1, 8–10, 12, 13, 24, 26, 29). Recently, we extended these findings to include spontaneous reactivation. We showed that deleting the LAT promoter and the first 1.6 kb of the 5' end of LAT reduced the rate of *in vivo* spontaneous reactivation in the rabbit ocular model of HSV-1 3- to 10-fold (16). In that study and the studies discussed in this report we were able to detect statistically significant changes in the rate of spontaneous reactivation, because we used the HSV-1 McKrae strain as the parental virus. McKrae has a higher spontaneous reactivation rate than other HSV-1 strains previously used for the construction of mutants. Without this high spontaneous reactivation rate, the range of changes we observed would not have reached statistical significance.

One approach to mapping functional regions of LAT involved in spontaneous reactivation is to delete different portions of LAT and examine the effect on spontaneous reactivation. However, this approach is complicated by the fact that much of LAT overlaps the important HSV-1 genes ICP0 and ICP34.5. ICP0 is essential for efficient virus replication at a low multiplicity of infection (MOI) (22), while ICP34.5 is essential for efficient replication in neurons (3). Thus, alterations to either gene would be likely to affect apparent reactivation

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rates. This would make LAT deletion mutants that also delete portions of either ICP0 or ICP34.5 difficult to assess for LAT's role in any observed phenotypic changes.

We recently constructed and studied a 0.9-kb deletion mutant in the long repeats in which both copies of ICP34.5 and the colinear regions of LAT from kb 6.2 to 7.1 were deleted (mutant *d34.5*) (18). Neurovirulence and spontaneous reactivation from latency were both dramatically reduced. In this report, we show that the apparent decrease in the spontaneous reactivation rate in *d34.5* was due not to the deletion in LAT but rather to the lack of ICP34.5. We inserted a copy of the ICP34.5 gene in the unique long region (U_L) of the ICP34.5-LAT kb 6.2 to 7.1 deletion mutant. This produced a virus with the ICP34.5 gene at a novel location and a deletion in both copies of LAT from kb 6.2 to 7.1. This mutant had a normal spontaneous reactivation rate in rabbits (162 of 1,144 [14%] compared with 100 of 668 [15%] for wild-type virus and 7 of 728 [1%] for the ICP34.5 deletion mutant). Thus, the deleted region (kb 6.2 to 7.1) of LAT was not required for the function of LAT involved in spontaneous reactivation.

MATERIALS AND METHODS

Virus and cells. All mutants were derived from HSV-1 strain McKrae. The parental McKrae virus and all mutants were triple plaque purified and passaged no more than two times prior to use. *d34.5* has a 0.9-kb deletion in both copies of LAT at the location of the ICP34.5 gene in the viral long repeats (18). *d34.5R*, marker-rescued *d34.5*, is identical to wild-type McKrae (18). *d34.5A*, the virus reported here, is *d34.5* with a copy of the ICP34.5 gene added into the U_L . Primary rabbit kidney cells, rabbit skin (RS) cells, and CV-1 cells were grown as monolayers in Eagle's minimal essential medium supplemented with 5% (RS) or 10% (primary rabbit kidney and CV-1) fetal calf serum.

Construction of *d34.5A* containing a single copy of ICP34.5. The parental virus for construct *d34.5A* was *d34.5* (see above) (18). The previously cloned *EcoRI*A fragment from HSV-1 strain McKrae (17) was digested with *Bam*HI. A 7.5-kb band containing the McKrae genomic region including UL37 and UL38 was isolated and cloned into the *Bam*HI site of plasmid pEV-vrf3 (5, 17) to produce the plasmid pV375. A unique *Afl*III site between the sequences for UL37 and UL38 within pV375 was converted to a unique *Pac*I site to produce pV375Pac. This was amplified by transformation into *Escherichia coli* RR1Δc1857 according to the standard protocol.

The ICP34.5 gene was isolated and prepared for insertion into the *Pac*I site of pV375Pac as follows. The previously cloned *Bam*HI SQ fragment of McKrae (17) was digested with *Dra*I and *Sph*I to produce a 1.5-kb DNA fragment containing the complete ICP34.5 open reading frame (ORF), the ICP34.5 gene poly(A) sequences, and part of the putative ICP34.5 promoter. This was cloned into the pUC19 *Sma*I-*Sph*I site. The resulting plasmid, designated pM34.5, was amplified, and a 1.5-kb fragment containing ICP34.5 was released from pM34.5 by digestion with *Eco*RI and *Bam*HI. The ends were filled in with Klenow fragment, *Pac*I linker (New England Biolabs, Beverly, Mass.) was added, and the fragment was digested with *Pac*I to produce *Pac*I ends and ligated into the *Pac*I site of the plasmid pNEB193 (New England Biolabs) to produce the plasmid pNEB193M34.5. The ICP34.5 gene was released from pNEB193M34.5 by digestion with *Pac*I and inserted into the *Pac*I site of pV375Pac. The resulting plasmid was designated pV375M34.5. pV375M34.5 contains the complete ORF of the HSV-1 McKrae ICP34.5 gene bounded by UL37 and UL38.

The single-copy ICP34.5 rescued virus was generated by homologous recombination as we previously described (16, 18). Briefly, pV375M34.5 was cotransfected with infectious *d34.5* (ICP34.5 deletion mutant) DNA by the calcium phosphate method. Viruses from the cotransfection were plated, and isolated plaques were picked and screened for insertion of the ICP34.5 gene between UL37 and UL38 by restriction digestion and Southern analysis. Selected plaques were triple plaque purified and reanalyzed by restriction digestion and Southern analysis to ensure that the ICP34.5 gene was present between UL37 and UL38 and that both long repeats retained the original ICP34.5-LAT deletion. A final plaque was purified and designated *d34.5A* (ICP34.5 deletion with an ICP34.5 addition).

Reverse transcription-PCR (RT-PCR). CV-1 cell monolayers (approximately 5×10^6 cells) were infected with *d34.5*, *d34.5R*, or *d34.5A* at an MOI of 10 PFU per cell. At 8 h postinfection, total RNA was isolated as previously described (19), treated with DNase I (12 U, 37°C, 30 min; Stratagene, La Jolla, Calif.), digested with proteinase K (200 μg/ml, 55°C, 1 h; Sigma), extracted with phenol-chloroform, and precipitated with 100% ethanol. Each RNA pellet was resuspended in diethyl pyrocarbonate-treated double-distilled water, and 5 μg was subjected to first-strand cDNA synthesis by superscript II (Gibco BRL, Grand Island, N.Y.) according to the manufacturer's protocol. The primer for first-strand cDNA synthesis from ICP34.5 mRNA was 5'-CGGAAGGCGGA

AGGGGCGGAGGGGGGTGGGA-3'. The cDNA product was then amplified by PCR with the primers 5'-TCGTCGGACGCGGACTCGGGAACGGTGGAGC-3' and 5'-CTCCACGCCCAACTCGGAACCCGCGGTGAC-3'. These primers generate a 134-bp product specific for ICP34.5. The PCRs were done in 100-μl reaction mixtures containing 5 μl of the first-strand synthesis mixture, 10 μl of 10× buffer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 2 μl of 100 mM deoxynucleoside triphosphate mix (New England Biolabs), 1 μl of each of the two primers, 1 μl of *Taq* polymerase (Boehringer Mannheim Biochemicals), and 80 μl of double-distilled water. This was overlaid with 100 μl of mineral oil. Cycling reactions were performed in a thermal cycler (Applied Biosystems, Pleasanton, Calif.) as follows: 1 cycle of denaturation at 95°C for 4 min; 30 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 30 s, and extension at 71°C for 2 min; and 1 cycle of extension at 74°C for 10 min. The amplified products were fractionated on a 4% NuSieve agarose gel running in Tris-borate-EDTA buffer, transferred to a nylon membrane, and hybridized to a ³²P-labeled internal probe.

