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Deletion of the S component inverted repeat sequence *c′* and the nonessential genes U_S1 through U_S5 from the herpes simplex virus type 1 genome substantially impairs productive viral infection in cell culture and pathogenesis in the rat central nervous system

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A distinctive feature of the genetic make-up of herpes simplex virus type 1 (HSV-1), a human neurotropic virus, is that approximately half of the 81 known viral genes are not absolutely required for productive infection in Vero cells, and most can be individually deleted without substantially impairing viral replication in cell culture. If large blocks of contiguous viral genes could be replaced with foreign DNA sequences, it would be possible to engineer highly attenuated recombinant HSV-1 gene transfer vectors capable of carrying large cellular genes or multiple genes having related functions. We report the isolation and characterization of an HSV-1 mutant, designated d311, containing a 12 kb deletion of viral DNA located between the L-S Junction *a* sequence and the U_S6 gene, spanning the S component inverted repeat sequence *c′* and the nonessential genes U_S1 through U_S5. Replication of d311 was totally inhibited in rat B103 and mouse Neuro-2A neuroblastoma cell lines, and was reduced by over three orders of magnitude in human SK-N-SH neuroblastoma cells compared to wild-type (wt) HSV-1 KOS. This suggested that the deleted genes, while nonessential for replication in Vero cells, play an important role in HSV replication in neuronal cells, particularly those of rodent origin. Unlike wt KOS which replicated locally and spread to other regions of brain following stereotactic inoculation into rat hippocampus, d311 was unable to replicate and spread within the brain, and did not cause any apparent local neuronal cell damage. These results demonstrate that d311 is highly attenuated for the rat central nervous system. d311 and other mutants of HSV containing major deletions of the nonessential genes within U_S have the potential to serve as useful tools for gene transfer applications to brain.

Keywords: HSV pathogenesis; central nervous system; gene transfer vector

Introduction

The 152 kb linear double-stranded DNA genome of herpes simplex virus type 1 (HSV-1) consists of a 108 kb long (L) and a 13 kb short (S) component, each of which are flanked by inverted repeat elements. The *b* inverted repeat sequences (9 kb) surround the unique long (U_L) component, while the *c* inverted repeats (6.5 kb) flank the unique short (U_S) component (Hayward *et al*, 1975; Sheldrick and Berthelot, 1975; Wadsworth *et al*, 1975). Each of the *b* inverted repeats contain a copy of the ICPO and γ34.5 genes (Chou and Roizman, 1986), as well as the latency-associated transcript (LAT) sequence and the newly discovered ORF P (Lagunoff and Roizman, 1994; 1995; Ward and Roizman, 1994). The *c*-inverted repeats each contain a copy of the ICP4 gene (Morse *et al*, 1978) and an origin of viral DNA replication (ori_S) (Deb and

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Doelberg, 1988; Stow, 1982; Stow and McMonagle, 1983; Vlazny and Frenkel, 1981; Ward and Roizman, 1994). A third origin of viral DNA replication, ori_L, has been mapped within the L component (Weller *et al*, 1985). Bracketing the *b* and *c* repeat elements is the *a* inverted repeat sequence (250–500 bp), contained in one to several copies at the L component terminus and at the L-S junction, but present as a single copy at the S component terminus (Locker and Frenkel, 1979; Wagner and Summers, 1978). The *a* sequence has been previously shown to promote standard homologous recombinational inversion of L and S components during viral DNA replication (Mocarski and Roizman, 1981; 1982a; Weber *et al*, 1988), and to contain a *cis*-acting site for cleavage of concatemeric DNA and packaging of unit-length genomes into capsids (Varmuza and Smiley, 1985; Vlazny *et al*, 1982).

The HSV-1 genome encodes at least 81 genes (Lagunoff *et al.*, 1996, Ward and Roizman, 1994). Forty-three of these are nonessential for productive viral infection in cell culture (Ward and Roizman, 1994). Nonessential or accessory genes map throughout the HSV-1 genome, in both U_L and U_S, and in the inverted repeats flanking U_L. The clustering of nonessential genes in U_S is particularly striking in that 13 of 14 U_S genes are nonessential. Moreover, only three essential genes encoding ICP27 (U_L54), ICP4, and glycoprotein D (U_S6), map to the right-hand 40 kb of the 152 kb viral genome. Replacement of this entire region of nonessential viral genes by foreign DNA would allow HSV-1 to be used as a carrier for complex genomic DNA sequences containing both promoter and noncoding introns.

Nonessential HSV-1 U_S genes encode diverse accessory functions. ICP22 (U_S1), an immediate-early (IE) regulatory protein, is involved in phosphorylation of the large subunit of host RNA polymerase II (Rice *et al*, 1995), and is itself phosphorylated by viral protein kinases encoded by U_L13 and U_S3 (Purves and Roizman, 1992). A novel HSV-1 IE gene, $U_S1.5$, whose open reading frame is contained within the carboxy-terminal 273 amino acids of ICP22, codes for the functions associated with optimal expression of ICPO and late genes, and replication in human primary or rodent cells (Carter and Roizman, 1996). The U_S3-encoded cytoplasmic kinase phosphorylates ICP22 and the non-glycosylated, membrane-associated virion protein encoded by U_L34 (Purves *et al*, 1991). gG, the U_S4 gene product, is a nonessential envelope glycoprotein involved in virion entry, egress, and cell-to-cell spread (Balan *et al*, 1994). Glycoproteins I(U_S7) and E (U_S8) facilitate neuron-to-neuron viral spread *in vivo* and across junctions of fibroblasts and neurons in culture (Dingwell *et al*, 1994, 1995). A complex composed of gI bind the Fc domain of immunoglobulin G (Johnson *et al*, 1988), potentially reducing immune cytolysis of virus-infected cells (Adler *et al*, 1978; Lehner *et al*, 1975). The U_S9 and U_S10 gene products are tegument proteins (Frame *et al*, 1986), and U_S11 encodes an RNA binding protein (Roller and Roizman, 1990, 1992). ICP47 (U_S12), a cytoplasmic IE protein, contributes to the escape of HSV from immune surveillance by down-regulating antigen presentation to major histocompatibility complex class I-restricted $CD8^+$ T cells (York *et al*, 1994) through inhibition of antigen peptide translocation from the cytosolic proteosomes into the lumen of the endoplasmic reticulum (Fruh *et al*, 1995; Hill *et al*, 1995). No functions have yet been ascribed to the gene products encoded by U_S2, U_S5, and $U_S8.5$.

HSV-1 mutants in which single or multiple nonessential U_S genes have been inactivated are viable in the absence of complementation in cell culture. These include single gene mutants in U_S2 (Weber *et al*, 1987), U_S3 (Longnecker and Roizman, 1987; Weber *et al*, 1987), U_S4 (Weber *et al*, 1987), U_S5 (Weber *et al*, 1987), U_S7 (Johnson *et al*, 1988; Longnecker *et al*, 1987), U_S8 (Dingwell *et al*, 1994; Johnson *et al*, 1988; Longnecker *et al*, 1987; Neidhardt *et al*, 1987), and U_S12 (Mavromara-Nazos *et al*, 1986; Umene, 1986); double gene mutants in U_S1 and $U_S1.5$ (Post and Roizman, 1981), U_S2 and U_S3 (Longnecker and Roizman, 1987), U_S3 and U_S4 (Longnecker and Roizman, 1987), U_S8 and $U_S8.5$ (Meignier *et al*, 1988), and U_S11 and U_S12 (Meignier *et al*, 1988); and a mulitple gene mutant from which U_S8, 9, 10, 11 and 12, as well as one copy of each of the three diploid genes mapping in inverted repeat sequences (ICPO, γ34.5, and ICP4) have been deleted (Longnecker and Roizman, 1986). Other HSV-1 mutants containing a deletion of one copy of the three genes in the inverted repeat sequences have also been isolated (Jenkins *et al*, 1985; Poffenberger and Roizman, 1985; Poffenberger *et al*, 1983), suggesting that single copies of these genes are sufficient for replication of HSV-1 in cell culture. In addition, both copies of ori_S have been shown to be dispensable for replication of the virus in culture (Igarashi *et al*, 1993).