Replication of virus in tissue culture. Cell monolayers at approximately 70 to 80% confluency were infected with virus at MOIs of 0.01 or 10 PFU per cell. At various times, virus was harvested for titration by two cycles of freeze-thawing the monolayers plus media (−80°C to room temperature). PFU per milliliter were determined by standard plaque assays on RS cells.

Rabbits. Eight- to ten-week-old New Zealand White female rabbits were used. The 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. The rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of HSV-1 into the conjunctival cul-de-sac per eye, closing the eye, and rubbing the lid gently against the eye for 30 s (21). At this dose of McKrae virus, virtually all of the surviving rabbits harbor a bilateral latent HSV infection in both trigeminal ganglia (TG), resulting in a high group rate of spontaneous reactivation with this strain of HSV-1 (16). Latency is assumed to have been established by 28 days postinfection. Acute infection of all eyes was confirmed by HSV-1-positive tear film cultures collected on days 3 and 4 postinfection.

Detection of spontaneous reactivation by ocular shedding. To test for spontaneously reactivated virus, beginning on day 30 postinfection, tear film specimens were collected daily from each eye with a nylon-tipped swab as previously described (15). The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect primary rabbit kidney cell monolayers. These cell monolayers were observed in a masked fashion by phase-light microscopy for up to 30 days for HSV-1 cytopathic effects. All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. DNA was purified from selected positive cultures derived from latently infected rabbits and analyzed by restriction enzyme digestion and Southern blots to confirm that the cytopathic effects were due to reactivated HSV-1 and that the reactivated virus was identical to the input virus. This was found to be the case in all instances (see Fig. 4).

Acute virus replication in rabbit eyes. Tear films were collected at various times as described above and assayed by plaque assay on RS cells.

Quantitation of latent HSV-1 DNA by competitive PCR. DNA was isolated from individual TG of latently infected rabbits as we previously described (16, 18). The primer pair used for the competitive PCR analyses amplify a 192-bp region of the HSV-1 glycoprotein B (gB) gene as previously reported (20). The competitor DNA corresponds to the same 192-bp region of gB but is missing 35 internal nucleotides. To construct the competitor DNA, the 192-bp wild-type region was amplified by PCR with the primers shown above. The PCR product was digested with *Bsa*JI and religated to delete 35 internal nucleotides. The resulting 157-bp product was cloned into the plasmid pGEM-T (Promega, Madison, Wis.) and transformed into *E. coli* RR1Δc1857. The resulting plasmid was designated pT157. When the pT157 plasmid was subjected to PCR with the gB primers mentioned above, a 157-bp product was generated. This was separated from the 192-bp McKrae viral DNA gB PCR product by electrophoresis on 4% NuSieve GTG agarose in 1× Tris-borate-EDTA (FMC Bioproducts, Rockland, Maine).

Competitive PCR assays were done as previously described (6, 20) with minor modifications. The DNA extracted from each TG was resuspended in 100 μl of double-distilled water, and 5-μl aliquots were placed into PCR tubes containing 10-fold serial dilutions of a known amount of competitor DNA (corresponding to 6×10^3 to 6×10^7 copies of HSV-1 DNA per TG). The competitive PCRs employed an excess of both primers and *Taq* polymerase. Each PCR was carried out in a final volume of 100 μl containing 10 μl of 10× PCR buffer (Boehringer Mannheim Biochemicals), 1 μl of deoxynucleoside triphosphates (100 mM [each] dCTP, dGTP, dATP, and dTTP) (New England Biolabs), 1 μl of the primer pair, and 0.5 μl (2.5 U) of *Taq* polymerase (Boehringer Mannheim Biochemicals). PCR amplification was carried out in a thermal cycler (Applied Biosystems) as we previously described (16, 18), with modifications. Briefly, 1 cycle was done at 95°C for 3 min; 30 cycles were done at 95°C for 15 s, 54°C for 15 s, and 71°C for 1.5 min; and this was followed by 1 extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 4% NuSieve GTG agarose. The DNA gel was denatured in 1.5 M NaCl and 0.5 M NaOH for 20 min, the DNA was transferred to a Hybond-N membrane (Amersham, Arlington, Ill.) and

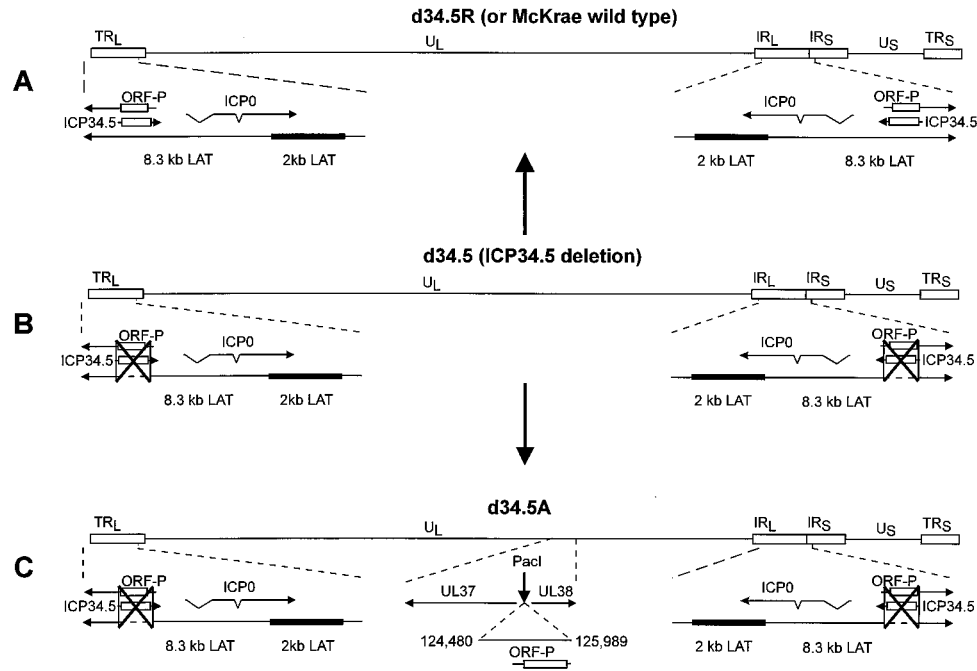


FIG. 1. Schematic representation of *d34.5*, *d34.5R*, and *d34.5A*. (A) Schematic representation of the genome of *d34.5R* and McKrae. *d34.5R* was rescued from the ICP34.5 deletion mutant (*d34.5*) by homologous recombination with a cloned HSV-1 restriction fragment as we previously described (18). This derivation is indicated by the large vertical arrow pointing from *d34.5* to *d34.5R*. The structure of *d34.5R* is identical to that of the parental McKrae virus genome. The prototypic orientation of HSV-1 shown here contains a U_L and a U_S , each bounded by inverted repeats. The unique regions are indicated by a solid line. The repeats are indicated by open rectangles. The lines with arrows under the genomes indicate the locations and directions of the ICP34.5, LAT, ORF-P, and ICP0 transcripts. The open rectangles within the ICP34.5 and ORF-P arrows show the relative sizes and locations of these ORFs. (B) ICP34.5 deletion mutant, *d34.5*, whose construction we previously described (18). Both copies of ICP34.5 (large X's) and the corresponding location in LAT (dashed line) are deleted. The extent of the deletion is shown by the vertical lines outside the large X. Both the ICP34.5 and the ORF-P ORFs are completely deleted. (C) The large vertical arrow pointing to the schematic for *d34.5A* signifies that *d34.5A* was derived from *d34.5*. *d34.5A* contains one copy of ICP34.5 inserted into the U_L between genes UL37 and UL38 (center, expanded portion of U_L) at genomic nucleotide position 84252. This corresponds to an *Afl*III site that we converted to a unique *Pac*I site. This site is 168 nucleotides from the 5' end of the UL37 ORF and 279 nucleotides from the 5' end of the UL38 ORF. Previous studies have shown that disruption of the genome at this location does not appear to alter any of the biological characteristics of the virus, including establishment and reactivation from latency (unpublished observations). The derivation of the inserted fragment is shown by the nucleotide positions this fragment has in its original position in the IR_L (124480 to 125989). *d34.5A* retains the LAT and ICP34.5 deletions of *d34.5*, as indicated by the large X's.

probed with a [32 P]dCTP-labeled cloned fragment within the 192-bp gB region and internal to the primers. The amount of radioactivity in each band was determined with a radioanalytic imaging detector (AMBIS Inc., San Diego, Calif.).