Although nonessential for viral growth in cell culture, deletion of one or more of these genes can reduce the efficiency of virus replication and alter viral pathogenesis or host range *in vivo*. For instance, virus mutants lacking the carboxy-terminal domain of ICP22, starting with amino acid 200, exhibit a reduced capacity to replicate in primary human cells or in cells of rodent origin, and show a reduced expression of ICPO and a subset of viral late genes (Carter and Roizman, 1996; Sears *et al*, 1985). Similarly, mutants in either gE or gI do not spread as efficiently as wt virus in cell culture, giving rise to phenotypically smaller plaques on Vero cells (Dingwell *et al*, 1994). In addition, these accessory genes may play an important role in the

complex life cycle of the virus in the infected host, affecting viral growth and spread in non-dividing cells such as neurons (Dingwell *et al*, 1995), and thereby contributing to neurovirulence. Single gene mutants in U_S1 (Meignier *et al*, 1988), U_S3 (Fink *et al*, 1992; Meignier *et al*, 1988), U_S4 (Weber *et al*, 1987), and a double mutant in both U_S11 and U_S12 (Meignier *et al*, 1988) have all been shown to be significantly attenuated for replication in the rodent central nervous system (CNS).

The sole essential gene within U_S is U_S6 which encodes glycoprotein D (gD), a component of the virion envelope required for post-attachment entry of the virus into cells (Ligas and Johnson, 1988). This gene is centered among a group of five glycoprotein-encoding genes, of which four (U_S4, 5, 7, and 8) code for accessory glycoproteins (gG, gJ, gI, gE, respectively). The host-range of HSV-1 is defined in part by the ability of the virus to adsorb to and penetrate cells, and these functions are carried out by viral envelope glycoproteins. Deletion of nonessential envelope glycoprotein genes and other accessory functions from the HSV-1 genome may be expected to alter the mutant virus host range and reduce viral pathogenesis for the nervous system. Moreover, construction of HSV-1 mutants containing deletions of nonessential U_S genes may provide molecular tools for analysis of the role of these genes in the viral life cycle and pathogenesis in the nervous system. It may eventually become possible to replace viral attachment and penetration functions with novel receptor-binding proteins or foreign glycoproteins, thus permitting targeting of the virus to particular cell types. Additionally, such deletions would allow the accommodation of at least 12 kb of foreign DNA sequences for construction of recombinant HSV vectors for gene therapy applications.

In order to determine the effect of deleting these accessory genes on viral pathogenesis *in vivo*, and to provide a viral genomic backbone devoid of nonessential U_S functions for construction of recombinant HSV-1 vectors to be used in gene transfer applications, we designed experiments to delete the entire group of 13 nonessential genes from the S component of the viral genome in a two-step fashion. In the first step, reported in this manuscript, we describe the deletion of a contiguous 12 kb segment of DNA located between the L-S junction *a* sequence and the U_S6 gene of the HSV-1 genome. The genome of the resulting recombinant virus, designated d311, carries a deletion spanning all but 84 nucleotides of the S component inverted repeat sequence *c′* along with nonessential genes U_S1 through U_S5. Thus, d311 contains a single copy of the essential ICP4 gene and a single ori_S present in the S component inverted repeat sequence *c*. Although the d311 genome is deleted for the *c′* inverted repeat sequence, its S component still undergoes genome isomerization events, suggesting that the *a* sequence alone is sufficient for the inversion process. We present data showing that the deletion substantially impairs productive viral infection in primate fibroblasts and neuronal cells, almost totally abolishes viral replication in neuronal cells of rodent origin, and highly attenuates replication and spread of the virus in the rat central nervous system.

Results

Homologous recombination of a plasmid containing U_S6 and the a *sequence into HSV-1 DNA*

We constructed KLP, a recombinant HSV-1 (strain KOS), by marker rescue of the ICP4 deletion mutant, d120 (DeLuca *et al*, 1985) with a recombinant plasmid, pICP4-lox-pac (Rasty and Glorioso, unpublished data). The KLP genome contains a unique *Pac*I restriction site and a bacteriophage P1 *lox*P recombination site (Sauer *et al*, 1987) inserted into the *Bam*HI site located 27 bp downstream of the ICP4 transcriptional initiation site and within the untranslated 5′-leader sequence of each copy of the ICP4 gene (m.u. 0.863 on the HSV-1 genome; Figure 1A) (Rasty and Glorioso, unpublished data). The insertions of *Pac*I and *lox*P sites flanking U_S at a benign location within the KLP genome were intended to allow future modification of KLP DNA by restriction digestion or Cre-*lox*P recombination of *lox*P plasmids and viral DNA (Rasty *et al*, 1995). We also generated a multigenic shuttle plasmid, pSR581 (Figure 1B), which contained the essential U_S6 gene, a reporter *lac*Z expression cassette, and the *a* sequence. We reasoned that homologous recombination between DNA sequences shared by the U_S6 gene in circular pSR581 plasmid DNA and the U_S6 gene in the U_S segment of the KLP genome would target the entire plasmid into the U_S6 locus in U_S (Figure 1C). Separate portions of the homologous DNA sequence on the plasmid (i.e. U_S6) would serve as flanks for the intervening sequence (i.e. the remainder of the plasmid), thus duplicating U_S6 in the recombinational product (Figure 1C). Because of the orientation of the U_S6 gene in pSR581 relative to the *a* sequence and the *lac*Z gene in the plasmid, such an homologous recombination event would insert *lac*Z and the plasmid-derived *a* sequence into U_S such that in an I_S arrangement of the recombinant viral genome the novel *a* sequence would be a direct repeat of the 3′-terminal *a* sequence in KLP viral DNA (Figure 1C). Earlier studies on isomerization of the HSV-1 genome had indicated that insertion of an additional copy of the *a* sequence into the nonessential thymidine kinase (U_L23) gene would cause rearrangements of the viral genome including deletion of sequences located between two directly repeated *a* sequences (Mocarski *et al*, 1980); Smiley *et al*, 1981). We theorized that introduction of an additional copy of the *a* sequence into the U_S segment of KLP through homologous recombination

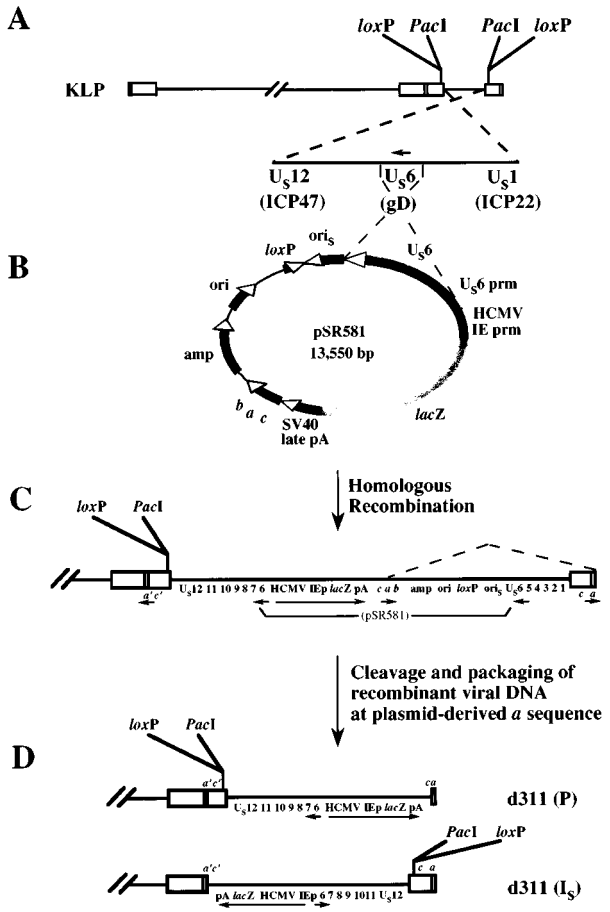


Figure 1 Strategy for generation of d311. (**A**) Schematic diagram of the KLP genome showing the location of the *Pac*/*lox*P sites upstream of the ICP4 structural gene in both copies of the inverted repeats flanking U_S. The S component of the viral genome in an I_S isomer of viral DNA is enlarged below. (**B**) Schematic diagram of pSR581, showing the relative orientation and location of *lox*P, ori$_S$, U_S6, HCMV IEp-*lac*Z-SV40 poly(A), and *c*-*a*-*b* elements on the 13.55 kb shuttle plasmid. Inclusion of the SV40 poly(A) signal downstream of the U_S6 coding sequences was necessary since the U_S6 transcription unit in the HSV-1 genome is co-terminal with that of U_S5 and U_S7, thus its natural transcriptional termination signals are located downstream of the U_S7 gene. (**C**) Intermediate recombinational product, generated through homologous recombination between U_S6 sequences in KLP viral DNA and those in plasmid pSR581. Either a deletion of DNA sequences between the two directly repeated copies of the *a* sequence (hashed lines) by homologous recombination, or a bona-fide genomic cleavage event at the pSR581-derived *a* sequence would delete a 12 kb viral DNA sequence located between the repeated *a* sequence, giving rise to the d311 mutant (**D**). HCMV IEp, human cytomegalovirus immediate-early promoter; P, prototype; I_S, inverted short.

with the U_S6 gene of pSR581 would similarly allow for deletion of sequences by either homologous recombination between the two directly repeated copies of the *a* sequence in the intermediate recombinational product (Figure 1C), or occurrence of genome cleavage events at the plasmid-derived *a* sequence prior to packaging of viral DNA. Either mechanism would thus remove the entire plasmid vector sequence, the second copy of U_S6, the original viral genes for U_S5, U_S4, U_S3, U_S2, U_S1.5, U_S1, and the terminal repeat of the KLP genome. This would delete a 12 kb viral DNA sequence located between the L-S Junction *a* sequence and the U_S6 gene in a P isomer of KLP DNA, precisely spanning the S component inverted repeat sequence *c*′ and the nonessential genes U_S1 through U_S5 (Figure 1D). The resulting mutant would contain a single copy of the ICP4 gene, a single ori$_S$, the *lac*Z cassette from pSR581, and genes encoding U_S6 through U_S12. Since mutants of HSV-1 bearing a single copy of the ICP4 gene had already been shown to be viable in cell culture without the need for complementation (Jenkins *et al*, 1985; Poffenberger and Roizman, 1985; Poffenberger *et al*, 1983), we expected the KLP-derived U_S mutant to plaque on Vero cells.

pSR581 plasmid and infectious KLP viral DNA were co-transfected into Vero cells, and *lac*Z-expressing progeny recombinant viruses obtained from the co-transfection were plaque-purified. Detailed analysis of genomic DNA from all such *lac*Z-expressing recombinants designated d311, indicated that their genomes differed from the parental KLP genome as predicted above. In summary, (i) the deletion in d311 DNA was a 12 kb segment of the viral genome located between the L-S Junction *a* sequence and the U_S6 gene. The deletion spanned all but 84 nucleotides of the S component inverted repeat sequence *c*′ along with the genes encoding U_S1 through U_S5. (ii) In place of the deleted viral DNA sequences, d311 had acquired the 5.5 kb HCMV IEp-*lac*Z-SV40 poly(A) cassette from the plasmid pSR581. (iii) d311 contained a single copy of the essential ICP4 gene and a single ori$_S$. These conclusions are supported by the folowing data.

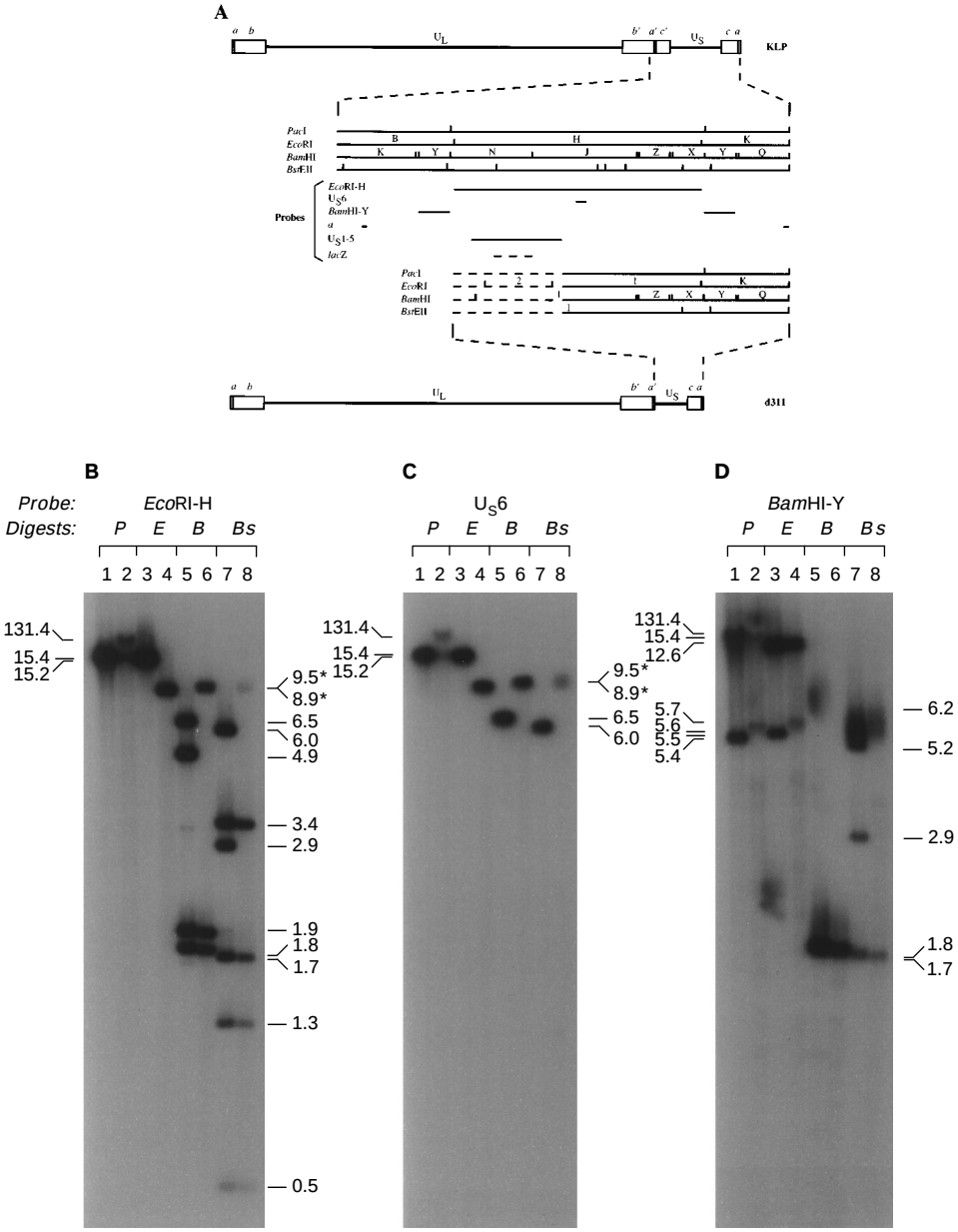
Southern hybridization analysis of the d311 genome

To analyse the structure of d311 DNA compared to that of the parental KLP genome, KLP and d311 viral DNA were digested with *Pac*I, *Eco*RI, *Bam*HI, and *Bst*EII, and Southern blotted with a panel of ^{32}P-labelled DNA probes spanning U_S and the joint sequences of the HSV-1 (KOS) genome (Figure 2A). To provide an overall picture of the rearrangements or deletions in the U_S-specific *Pac*I, *Eco*RI, *Bam*HI, and *Bst*EII DNA fragments of d311 compared to those derived from KLP, the *Eco*RI-H fragment containing the entire HSV-1 U_S DNA sequence (Goldin *et al*, 1981) was used as a probe in the Southern analysis. This was followed by hybridization to a series of smaller probes originating within U_S, its inverted repeats, or the *lac*Z transgene in the d311 genome.

In *Pac*I digests of d311 DNA two separate viral DNA fragments, 131.4 and 15.4 kb in size, hybridized to either of the *Eco*RI-H, U_S6, and *lac*Z probes,

all containing unique sequences (Figure 2B, C, G, lanes 2), while only the 15.4 kb *Pac*I fragment of KLP DNA was detected by the *Eco*RI-H and U_S6 probes (Figure 2B and C, lanes 1). This suggested that d311 had retained only one of the two *Pac*I sites present in the KLP genome and that the two *Pac*I fragments of d311 DNA appeared to originate from two viral DNA isomers differing in the orientation of their S components. Both *Bam*HI-Y and *a* sequence probes, containing sequences from the inverted repeats flanking U_S, hybridized to 131.4 and 5.6 kb *Pac*I fragments of d311, while they detected 131.4 and 5.4 kb *Pac*I fragments in KLP DNA (Figure 2D and E, lanes 1 and 2). This indicated that the 3′-terminus of the d311 S component had increased in size by approximately 0.2 kb, possibly as a result of incorporation of the pSR581-borne *a* sequence which was originally derived from HSV-1 (F), and is approximately 0.2 kb larger than that present in the KOS-derived KLP genome (Mocarski and Roizman, 1981; Smiley *et al*, 1990). A probe containing sequences from U_S1-U_S5 did not hybridize to any of the restriction fragments from d311 DNA (Figure 2F, lanes 2, 4, 6, and 8), while it detected the expected *Pac*I, *Eco*RI, *Bam*HI, and *Bst*EII fragments of KLP DNA (Figure 2F, lanes 1, 3, 5, and 7). This indicated that the 4.5 kb U_S1-U_S5 DNA sequence contained within this probe was deleted from the d311 genome.