For each experimental sample, the number of HSV-1 DNA copies in the original TG was determined from the dilution of the competitor at which the competitor and viral PCR products were present in equal amounts. If the intensities of the competitor and latent DNA PCR products did not exactly coincide at any of the competitor DNA dilutions, the competitor DNA dilution at which the PCR products would have been identical was estimated from a linear regression plot of the two PCR products with the computer program Graph Pad. The point at which the plots cross indicates the point of equivalence of the competitor and viral PCR products.

Statistical analyses. Statistical analyses were performed with Instat, a personal computer software program. For analyses using either the Student *t* test, the Mann-Whitney rank sum test, the chi-square test, or the Fisher exact test, results were considered statistically significant when the *P* value was <0.05.

RESULTS

Structure of ICP34.5-deleted and -rescued viruses. McKrae was used as the parental virus for all of the mutants in this study. The McKrae strain of HSV-1 has the highest spontaneous reactivation rate of any of the commonly used HSV-1 laboratory strains. This previously allowed us to make LAT and ICP34.5 McKrae-based mutants that demonstrated significantly reduced spontaneous reactivation rates. The genomic structures of the three viruses used in this report are shown schematically in Fig. 1. Figure 1A illustrates the wild-type

McKrae and the *d34.5R* rescued viruses, which are identical. The HSV-1 genome contains a unique long region (U_L) and a unique short region (U_S), both of which are flanked by inverted repeats (terminal and internal long repeats [TR_L and IR_L , respectively] and terminal and internal short repeats [TR_S and IR_S , respectively]). Portions of the repeats are expanded under each genome to show the status of the 8.3-kb LAT, ICP34.5, and ORF-P RNAs. ORF-P (11) is a recently described potential ORF with the same sense as LAT whose mRNA may be transcribed independently from the 8.3-kb LAT. The location of the U_L containing genes UL37 and UL38 is expanded in Fig. 1C (*d34.5A*) to show the novel location into which a copy of ICP34.5 was inserted in that virus.

The ICP34.5-deleted virus, *d34.5* (Fig. 1B), is the precursor for both the *d34.5R* virus (Fig. 1A) and the *d34.5A* virus (Fig. 1C). We have previously described the construction of *d34.5* and *d34.5R* (18). In *d34.5*, both copies of the ICP34.5 ORF (one in each long repeat) are deleted (Fig. 1B and C). The colinear kb 6.2 to 7.1 region of LAT in each long repeat and the ORF-P ORF are also deleted in these viruses (Fig. 1B and C). *d34.5R* (Fig. 1A) was rescued from *d34.5* by homologous recombination with a cloned restriction fragment and shown to be restored to the wild-type (McKrae) phenotype (18). The structures of *d34.5* and *d34.5R* were confirmed by Southern analyses (18).

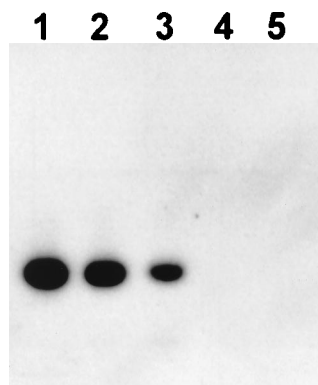


FIG. 2. Expression of ICP34.5 mRNA by *d34.5A*. Infected cells were harvested; RNA was isolated, digested with DNase, and subjected to RT-PCR for ICP34.5 mRNA; and the products were visualized by Southern analysis with a labeled probe internal to the original PCR primers as described in Materials and Methods. Lane 1, marker DNA generated by PCR with the primers used for RT-PCR; lane 2, RT-PCR product corresponding to ICP34.5 mRNA from McKrae; lane 3, *d34.5A*; lane 4, *d34.5*; lane 5, uninfected cells.

The virus shown in Fig. 1C (*d34.5A*) was constructed from *d34.5* by the addition (hence the "A" in *d34.5A*) of one copy of the ICP34.5 ORF into the gap between genes UL37 and UL38 in the viral U_L . This insertion was at the location of an *Afl*II site that we had converted to a unique *Pac*I site as described in Materials and Methods (Fig. 1C). The ICP34.5 addition (HSV-1 nucleotides 125989 to 124480) does not include all of the predicted ICP34.5 promoter (23). This is because the 5' portion of the promoter is located within the "a" region of the long repeats, and it is extremely difficult to construct a stable HSV-1 mutant that contains an extra copy of "a" in the U_L . This would have been required if we had attempted to include the entire ICP34.5 promoter. We therefore included in the insert only that portion of the ICP34.5 promoter that we expected would not unduly complicate construction of the virus. We then determined that ICP34.5 expression from this partial promoter was sufficient to restore biological function (see below). This insert also contains the complete sequence for the postulated ORF-P and its postulated promoter but not the 3' end and poly(A) sequence of the postulated ORF-P mRNA which is the same as the 3' end of the 8.3-kb LAT (35). Further details of the construction of *d34.5A* are given in Materials and Methods. *d34.5A* is identical to *d34.5* except that it contains a copy of ICP34.5 (and ORF-P) introduced into the U_L of the virus rather than the normal ICP34.5-ORF-P location. For simplicity (and because ORF-P has not been conclusively shown to be independently transcribed or translated except in ICP4-deleted mutant viruses), in the remainder of this section of this report we will refer only to ICP34.5. The structure of *d34.5A* was confirmed by Southern analysis (see Fig. 4, below).

Expression of ICP34.5 mRNA in cells acutely infected with *d34.5A*. CV-1 cells were infected with 10 PFU of wild-type McKrae, *d34.5*, or *d34.5A* per cell. Total RNA was harvested, digested with DNase I, and analyzed by RT-PCR as described in Materials and Methods with primers that are within the ICP34.5 gene (Fig. 2). The PCR primer pair produces a 134-nucleotide PCR product corresponding to a portion of the ICP34.5 mRNA. The ICP34.5 mRNA-specific RT-PCR products were visualized by Southern hybridization with a labeled probe internal to the primers. The RT-PCR product representing ICP34.5 mRNA from *d34.5A* is clearly visible in the autoradiogram (Fig. 2, lane 3). This band comigrated with the RT-PCR product representing ICP34.5 mRNA from the pa-

rental wild-type McKrae virus (Fig. 2, lane 2) and with the size marker PCR product generated with the same primer set from purified viral DNA (Fig. 2, lane 1). No ICP34.5-related product was detected following RT-PCR of RNA from uninfected cells (Fig. 2, lane 5) or of RNA from cells infected with the ICP34.5 deletion mutant *d34.5* (lane 4). In addition, PCR analysis confirmed that the original RNA preparations did not contain any contaminating HSV-1 DNA (not shown). Although the relative amounts of the RT-PCR products shown here cannot be accurately quantitated, the *d34.5A* RT-PCR product was readily apparent, and the quantity of this product appeared to be approximately half that of the McKrae RT-PCR product. These results demonstrate that *d34.5A*, despite not containing the complete ICP34.5 gene promoter (23) and containing only one copy of the ICP34.5 gene, nonetheless appeared to express a significant amount of ICP34.5 mRNA.