To further examine the extent of the rearrangements in d311 DNA, *Eco*RI digests of d311 and KLP DNA were compared. An 8.9 kb d311 *Eco*RI



fragment was detected by the *Eco*RI-H and U_S6 probes (Figure 2A, d311 *Eco*RI fragment-1; Figure 2B and C, lanes 4), while the same two probes had hybridized to the 15.2 kb *Eco*RI fragment of KLP DNA (Figure 2B and C, lanes 3). This indicated that either a deletion event had removed 6.3 kb from the KLP *Eco*RI-H fragment during generation of d311, or that a rearrangement event had introduced a novel *Eco*RI site 8.9 kb from either terminus of the KLP *Eco*RI-H in generating d311. Both *Bam*HI-Y and *a* sequence probes detected a terminal *Eco*RI-K fragment in d311 DNA that was approximately 0.2 kb larger than that of KLP, migrating at approximately 5.6 kb (Figure 2D and E, lanes 3 and 4), confirming the previous observation related to the sequence rearrangement at the 3′-terminus of the d311 genome. In addition, the *a* probe also hybridized to a novel 1.1 kb *Eco*RI fragment of d311 DNA (Figure 2E, lane 4), suggesting that a newly acquired *Eco*RI site in d311 DNA had released a fragment approximately 1.1 kb in size, which contained all or a part of the *a* sequence, and originated from either the joint or the termini of the d311 genome. The *lac*Z probe hybridized to a 4.1 kb d311 *Eco*RI fragment (Figure 2A, d311 *Eco*RI fragment-2; Figure 2G, lane 4), also detected in *Eco*RI digests of plasmid pSR581 (data not shown).

In *Bam*HI digests of d311 DNA, the *Eco*RI-H probe did not detect the 6.5 kb *Bam*HI-J and the 4.9 kb *Bam*HI-N fragments. Instead, it hybridized to a novel 9.5 kb *Bam*HI fragment (Figure 2A, d311 *Bam*HI fragment-1; Figure 2B, lane 6). Thus, sequences contained in all or a part of wt *Bam*HI-J and -N fragments were missing from d311 DNA. The same 9.5 kb d311 *Bam*HI fragment was also detected by the U_S6 (Figure 2C, lane 6) and *lac*Z probes (Figure 2G, lane 6), indicating that this DNA fragment contained sequences from U_S6 as well as those from *lac*Z. Several observations were made from the differential hybridization patterns of the *a* probe to *Bam*HI digests of KLP and d311 DNA's. The

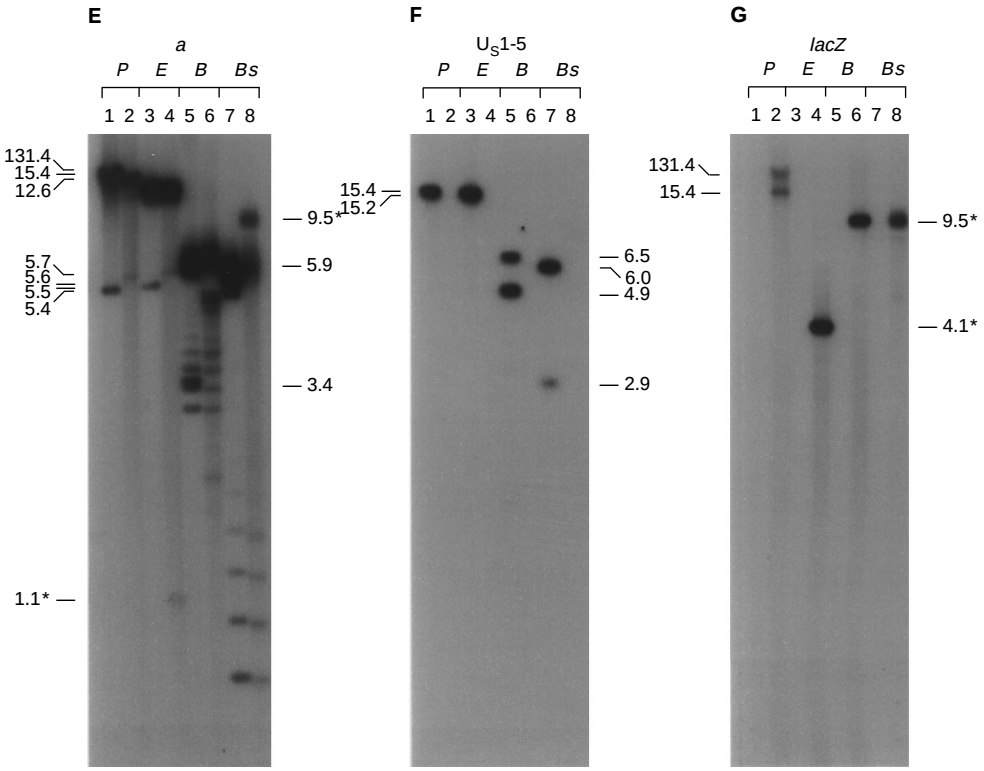


Figure 2 (**A**) Schematic diagram of the HSV-1 genome and the location of the probes used for comparitive Southern blot analysis of KLP and d311 viral DNA. Top, KLP; bottom, d311. The enlarged *Pac*I, *Eco*RI, *Bam*HI, and *Bst*EII restriction maps within the U_S component and its inverted repeats in viral DNA are pictured above (for KLP) and below (for d311) the genomic location of the probes used for Southern blot analyses. The dashed lines in restriction maps of d311 DNA refer to sequences absent from KLP parental DNA. The probes included the 15.2 kb *Eco*RI-H fragment, containing the entire U_S component and 1.1 kb of the inverted repeats flanking U_S; a 0.47 kb *Nae*I fragment internal to U_S6 coding sequences; the 1.8 kb *Bam*HI-Y fragment of ICP4 coding sequences; a 318 bp *Bam*HI fragment from plasmid pA23 (69) encompassing the entire HSV-1 KOS *a* sequence; a 4.5 kb *Hind*III-*Sac*I subfragment of *Hind*III-N encompassing sequences from U_S1-U_S5; and a 2.5 kb fragment from the *lac*Z gene in plasmid pSR581. (**B**–**G**) Southern blots of KLP (lanes 1, 3, 5, 7) or d311 (lanes 2, 4, 6, 8) viral DNA digested with *Pac*I (*P*; lanes 1 and 2), *Eco*RI (*E*; lanes 3 and 4), *Bam*HI (*B*; lanes 5 and 6) and *Bst*EII (*Bs*; lanes 7 and 8), and hybridized to *Eco*RI-H (B), U_S6 (**C**), *Bam*HI-Y (**D**), a (**E**), U_S1-U_S5 (**F**), and *lac*Z (**G**) probes. Numbers on either the left or the right side of each blot refer to the sizes of viral DNA fragments hybridizing to each probe in kb. *: novel fragments detected only in d311 DNA.

probe hybridized to the joint-spanning *Bam*HI-K fragments, ranging in size from approximately 5.7 to 7.0 kb due to the presence of multiple *a* sequences at the joint (Wagner and Summers, 1978), in *Bam*HI digests of DNA from either virus (Figure 2E, lanes 5 and 6). The 2.9 kb *Bam*HI-S fragment, originating from the 5′-terminus of the L component *ab* repeat, and its ladder of bands containing multiple copies of *a* (Locker and Frenkel, 1979; Wagner and Summers, 1978), were detected in DNA from either virus (Figure 2E, lanes 5 and 6). However, the 3.4 kb *Bam*HI-Q fragment, originating from the 3′-terminus of the S component *ca* repeat, was detected only in DNA from KLP but not from d311 (Figure 2E, lanes 5 and 6). This could be explained by the 0.2 kb increase in size at the 3′-terminus of the d311 genome as described above, which would shift the size of the *Bam*HI-Q fragment of d311 to 3.6 kb, possibly causing it to co-migrate with one of the DNA bands in the ladder of d311 *Bam*HI-S fragments during electrophoresis.

The d311 genome was found to be deleted of two wt *Bst*EII fragments: a joint-derived 2.9 kb fragment, containing 5′-sequences of the ICP4 gene, ori_S, and the entire U_S1 coding sequence; and a 6.0 kb *Bst*EII fragment encompassing U_S2-U_S7 (Figure 2B, lanes 7 and 8). The 2.9 kb *Bst*EII fragment was detected in DNA from KLP but not from d311 by either of the *Eco*RI-H, *Bam*HI-Y, and U_S1-5 probes (Figure 2B, D, F, lanes 7 and 8). The 6.0 kb *Bst*EII fragment was similarly detected only in KLP DNA by either of the *Eco*RI-H, U_S6, and U_S1-5 probes (Figure 2B, C, F, lanes 7 and 8). Instead of these two missing *Bst*EII fragments, a number of closely migrating, novel d311-derived *Bst*EII fragments, ca. 9.5 kb in size (Figure 2A, d311 *Bst*EII fragments-1), hybridized to either of the *Eco*RI-H, U_S6, *a* or *lac*Z probes (Figure 2B, C, E, G, lanes 8). Additionally, it was apparent that the *Bam*HI-Y probe had hybridized with greater intensity to the 5.2 kb U_S inverted repeat *c*-derived *Bst*EII fragment in DNA from KLP than that from d311 (Figure 2D, lanes 7 and 8). Similarly, the probe hybridized more strongly to the joint-spanning *Bst*EII fragments of KLP than those of d311, ranging in size from approximately 5.4 to 6.2 kb (Figure 2D, lanes 7 and 8). This differential intensity of the hybridization signals indicated that sequences homologous to *Bam*HI-Y were present in the L-S junction and S-terminal *Bst*EII fragments of d311 at a molar ratio less than that present in KLP DNA. In contrast, the 1.7 kb *Bst*EII fragment, containing a 268 bp sequence, which overlaps U_S11 and U_S12, followed by a 1.45 kb sequence from the *c* inverted repeat containing ori_S and the 5′-untranslated leader sequence of the ICP4 gene, was common to both viruses and was present at the same molar ratio in either viral genome (Figure 2D, lanes 7 and 8).

Because one to multiple copies of the *a* sequence are present at the 5′-terminus of the HSV-1 L component (Locker and Frenkel, 1979; Wagner and Summers, 1978), digestion of viral DNA within the U_L inverted repeat sequence *b* would generate a ladder of DNA fragments containing one to multiple copies of the *a* sequence. Due to the presence of a *Bst*EII restriction site at position 596 of the viral genome (McGeoch *et al*, 1991), such a ladder was detected following hybridization of *Bst*EII digests of either KLP or d311 viral DNA to the *a* probe (Figure 2E, lanes 7 and 8). The slight difference in the electrophoretic mobility of the ladder fragments, derived from KLP and d311 was not unexpected, though, since variations in *a* sequence size occur naturally depending on the number of reiterations of internal repeats (Mocarski and Roizman, 1981). However, the 5′-terminus of the d311 genome's L component had apparently not increased in size by approximately 0.2 kb as had its S component 3′-terminus. This suggested that rearrangements at the 3′-end of the d311 genome, possibly occurring as a result of incorporation of the pSR581-derived, strain (F) *a* sequence, had not duplicated themselves in the 5′-end of d311 genomic DNA by recombination.

Taken together, the results of the Southern blot analysis of d311 DNA confirmed the genomic structure depicted in Figure 1D, consisting of the deletion of a 12 kb viral DNA sequence located between the L-S Junction *a* sequence and the U_S6 gene, precisely spanning the inverted repeat sequence *c*′ and the nonessential genes U_S1 through U_S5. In place of the deletion, the d311 genome has acquired a 5.5 kb *lac*Z expression cassette from pSR581, resulting in a viral genome size of approximately 144 kb. It is worth noting that the homologous recombination event shown in Figure 1 predicts that all bacterial plasmid vector sequences would be removed from d311 DNA due to cleavage and packaging of the recombinant genome at the plasmid-derived *a* sequence. This prediction was confirmed by the finding that d311 DNA did not hybridize to a full-length pBSIISK$^-$ plasmid probe (data not shown).

Sequencing of DNA across the junctions of deletion in the d311 genome

To precisely determine the boundaries of HSV-1 nucleotide sequences deleted from the d311 genome, two *Dra*I subfragments of d311 DNA, approximately 1.2 and 4.5 kb in size, were subcloned from viral DNA into plasmid pBSIISK$^-$ and subjected to DNA sequencing. The 1.2 kb fragment, absent in KLP DNA (data not shown), was generated due to *Dra*I restriction at a *Dra*I site located 1.2 kb upstream of the 3′-terminus of d311 DNA, derived from pSR581-borne SV40 poly(A) sequences. We reasoned that sequencing of this DNA fragment using a primer complementary to SV40 poly(A) sequences would precisely define the 5′-terminus of the deletion in an I_S isomer of d311 DNA (Figure 1D). As shown in Figure 3A, the nucleotide

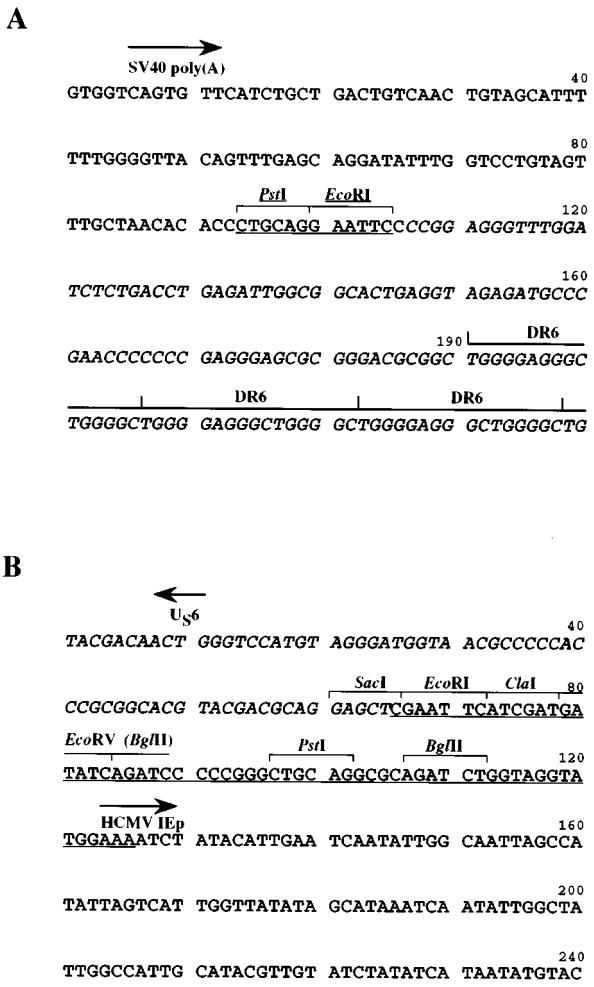


Figure 3 DNA sequences across the 5′- and 3′-junctions of the deletion in d311 DNA. (**A**) Sequences starting upstream of the 3′-terminus of the SV40 poly(A) site (plain-text letters), followed by pSR581-derived *Pst*I and *Eco*RI sites (underlined letters), and extending into 84 nucleotides of the *c*′ repeat followed by the DR6 repeats of the HSV-1 (F) *a*′ sequence in d311 DNA (italicized letters). (**B**) Sequences starting within the 5′-promoter-regulatory sequences of U_S6, derived from the 2.9 kb *Sac*I fragment of *Bam*HI-J in pSR581 (italicized letters), followed by pSR581-derived polylinker sequences (underlined letters), and extending into the 5′-promoter-regultory sequences of HCMV IEp (plain-text letters). (*Bgl*II) refers to the original *Bgl*II site at the 5′-terminus of the U_S6-poly(A)-containing fragment in pgD-PA which was destroyed after ligation to the unique *Bam*HI site in the pSR521 polylinker (see Materials and methods). Arrows denote the 5′ to 3′ direction of the various DNA elements in viral DNA.

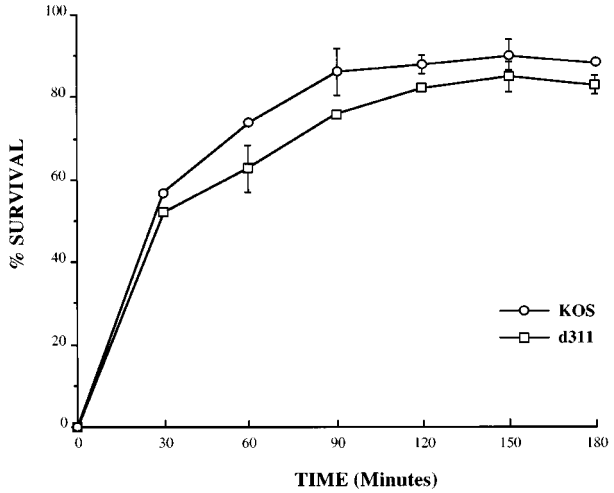


Figure 4 Assay for kinetics of virus penetration into Vero cells. 400 PFU of either KLP or d311 was used to adsorb virus onto confluent monolayers of Vero cells. Following 2 h of virus adsorption at 4°C, monolayers were washed with PBS-$MgCl_2$, overlaid with 0.5 ml of DMEM-10% FBS, and shifted to 37°C to allow virus penetration. At 30 min intervals following the temperature shift, media was aspirated and cells were treated for 1 min with 1 ml of citrate buffer (pH 3.0) to inactivate unpenetrated viruses, washed twice with PBS-$MgCl_2$ and overlaid with growth medium containing 1% methylcellulose. % Survival: percent of viral PFU surviving citrate treatment at a given time, where 100% is the number of plaques formed on a monolayer treated with PBS-$MgCl_2$ only.

sequence of this fragment indicated the presence of SV40 poly(A) sequences terminating at a *Pst*I site, immediately followed by an *Eco*RI site. As expected, both of these restriction sites were derived from pSR581, as the *Pst*I site was the 3′-terminus of SV40 poly(A) sequences on the plasmid, and the joint-spanning, (F)-derived *c-a-b* fragment (Mocarski and Roizman, 1982a) had been subcloned immediately downstream of this site in the pSR581 polylinker as an *Eco*RI fragment. The sequence following the *Eco*RI site included 84 nucleotides belonging to the *c* element from the pSR581-derived *c-a-b* fragment, followed by the DR6 repeats of the (F) *a* sequence (Mocarski and Roizman, 1981). The results indicated that in this segment of the d311 genome all but 84 nucleotides of the *c*′ inverted repeat sequence were deleted, and that SV40 poly(A) sequences were juxtaposed to these 84 nucleotides followed by the *a* sequence. This confirmed previous Southern blot data which had indicated the presence of half-molar viral DNA fragments spanning the *c*′ inverted repeat sequence and the deletion of the joint copy of ori_S from d311 DNA (Figure 2).