Spontaneous reactivation of *d34.5A* from latently infected rabbits. We recently showed that a LAT deletion mutant (*dLAT2903*) had significantly reduced spontaneous reactivation in latently infected rabbits (16). We also recently reported that the ICP34.5 deletion mutant *d34.5* (18) had significantly reduced spontaneous reactivation under the same conditions. Since the 0.9-kb deletion that produced *d34.5* also deleted 0.9 kb of the overlapping LAT gene, it was not clear if the reduction in spontaneous reactivation was due to the loss of the ICP34.5 gene, the kb 6.2 to 7.1 colinear deletion in LAT, or a combination of the two. We therefore constructed *d34.5A* and examined its ability to reactivate spontaneously. As described above, *d34.5A* retains the 0.9-kb deletion in both copies of LAT and contains a functional copy of ICP34.5 in the U_L of the viral genome (Fig. 1C).

Rabbit eyes were infected with 2×10^5 PFU of *d34.5A*, *d34.5*, or *d34.5R*. Beginning 30 days postinfection (at which time latency had already been established), all eyes were swabbed once a day to collect tear films for analysis of spontaneously reactivated virus as described in Materials and Methods. Two independent experiments were done. The numbers of rabbits per group examined for spontaneous reactivation in experiments 1 and 2 were 17 and 7 for *d34.5A*, 8 and 7 for *d34.5*, and 17 and 10 for *d34.5R*, respectively.

The cumulative number of virus-positive tear film cultures during 26 days in each experiment is shown in Fig. 3. Because of the different numbers of rabbits (and eyes) in the different groups, the data were standardized to represent cumulative positive cultures per eye. In both experiments, the ICP34.5 deletion mutant *d34.5* had a very low level of spontaneous reactivation (Fig. 3). This was in agreement with our previous findings (18). In each experiment, the cumulative spontaneous reactivation rate in rabbits latently infected with *d34.5A* and *d34.5R* appeared to be very similar (Fig. 3A and B).

A statistical analysis of positive (spontaneously reactivated) cultures versus negative cultures is shown in Table 1. In experiment 1, about 16.1% (134 of 832) of the tear films from rabbits latently infected with *d34.5A* contained spontaneously reactivated virus. This was significantly higher than the 1.4% (6 of 416) positive tear film cultures from rabbits latently infected with *d34.5* ($P < 0.0001$; Fisher's exact test) and not significantly different from the 19.6% (80 of 408) positive cultures from rabbits latently infected with *d34.5R* ($P = 0.13$). The results for experiment 2 were similar. Only 0.3% (1 of 312) of the cultures from rabbits infected with *d34.5* were positive, compared with 7.7% (20 of 260) and 9.0% (28 of 312) positive cultures for rabbits latently infected with *d34.5R* and *d34.5A*, respectively. Again, the difference between *d34.5* and *d34.5A* was highly significant ($P < 0.0001$), while the results for *d34.5A* and *d34.5R* were not significantly different ($P = 0.67$). Thus, de-

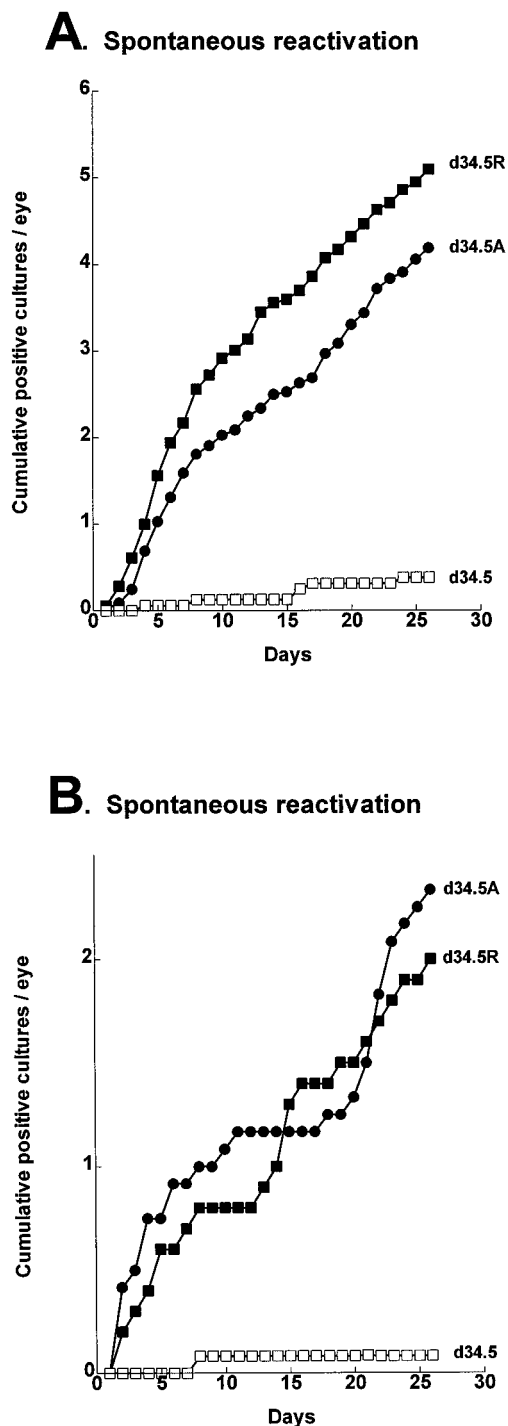


FIG. 3. Spontaneous reactivation in rabbits latently infected with *d34.5A*. As described in Materials and Methods, rabbits were ocularly infected with 2×10^5 PFU of *d34.5*, *d34.5R*, or *d34.5A* per eye. Beginning at thirty days postinfection (day 0), eye swabs were taken daily to collect tear films for the detection of HSV-1. The collected tear films were plated on primary rabbit kidney cells and observed for up to 30 days for the presence of cytopathic effects indicative of the presence of spontaneously reactivated virus in the corresponding eye. All positive cultures were confirmed by passage, and most were also confirmed by Southern analysis (Fig. 4). The y axis represents the cumulative number of HSV-1-positive cultures for each virus group divided by the number of eyes in the group. Panels A and B show results from independent experiments. A statistical analysis is shown in Table 1.

spite the fact that *d34.5A* contained only a single copy of ICP34.5, the spontaneous reactivation rate of *d34.5A* appeared to have been completely rescued back to wild-type virus (*d34.5R*) levels.

Because the analyses discussed above do not take into account the number of eyes in each of the groups, the data were analyzed by additional methods. The fraction of virus-positive cultures for each eye in each group (i.e., the fraction of time each eye was virus positive) was calculated, and these fractions were analyzed (Table 1). In both experiments, the level of spontaneous reactivation with the *d34.5A* virus was significantly higher than that of *d34.5* ($P < 0.0001$ and $P = 0.007$, respectively; Mann-Whitney rank sum test) and similar to that of *d34.5R* ($P = 0.3$ and $P = 0.75$, respectively; Student's *t* test).

The number of eyes in each group that had at least one spontaneous reactivation was also analyzed (Table 1). Again, in both experiments the results for the *d34.5A* virus were significantly greater than the results for the *d34.5* virus (94 versus 25%, $P < 0.0001$, and 67 versus 8%, $P = 0.01$) and similar to the wild-type results (94 versus 94%, $P = 1.0$, and 67 versus 50%, $P = 0.67$).

Another method of examining the effect of one copy of ICP34.5 on spontaneous reactivation is to analyze the number of times spontaneous reactivation is detected in each eye, regardless of the length of time the virus is present. This is equivalent to the number of episodes in which reactivated virus is detected in the tears, with consecutive days of positive cultures being treated as a single event. Thus, an eye that sheds virus for a single day and an eye that sheds virus for 5 consecutive days would both constitute one episode of spontaneous reactivation. The average number of spontaneous reactivations per eye in each group calculated in this manner is shown in Table 1. The numbers of episodes of spontaneous reactivation in *d34.5A*- and *d34.5R*-infected eyes were similar (2.4 and 2.1, respectively, $P = 0.33$, in experiment 1 and 1.4 and 1.2, respectively, $P = 0.63$, in experiment 2). In contrast, the numbers of episodes in *d34.5A*- and *d34.5*-infected eyes were significantly different in both experiments (Table 1, $P < 0.0001$ and $P = 0.007$, respectively). All of the analyses discussed above indicate that *d34.5A* (containing one copy of ICP34.5 in a novel location and a 0.9-kb deletion from kb 6.2 to 7.1 in both copies of LAT) reactivated spontaneously at a rate indistinguishable from that of *d34.5R* (wild-type virus).

Southern analysis of spontaneously reactivated virus. To eliminate the unlikely possibility that the spontaneously reactivated virus recovered from the eyes of rabbits latently infected with the *d34.5A* mutant viruses was contaminating wild-type virus or mutant virus that had somehow reverted back to wild-type virus, Southern analyses with selected spontaneously reactivated viruses were done. Examples of some of these Southern analyses are shown in Fig. 4. Purified viral DNAs were obtained prior to infection (lanes I) and from virus in tears after spontaneous reactivation (lanes S). The DNAs were individually digested with *Bam*HI and subjected to Southern analysis by using a probe that spans the ICP34.5 gene and is completely within the viral long repeat. As we previously reported for wild-type virus (or *d34.5R*) (18), the 5.9- and 2.9-kb bands seen in Fig. 4, lane *d34.5R*-I (solid arrows), represent *Bam*HI fragments containing the ICP34.5 gene from the IR_L and the TR_L, respectively. The additional fainter bands forming a ladder above the 5.9- and 2.9-kb bands represent *Bam*HI fragments from long repeats containing multiple copies of the "a" repeat (18).

As we previously reported (18), in *d34.5* both the 5.9- and 2.9-kb bands (and their ladder bands) have apparent sizes which are approximately 0.9 kb less than those of their wild-

TABLE 1. Spontaneous reactivation rate of *d34.5A* is normal^a

Expt no. and virus	Copies of ICP34.5	No. of positive cultures/total no. (%)	<i>P</i>	Fraction of positive cultures/eye ^b	<i>P</i>	No. of positive eyes/total no. (%) ^c	<i>P</i>	No. of episodes of spontaneous reactivation/eye (<i>n</i>) ^d	<i>P</i>
1									
<i>d34.5</i>	0	64/416 (1.4)	<0.0001 ^e	0.01	<0.0001 ^f	4/16 (25)	<0.0001 ^e	0.31 (16)	<0.0001 ^f
<i>d34.5A</i>	1	134/832 (16.1)		0.16		30/32 (94)		2.38 (32)	
<i>d34.5R</i>	2	80/408 (19.6)	0.13 ^e	0.19	0.3 ^g	17/18 (94)	1.0 ^e	2.1 (18)	0.33 ^f
2									
<i>d34.5</i>	0	1/312 (0.3)	<0.0001 ^e	0.003	0.007 ^f	1/12 (8)	0.01 ^e	0.08 (12)	0.007 ^f
<i>d34.5A</i>	1	28/312 (9.0)		0.089		8/12 (67)		1.42 (12)	
<i>d34.5R</i>	2	20/260 (7.7)	0.67 ^e	0.077	0.75 ^g	5/10 (50)	0.67 ^e	1.2 (10)	0.63 ^f

^a Rabbits were infected in both eyes with *d34.5* (no copies of ICP34.5), *d34.5A* (one copy of ICP34.5 in the U_L), or *d34.5R* (rescued *d34.5*; two copies of ICP34.5 in their normal location in the long repeat) as described in Materials and Methods. Experiments 1 and 2 were done independently, at different times, and with different groups of rabbits. Spontaneous reactivation was assessed beginning on day 30 postinfection by culturing tear films collected daily for 26 days as described in Materials and Methods. *P* values reported are relative to *d34.5A*.

^b Average for all eyes of the total HSV-1-positive cultures for eye "x" per total number of cultures for eye "x".

^c The total number of eyes that spontaneously reactivated at least once per the total number of eyes.

^d The total number of reactivations in each group, assuming that consecutive days of HSV-1-positive tear film cultures from a single eye are the result of a single reactivation event or episode. *n*, number of eyes.

^e Fisher's exact test; two sided. The groups are considered significantly different if *P* is <0.05.

^f Mann-Whitney rank sum test; two sided. The groups are considered significantly different if *P* is <0.05.

^g Student's *t* test; two sided. The groups are considered significantly different if *P* is <0.05.

type counterparts (Fig. 4, lane *d34.5-I* [open arrows]), consistent with the 0.9-kb deletion of ICP34.5 in each repeat. As with the *d34.5* virus DNA, the bands corresponding to the region of the repeats are also reduced in size in the *d34.5A* DNA (Fig. 4,

lane *d34.5A-I*). Again, this reduction in size is consistent with deletion of ICP34.5 from both regions and confirms that *d34.5A* has retained the *d34.5* ICP34.5-LAT deletion in both long repeats. The *d34.5A* DNA also contains a large band of approximately 9 kb (Fig. 4, lane *d34.5A-I*) to which the labeled probe hybridized. This band is consistent with the size of the *Bam*HI band (7.5 kb) containing the ICP34.5 gene (1.5 kb) that was inserted into the U_L and confirms that *d34.5A* contains this unique copy of the ICP34.5 gene. In addition, digestion of *d34.5A* DNA with *Pac*I converted this band to a size identical to that of the original ICP34.5 insert (1.5 kb) (data not shown). This Southern analysis therefore confirms that the *d34.5A* virus contains a copy of ICP34.5 in the U_L, while retaining the ICP34.5-LAT deletion in both repeats.

Lanes *d34.5-S*, *d34.5R-S*, and *d34.5A-S* (Fig. 4) show Southern analyses of DNAs isolated from spontaneously reactivated virus grown from tears. With all three viruses, the spontaneously reactivated (lanes S) viruses *d34.5*, *d34.5R*, and *d34.5A* have Southern patterns identical to those of the infecting viruses (lanes I), indicating that for each virus the organization of the genomic DNA has been retained. Specifically, the spontaneously reactivated *d34.5A* virus (Fig. 4, lane *d34.5A-S*) retained the ICP34.5-LAT deletion in both repeats as well as a copy of the ICP34.5 gene in the U_L. This was found to be the case for all of the spontaneously reactivated *d34.5A* virus examined. Thus, the ability of *d34.5A* to reactivate spontaneously was due to inherent properties of the virus and not to rescue of the ICP34.5-LAT deletions with the ICP34.5 gene sequences inserted into the U_L.

Virulence in rabbits. We previously found that *d34.5* was avirulent in rabbits (18). To determine if virulence was restored in *d34.5A*, rabbits were infected ocularly. The combined survival results of two experiments are shown in Fig. 5. Ninety-two percent (22 of 24) of the rabbits infected with *d34.5A* survived. This level of survival was significantly greater than that seen with *d34.5R* (13 of 27, 48%, *P* = 0.0009; Fisher's exact test). One hundred percent (15 of 15) of the *d34.5*-infected rabbits survived. This was not significantly different from the results obtained with *d34.5A* (*P* = 0.51). These results indicate that the addition of a single copy of ICP34.5 to *d34.5* did not restore virulence to wild-type levels.