The 4.5 kb *Dra*I subfragment of d311 DNA, also absent in KLP DNA (data not shown), was generated due to *Dra*I restriction of d311 DNA at a *Dra*I site located upstream of the U_S6 promoter [position 138,311 on the HSV-1 genome (McGeoch *et al*, 1985)] and at a *Dra*I site located 4.5 kb upstream near the 3′-terminus of *lac*Z sequences. Similar to sequencing of the 1.2 kb fragment, we reasoned that sequencing of the 4.5 kb *Dra*I fragment using a primer complementary to U_S6 promoter sequences would precisely define the nucleotide sequence of the segment of d311 genome where U_S6 promoter sequences would be juxtaposed next to those from HCMV IEp, thus defining the 3′-terminus of the

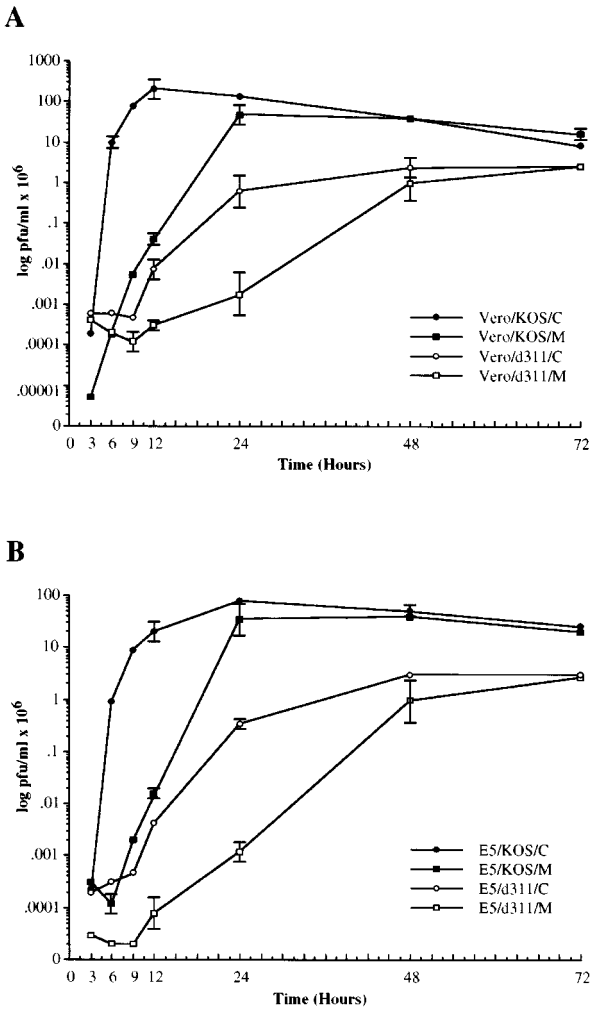


Figure 5 Single-step growth curves of KOS and d311 in Vero (**A**) or EB (**B**) cells. C, cell-associated virus. M, virus released into the media supernatant.

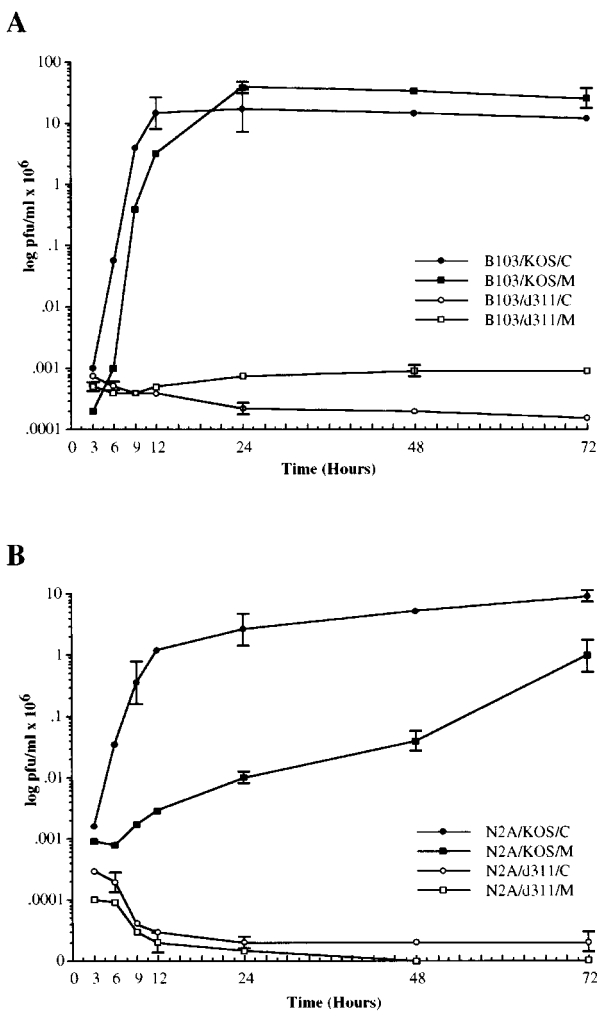


Figure 6 Single-step growth curves of KOS and d311 in rat B103 (**A**) or mouse Neuro-2A (N2A) (**B**) neuroblastoma cells. C, cell-associated virus. M, virus released into the media supernatant.

deletion. As shown in Figure 3B, the nucleotide sequence of this fragment indicated the presence of HSV-1 sequences upstream of the U_S6 promoter and within the 5′-end of the 2.9 kb *Sac*I subfragment of *Bam*HI-J (originally subcloned into pSR581), followed by polylinker sequences from plasmid pSR581 and sequences corresponding to the 5′-terminus of the HCMV IEp. This indicated that the 3′ boundary of the deletion in d311 DNA was exactly as that predicted by Southern blot analysis (Figure 2), where sequences corresponding to U_S1-5 were deleted from the viral genome, and the HCMV IEp sequences were immediately followed by the U_S6 promoter sequence, precisely as they were engineered into pSR581.

Replication kinetics of d311 in cell culture

During the isolation of d311 on Vero cells we had observed a dramatic decrease in the size of plaques produced by the recombinant compared to those arising from either the parental wt KLP or KOS viruses (data not shown). This suggested that certain aspects of d311 replication were inhibited in Vero cells. Either the mutant was less efficient in its attachment and penetration into cells, progeny d311 virions were produced more slowly, or d311 could not spread and infect neighboring cells as efficiently as wt virus.

To examine the kinetics of virus entry into Vero cells, we used an assay designed to inactivate extracellular virus by exposure to low pH (Highlander *et al*, 1987; Huang and Wagner, 1964). As shown in Figure 4, there were no significant differences in the kinetics of entry of d311 into Vero cells compared to wt HSV-1 (KOS), suggesting that the small-plaque phenotype of d311 was not due to impairment in attachment and penetration functions.

To determine whether progeny d311 viruses were produced more slowly or less efficiently in Vero cells, we compared d311 to wt KOS following a single round of viral replication after infection at a multiplicity of infection of 5 PFU per cell. The titer of infectious virus present in cells and culture media collected separately at various times post-infection was determined in three separate experiments. In a single round of viral replication on Vero cells the yield of infectious d311 virus was over one order of magnitude lower than that of wt KOS, either in cells or released into the culture media (Figure 5A). Since similar yields of virus were detected that were either cell-associated or were released into media, we concluded that the growth defect of the mutant on Vero cells was probably not due to any functions associated with viral egress. Because the d311 genome was shown to contain a single copy of the ICP4 gene, we performed similar studies in Vero-derived, ICP4-complementing E5 cells (DeLuca and Schaffer, 1987). Similar results were observed in E5 cells (Figure 5B), suggesting that the missing copy of the ICP4 gene in d311 was not responsible for the growth defect on Vero cells, and that the mutant's impairment in growth was most probably a result of the missing copy of ori_S, the deleted genes U_S1 through U_S5 or both.