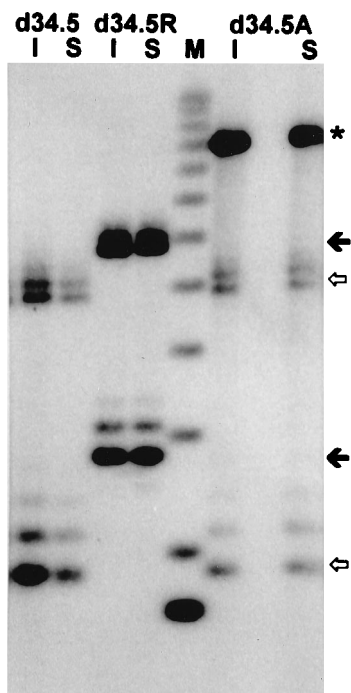


FIG. 4. Southern analysis of spontaneously reactivated *d34.5A*. DNA was isolated from virus from selected positive tear film cultures from Fig. 3, digested with *Bam*HI, run on an agarose gel, transferred to a nylon membrane, and hybridized with a ³²P-labeled probe from the long repeat region of the virus that spans the ICP34.5 gene (or deletion) as described in Materials and Methods. The solid arrows indicate the locations of the 5.9- and 2.9-kb ICP34.5 gene-related bands from wild-type virus. The open arrows indicate the locations of the corresponding bands from *d34.5* and *d34.5A* that are missing in the 0.9-kb ICP34.5 region. The star indicates the location of the *Bam*HI band that contains the ICP34.5 gene inserted into the U_L of *d34.5A*. Lanes: I, infecting virus; S, spontaneously reactivated virus; M, DNA markers.

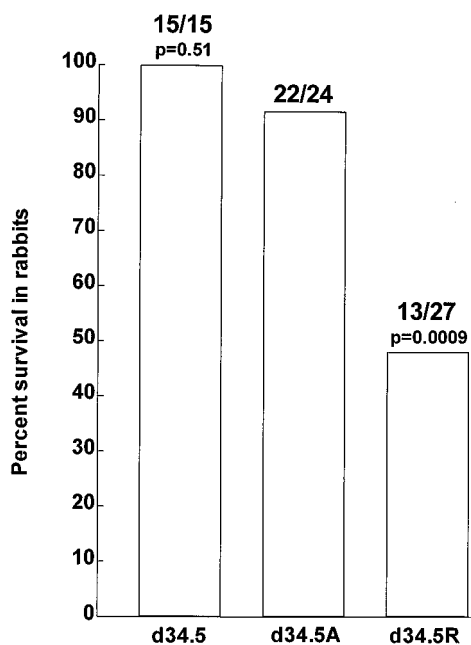


FIG. 5. Virulence of *d34.5A* in rabbits. Rabbits were infected ocularly with 2×10^5 PFU of each virus per eye. Survival was determined 21 days postinfection. *P* values are in comparison to *d34.5A*.

Replication of *d34.5A* in rabbit eyes. We previously reported that *d34.5* does not replicate well in rabbit eyes (18). To determine if *d34.5A* was fully rescued for this defect, rabbits were infected with 2×10^5 PFU per eye with either *d34.5*, *d34.5R*, or *d34.5A* as described in Materials and Methods. Tears were collected at various times during the acute phase of infection and the titers of the virus were determined. The average virus titers from six eyes per group on days 3, 5, 7, and 10 are shown in Fig. 6. As determined previously, *d34.5* grew to only very low titers (<100 PFU per eye). In contrast, *d34.5R* and *d34.5A* grew to high titers (5×10^5 to 9×10^5 PFU per eye). There were no significant differences in the titers of *d34.5R* and *d34.5A* on any given day ($P > 0.05$, Student's *t* test). In comparison, eyes infected with either *d34.5R* or *d34.5A* had significantly higher virus titers than eyes infected with *d34.5* ($P < 0.05$, days 3, 5, 7, and 10; Student's *t* test). Thus, it appeared that a single copy of ICP34.5 in a novel location was sufficient to fully restore wild-type levels of viral replication in rabbit eyes.

Replication of *d34.5A* in tissue culture. We previously reported that despite *d34.5* having normal replication kinetics in RS cells, replication of *d34.5* in CV-1 cells was restricted (18). LAT did not appear to be involved in this phenotype, since our null LAT mutant replicated normally in CV-1 cells (16). Nonetheless, it was of interest to determine if the ability to replicate in CV-1 cells could be restored by insertion of one copy of ICP34.5 in a novel location. We therefore infected confluent monolayers of CV-1 cells with *d34.5*, *d34.5R*, or *d34.5A*, each at an MOI of 10 or 0.01 PFU per cell (Fig. 7). At an MOI of 0.01, *d34.5R* and *d34.5A* had similar replication kinetics, with peak titers of just over 1×10^8 PFU/ml (Fig. 7A). In comparison, at this low MOI, *d34.5* replicated very poorly, reaching a maximum titer of less than 1×10^3 PFU/ml, or 100,000-fold less. At an MOI of 10 PFU per cell (Fig. 7B), *d34.5R* and *d34.5A* again had similar replication kinetics, with peak titers of over 10^7 PFU/ml. As before, *d34.5* appeared to

replicate much less well; however, at this higher MOI, the difference was less pronounced (approximately 100-fold; Fig. 7B). These results indicate that a single copy of ICP34.5 in a novel location appeared to fully restore the ability of the *d34.5* ICP34.5 null mutant to replicate in CV-1 cells.

HSV-1 DNA in TG of latently infected rabbits. The number of HSV-1 genomic copies per TG in rabbits latently infected with *d34.5*, *d34.5A*, or *d34.5R* was estimated by competitive PCR assays as described in Materials and Methods. The PCR primer pair produces a 192-nucleotide PCR product from HSV-1 genomic DNA corresponding to a portion of the HSV-1 glycoprotein B (gB) gene. The competitor DNA is identical to this gB region except for a 35-nucleotide deletion internal to the primers that results in a PCR product of 157 nucleotides. The genomic and competitor PCR products were separated, visualized, and quantitated by Southern analysis with a cloned internal probe that is outside the 35-nucleotide deletion. Representative results of such competitor PCR assays are shown in Fig. 8A. In all three panels, as the intensity of the competitor PCR product decreases from left to right in conjunction with the decreasing amount of input competitor DNA, the intensity of the HSV-1 DNA PCR product increases. For these particular TG, the competitor and HSV-1 DNA band intensities appear equivalent at approximately 6×10^4 competitor copy equivalents per TG. Thus, these three samples all appear to contain approximately 6×10^4 genomic copies of HSV-1 DNA per TG.

Identical competitive PCR assays were performed on 11 TG from rabbits latently infected with *d34.5R*, 14 TG from rabbits

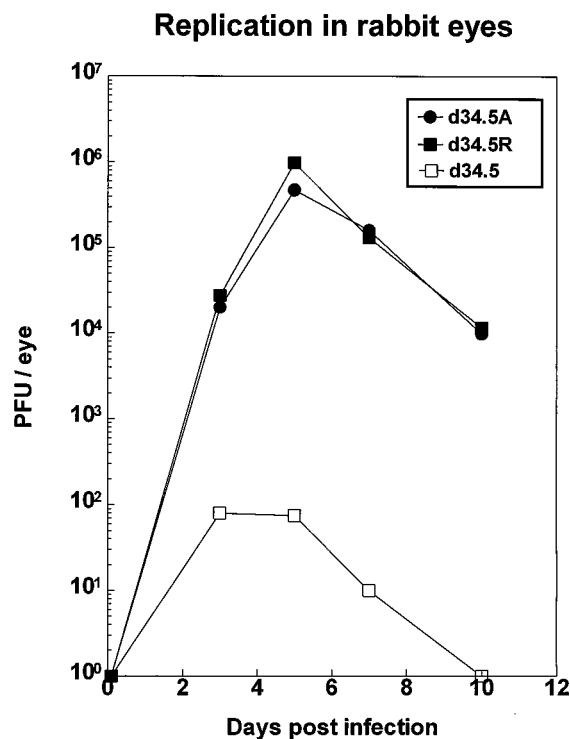


FIG. 6. Replication of *d34.5A* in rabbit eyes. Rabbits were infected with 2×10^5 PFU of *d34.5*, *d34.5R*, or *d34.5A* per eye as described in Materials and Methods. Tears were collected at the times indicated, and the amount of HSV-1 present was determined by titration with a standard plaque assay as described in Materials and Methods. Each time point represents the average virus titer from six eyes per group. The titers for *d34.5R* and *d34.5A* were not different on any day ($P > 0.05$; Student's *t* test). On all days the *d34.5* virus titers were lower than the *d34.5R* and *d34.5A* virus titers ($P < 0.05$; Student's *t* test).

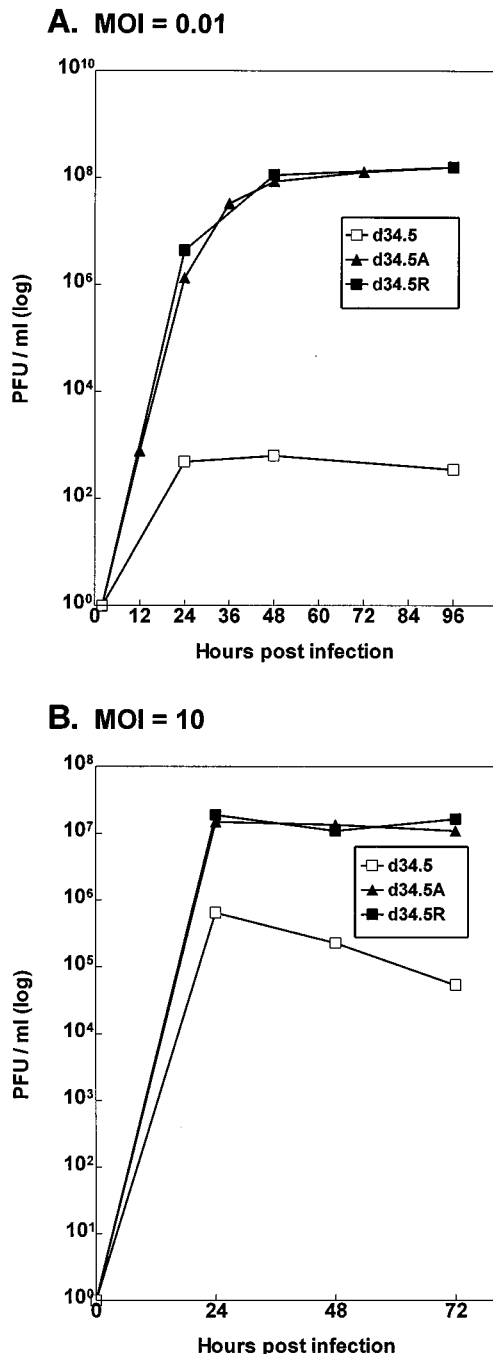


FIG. 7. Replication of *d34.5A* in CV-1 cells in culture. Subconfluent monolayers of CV-1 cells in 60-mm-diameter tissue culture plates were infected with 10 or 0.01 PFU of *d34.5*, *d34.5R*, or *d34.5A* per cell. At the times indicated, the monolayers were harvested and the amount of virus was determined by plaque assay.

latently infected with *d34.5*, and 16 TG from rabbits latently infected with *d34.5A*. The estimated number of HSV-1 DNA copies for each of the TG is shown in a scattergram (Fig. 8B). Each symbol represents the estimated number of copies for one TG. The scattergram therefore shows the distribution and spread of the results. The ranges and distributions for all three viruses appear similar. The average number of genomic copies was approximately 5×10^5 per TG for *d34.5R*, 7.1×10^5 per

TG for *d34.5*, and 1.5×10^5 per TG for *d34.5A* (Table 2). Compared with *d34.5R*, both *d34.5* and *d34.5A* appeared to have similar numbers of HSV-1 genomes per TG ($P = 0.68$ and $P = 0.07$, respectively; Student's *t* test). Thus, if the relative amount of HSV-1 DNA as determined by PCR analysis of latently infected TGs reflects the rate of establishment of latency, it would appear that all three viruses established latency at similar rates.

DISCUSSION

Recently we reported an ICP34.5 deletion mutant of HSV-1 strain McKrae (*d34.5*) that was severely restricted in its ability to spontaneously reactivate in the rabbit eye model of HSV-1 latency (18). Because of the colinearity of ICP34.5 with a portion of LAT, *d34.5* also contains a deletion in the 8.3-kb LAT from kb 6.2 to 7.1. Since we had previously shown that LAT is required for efficient spontaneous reactivation (16), it was of interest to determine if the reduced spontaneous reactivation of *d34.5* was due to the kb 6.2 to 7.1 deletion in LAT or to deletion of ICP34.5. Therefore, in the current study, we reintroduced back into the *d34.5* mutant a functional copy of the ICP34.5 gene at a novel location. This mutant, designated *d34.5A*, contains the original 917-nucleotide deletion in LAT (in both viral long repeats) and a single copy of the ICP34.5 gene in the U_L of the virus between genes UL37 and UL38. *d34.5A* thus has been repaired, or rescued, for one of the two copies of ICP34.5 while retaining the original kb 6.2 to 7.1 LAT deletion.

In two independent experiments, the spontaneous reactivation rate for *d34.5A* (9 to 16.1%) was the same as the spontaneous reactivation rate for wild-type rescued virus (7.7 to 19.6%). This demonstrated that the kb 6.2 to 7.1 region of the 8.3-kb LAT was not required for efficient spontaneous reactivation.

The original ICP34.5 deletion mutant, *d34.5*, had additional defects. Virulence in rabbits was dramatically decreased, as was replication in rabbit eyes and CV-1 cells. All of these defects were due to deletion of ICP34.5, since our LAT mutant, containing a complete deletion of the LAT promoter and constructed from the same McKrae parental virus, did not have any of these defects (16). The results reported here indicate that spontaneous reactivation and replication in rabbit eyes and CV-1 cells was completely restored by the single copy of the ICP34.5 gene in *d34.5A*. In contrast, virulence in rabbits was not completely restored in *d34.5A*. This suggests either that virulence in rabbits requires two copies of the ICP34.5 gene or that the ICP34.5 gene must be in its normal location in the long repeat.

It is of particular interest that in *d34.5A* the spontaneous reactivation phenotype was completely restored to wild-type levels while virulence remained very low. These results appear to at least partially separate spontaneous reactivation from virulence. They therefore strongly suggest that, in contrast to what has often been assumed, the ability of a particular HSV strain to spontaneously reactivate is not directly related to the virulence of the virus.

We previously reported that the results of a noncompetitive PCR assay appeared to show a trend toward less viral DNA in the TG of rabbits latently infected with *d34.5* versus *d34.5R*. However, the differences were not statistically different. In the current study, our competitive PCR assays also showed a lack of statistical significance. Thus, it appeared that rabbits latently infected with *d34.5A* had levels of viral DNA in their TG comparable to those in rabbits latently infected with *d34.5* or *d34.5R*. Thus, the ICP34.5 gene did not appear to be required