Because a natural site of HSV infection *in vivo* is the nervous system, we also examined the growth properties of the d311 mutant in neuronal cell lines. Replication of d311 was almost totally inhibited in rat B103 or mouse Neuro-2A neuroblastoma cell lines (Figure 6A and B). In Neuro-2A cells (Figure 6B), this inhibition of d311 replication was more strongly pronounced than that in B103 cells (Figure 6A).

HSV-1 mutants in $U_S1.5$ have previously been shown to be restricted for replication in cells of rodent origin (Carter and Roizman, 1996; Sears *et al*, 1985), and this gene is deleted from the d311 genome. To determine whether inhibition of d311 replication in B103 or Neuro-2A cells represented a rodent host-range effect or a neuronal cell-specific restriction, we examined its growth properties in human SK-N-SH neuroblastoma cells. Titers of d311 obtained following a single round of replication in SK-N-SH cells were over three orders of magnitude lower than those reached following wt KOS infection (data not shown). However, replication of the mutant was not totally restricted in this human cell line as it had been in neuronal cell lines of rodent origin, suggesting that the growth restriction of d311 in B103 or Neuro-2A cell lines was due to a combination of neuronal cell restriction and host-range effect.

Replication and spread of the d311 mutant in the rat brain

The ability of the d311 mutant to replicate in neurons and to spread in brain after focal intracranial inoculation was compared to wt KOS. 1.5×10^6 pfu of either KOS or d311 was injected stereotactically into the hippocampus of Sprague Dawley rats. At 2 or 7 days post-inoculation (dpi) the rats were sacrificed, the expression of β-galactosidase from the *lac*Z transgene in the d311 recombinant was assayed by X-gal staining and the presence of virus determined by immunocytochemical staining using a polyclonal rabbit anti-HSV antibody.

At 2 dpi, expression of β-galactosidase by d311 could be detected in a large number of neurons of the dentate gyrus by X-gal staining (Figure 7A and B). This indicated that the deletion in d311 had not compromised its ability to infect neurons *in vivo*. By 7 dpi, no β-galactosidase activity could be seen in cells of the injected hippocampus, nor in other regions of brain (data not shown). The *lac*Z transgene is not present in wt HSV-1 (KOS), so no blue cells were seen following infection with that virus (data not shown), but viral spread could be followed by immunocytochemistry for HSV antigen (Figure 8A). By 7 dpi, wt KOS infection had destroyed the dentate gyrus of infected hippocam-

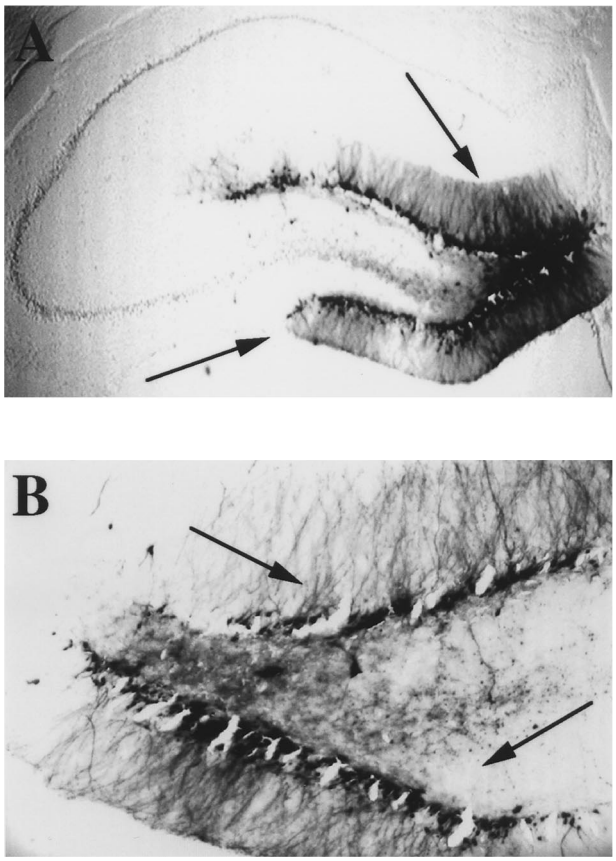


Figure 7 Hippocampal expression of β-galactosidase from the *lac*Z transgene in the d311 recombinant. X-gal-positive staining (arrows) predominantly of granule cells in the dentate gyrus of hippocampus 2 days after stereotactic inoculation of d311. (**A**) $10\times$ magnification. (**B**) $20\times$ magnification of a different section of the injected hippocampus from the same brain.

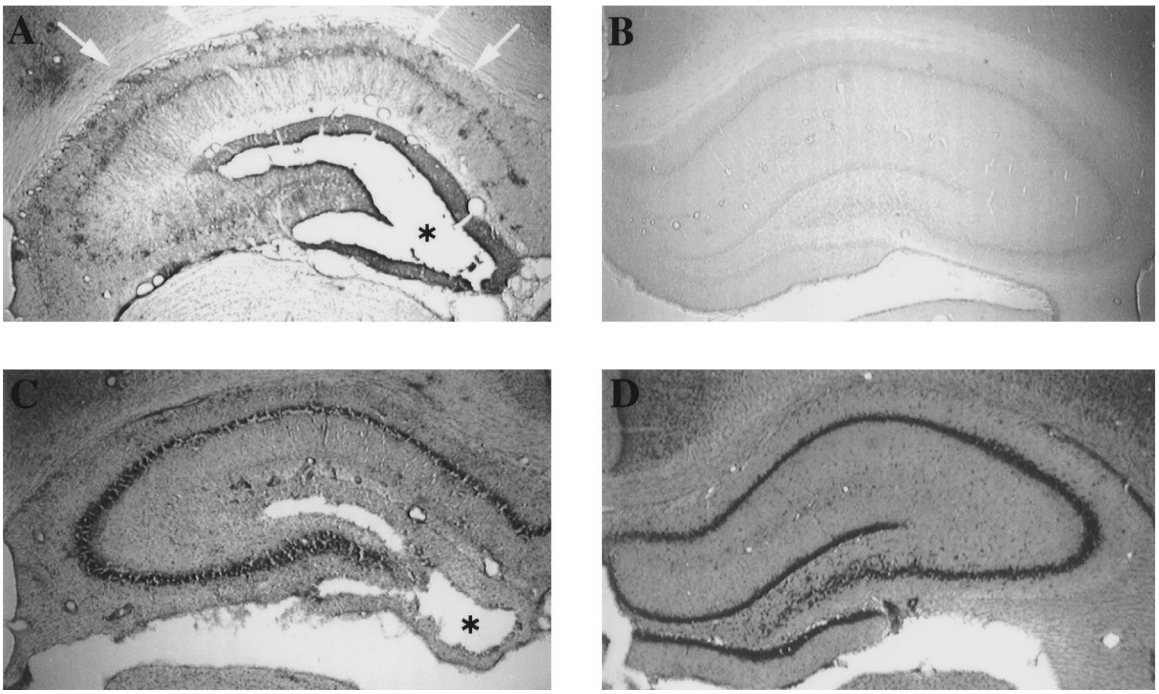


Figure 8 Immunostaining with anti-HSV antibodies (**A** and **B**) and Nissl staining (**C** and **D**) of the injected hippocampus 7 days after stereotactic inoculation with wt KOS (**A** and **C**) or the d311 mutant (**B** and **D**). Arrows: immunostained pyramidal cells of the hippocampus. *: destruction of dentate gyrus.

pus (Figure 8A and C), with extensive infection of pyramidal cells of the hippocampus proper evident by immunocytochemistry. In contrast, the d311-injected hippocampus showed no evidence of HSV antigen expression by immunostaining (Figure 8B), and appeared intact by Nissl stain (Figure 8D). At 7 dpi, the KOS-infected brain also showed spread of viral infection to many brain regions including the contralateral hippocampus, entorhinal cortex bilaterally, septal nuclei and the supramammilary nucleus (Figure 9A), as well as several areas of cortex (Figure 9B). Both HSV-positive cells and obvious damage were observed. On the other hand, at 7 dpi the d311-injected brain was negative for HSV antigen by immunostaining (data not shown), and showed no evidence of damage by Nissl stain (Figure 8D).