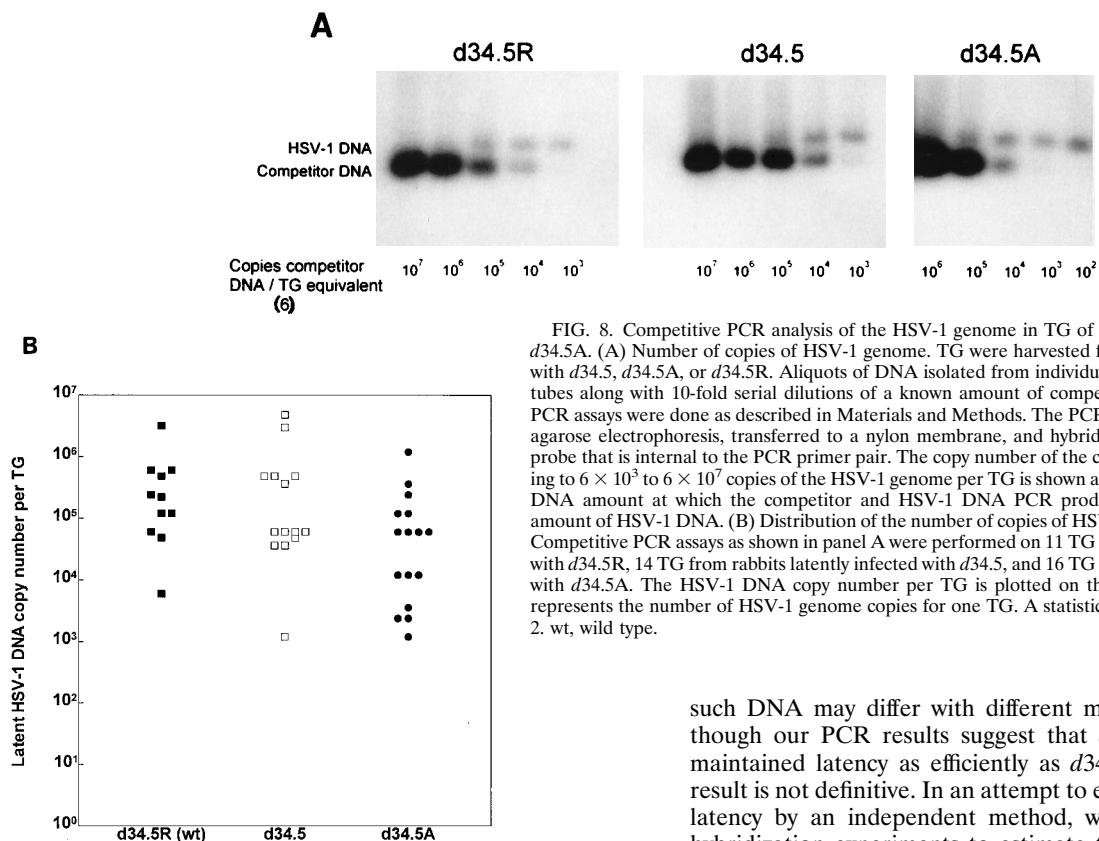


FIG. 8. Competitive PCR analysis of the HSV-1 genome in TG of rabbits latently infected with *d34.5A*. (A) Number of copies of HSV-1 genome. TG were harvested from rabbits latently infected with *d34.5*, *d34.5A*, or *d34.5R*. Aliquots of DNA isolated from individual TG were placed into PCR tubes along with 10-fold serial dilutions of a known amount of competitor DNA, and competitive PCR assays were done as described in Materials and Methods. The PCR products were separated by agarose electrophoresis, transferred to a nylon membrane, and hybridized to a ³²P-labeled cloned probe that is internal to the PCR primer pair. The copy number of the competitor DNA corresponding to 6 × 10³ to 6 × 10⁷ copies of the HSV-1 genome per TG is shown at the bottom. The competitor DNA amount at which the competitor and HSV-1 DNA PCR products are equal indicates the amount of HSV-1 DNA. (B) Distribution of the number of copies of HSV-1 genomes in different TG. Competitive PCR assays as shown in panel A were performed on 11 TG from rabbits latently infected with *d34.5R*, 14 TG from rabbits latently infected with *d34.5*, and 16 TG from rabbits latently infected with *d34.5A*. The HSV-1 DNA copy number per TG is plotted on the scattergram. Each symbol represents the number of HSV-1 genome copies for one TG. A statistical analysis is shown in Table 2. wt, wild type.

for HSV-1 to efficiently establish latency. Since *d34.5R* and *d34.5A* reactivated spontaneously at statistically higher levels than *d34.5*, it is therefore likely that ICP34.5 is either (i) involved in reactivation from latency or (ii) required for the virus to replicate efficiently and be detected in tear films following reactivation in the neuron.

The discussion in the preceding paragraph contains an implicit assumption for which there is currently no proof, namely, that the total amount of HSV-1 viral DNA present in the TG as determined by PCR assays is a direct and accurate measure of the relative amount of HSV-1 latency. However, PCR assays cannot distinguish between biologically active and biologically inactive HSV-1 DNA. Thus, some of the HSV-1 DNA detected by PCR may be degraded viral DNA that does not represent latent DNA capable of reactivation. The amount of

such DNA may differ with different mutants. Therefore, although our PCR results suggest that *d34.5* established and maintained latency as efficiently as *d34.5A* and *d34.5R*, this result is not definitive. In an attempt to estimate the amount of latency by an independent method, we have begun in situ hybridization experiments to estimate the amount of latency on the basis of the percentage of neurons containing the 2-kb LAT RNA. Preliminary results indicate that rabbits latently infected with *d34.5* may contain fewer LAT-positive neurons than other latently infected rabbits. Thus, in contrast to the interpretation that would normally be given to our PCR results (i.e., no differences in establishment of latency), the interpretation that would normally be given to our preliminary in situ hybridization results suggests that *d34.5A* may establish latency more efficiently than *d34.5*.

The apparent complexity of the kb 6.2 to 7.1 LAT region in the long repeat has recently been increased by reports suggesting that, in addition to the colinear but antisense ICP34.5 gene, this region contains an independent LAT-sense transcript containing a potential ORF that has been designated ORF-P (2, 11, 35). ORF-P is deleted from *d34.5* because it is almost completely colinear (but antisense) to the ICP34.5 ORF (Fig. 1B and C). Furthermore, the postulated ORF-P and its postulated promoter are, along with the ICP34.5 ORF, completely contained within the LAT region relocated to the U_L of *d34.5A*.

ORF-P is potentially of great interest, although its function remains unknown. Formally, some or all of the results in this report that have been attributed to ICP34.5 may instead be due to ORF-P. It should be stressed, however, that the mapping results reported here are independent of whether the rescued phenotypes in *d34.5A* were due to expression of ICP34.5 or ORF-P. In either case, our results clearly demonstrate that deletion of the kb 6.2 to 7.1 region from the 8.3-kb LAT primary transcript did not significantly affect spontaneous reactivation in the rabbit.

In conclusion, genetic mapping of the functional portion(s) of the 8.3-kb LAT gene has been complicated by the colinearity with portions of LAT of two important viral genes, ICP0

TABLE 2. Estimated number of HSV-1 genomes in TG of latently infected rabbits^a

Virus	Avg no. of genomic copies/TG ± SD (n) ^b	P ^c
<i>d34.5R</i> (wt) ^d	5 × 10 ⁵ ± 2.8 × 10 ⁵ (11)	
<i>d34.5</i>	7.1 × 10 ⁵ ± 3.8 × 10 ⁵ (14)	0.68
<i>d34.5A</i>	1.5 × 10 ⁵ ± 7.5 × 10 ⁴ (16)	0.07

^a Total DNA was isolated from individual TG of rabbits latently infected with either *d34.5R*, *d34.5*, or *d34.5A*. The number of HSV-1 genomic copies per latently infected TG was estimated by competitive PCR assays as described in Materials and Methods.

^b SD, standard deviation. n, number of TG.

^c Student's *t* test; double sided. A *P* value of >0.05 indicates that compared with the number of HSV-1 genomic copies in the *d34.5R* group, the number of HSV-1 genomic copies in the indicated group is not significantly different.

^d wt, wild type.

and ICP34.5. We have reported here an approach that allows functional genetic mapping of the LAT region that is colinear with ICP34.5. Insertion of a complete functional copy of the ICP34.5 gene in a novel location allowed us to determine that the corresponding (kb 6.2 to 7.1) to LAT region was not involved in the function of LAT responsible for enhancing spontaneous reactivation. A similar approach with the ICP0 gene should allow us to construct a series of deletions in regions of LAT that are normally colinear with ICP0. Combined with similar mapping of LAT regions that do not overlap ICP0, this should allow us to map the functional LAT region(s) and determine the coding region(s) of a potential LAT protein(s) and should ultimately lead to an understanding of the molecular mechanism by which LAT enhances spontaneous reactivation.

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