Discussion

Recombinant viral vectors as vehicles for gene transfer and therapy may require the ability to accommodate large segments of foreign DNA and to stably infect specific cells without virus-associated cytotoxicity. HSV-1 should fill a unique niche for gene transfer to the nervous system because it is naturally neurotropic and can persist long-term in a non-integrated latent state. Moreover, its large 152 kb double stranded DNA genome encodes a substantial number of accessary functions, which although nonessential for viral replication in cell culture contribute to viral pathogenesis *in vivo*. Since 13 of the 14 genes located within the U_S component of the viral genome fall into this category, construction of an HSV-1 mutant deleted of all nonessential U_S genes offers the possibility of developing a recombinant vector that is highly attenuated for replication and spread *in vivo*, is capable of packaging large (14–20 kb) segments of foreign DNA, and may show an altered host-range or tissue specificity since four nonessential glycoproteins (gG, gJ, gI, and gE) would be removed from the viral envelope. For example, vector mutants may replicate in rapidly dividing but not in post-mitotic cells.

As the first component of a two-step strategy for deletion of the entire group of nonessential genes from the HSV-1 U_S, we have described the isolation, characterization and properties of a U_S deletion mutant of HSV-1, d311. The mutant contains a 12 kb deletion of viral DNA located between the L-S Junction *a* sequence and the U_S6 gene. The deletion spans the S component inverted repeat sequence *c′* and the nonessential genes U_S1 through U_S5, leaving a single copy of the essential ICP4 gene and a single ori_S within the *c* inverted repeat sequence of the S component.

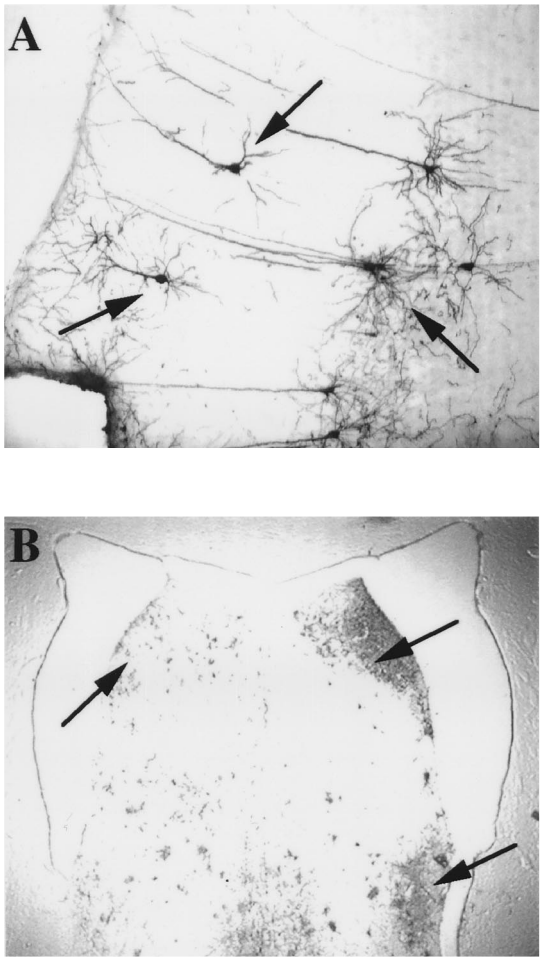


Figure 9 (**A**) HSV-positive immunostained neurons in entorhinal cortex (arrows), and (**B**) HSV-positive immunostaining in the septum of KOS-infected brain (arrows) 7 days after stereotactic inoculation of wt KOS into the hippocampus.

Implications for HSV-1 genome isomerization and the role of the a *sequence*

Southern blot analysis of *Pac*I digests of d311 DNA confirmed the presence of two viral DNA isomers differing in the orientation of their S component. Since we showed that all but 84 nucleotides of the *c*′ inverted repeat sequence have been deleted from the d311 genome, the observation that the S component of d311 DNA underwent isomerization events supports evidence from previous studies which suggested that the *a* sequence alone, and not the sequence homology between the inverted repeats *per se*, is sufficient for the inversion process (Davison and Wilkie, 1983; Mocarski *et al*, 1980; Mocarski and Roizman, 1982b; Smiley *et al*, 1990). Our Southern blot data indicated that the 3′-terminus of the d311 genome acquired the pSR581-derived, HSV-1 (F) *a* sequence that was approximately 0.2 kb larger than the KOS-derived *a* sequence at the 5′-terminus of d311 DNA. This suggested that the heterogeneity in direct repeat (DR) sequences within the *a* sequence were not only tolerated within the same viral genome, but existed independent of viral genome isomerization events.

Growth phenotype of d311 in vitro

In single-step growth experiments in Vero or Vero-derived, ICP4-complementing E5 cells (DeLuca and Schaffer, 1987), d311 grew to titers that were over one order of magnitude lower than those reached by wt KOS. This suggested that the growth defect of d311 was not due to the missing copy of the ICP4 gene, and was most probably a result of the deletion of one copy of ori_S, the deletion of U_S1-U_S5, or both. Furthermore, d311 penetrated Vero cells with nearly the same efficiency as wt KOS, and yields of infectious virus that was either cell-associated or released into the culture media were similar. This indicated that the growth impairment of d311 in Vero cells was associated with the loss of viral functions required for viral replication at some step post-penetration into cells but prior to egress. Growth kinetics of d311 in Vero cells reported here are very similar to those of an HSV-1 (KOS) mutant whose U_S1-$U_S1.5$ genes have been replaced by a *lac*Z cassette (D Krisky and JC Glorioso, unpublished data), suggesting that it is the absence of one or both of these accessory genes which contributes to the growth impairment of d311 in Vero cells. Compared to wt virus, replication of d311 was totally inhibited in rat B103 or mouse Neuro-2A neuroblastoma cell lines, while the mutant could replicate, albeit poorly, in human SK-N-SH neuroblastoma cells. These results suggested that the nonessential functions missing from the d311 genome play an important role in HSV replication in neuronal cells, particularly those of rodent origin. The latter observation confirmed previous studies which showed HSV-1 U_S1-$U_S1.5$ mutants are restricted for replication in rodent cells (Carter and Roizman, 1996; Sears *et al*, 1985).

Growth phenotype of d311 in vivo

The lack of replication and spread of d311 in the rat CNS shown here is similar to the growth phenotype of a U_S3 mutant of HSV-1 previously reported to be highly attenuated for the rat CNS (Fink *et al*, 1992). It is unclear as to what role, if any, the larger deletion in d311 compared to inactivation of U_S3 alone may play in attenuation of d311 *in vivo*. It is reasonable to predict that the loss of U_S1-$U_S1.5$ alone may further attenuate d311 in the rodent CNS. However, the deletion of U_S4 and 5, encoding accessory glycoproteins which may play a role in HSV attachment or cell-to-cell spread functions, would suggest that d311 is even more highly attenuated *in vivo* than single gene mutants in either U_S3 or U_S1-$U_S1.5$.

Deletion of the remaining U_S genes from the d311 genome

Because a *lac*Z expression cassette has replaced the deleted sequences in d311 DNA, it provides a convenient phenotypic screen for isolation of *lac*Z deletion mutants derived from d311. Coupled to a genetic screen based on deletion of the essential U_S6 gene, a second homologous recombination scheme can be utilized to delete the remaining U_S genes (including U_S6) from d311 DNA. d311 genomic DNA could be co-transfected into U_S6-complementing VD60 cells (Ligas and Johnson, 1988) with a recombinant DNA fragment which would juxtapose ICP4 coding sequences in the *c* repeat (3′ of U_S) to those of ICPO in the *b*′ repeat (3′ of U_L). Since sequences coding for U_S6 through U_S12, as well as the *lac*Z cassette, would be deleted from progeny viruses arising from a homologous recombination event occurring between this construct and d311 DNA, one may be able to isolate a deletion mutant lacking all U_S and *a*′-*c*′ sequences based on the mutant's ability to form white plaques (in the presence of chromogen) only on VD60 but not on Vero cells. For efficient virus growth in cell culture, it is possible that deletion of the entire group of nonessential U_S genes from d311 DNA may require complementation of some of the nonessential functions through construction of complementing cell lines expressing those genes.

It may ultimately be possible to replace 40 kb at the 3′-terminus of the HSV-1 genome with foreign sequences. Such mutants will be particularly useful for accommodating multiple or genomic genes and promoters, raising the possibility that foreign promoters integrated into the viral genome may respond to cellular signals for gene expression.

Materials and methods

Plasmids

Using synthetic linkers, the polylinker of the *lox*P-containing vector pBS64 (Sauer *et al*, 1987) was modified by converting the *Hind*III site to *Bgl*II, and the *Pst*I site to *Pac*I (New England Biolabs, Beverly, MA) (Sambrook *et al*, 1989). The new plasmid, pBS64-BP, contained a *lox*P site flanked by novel restriction sites in the following arrangement: 5′-*Bam*HI-*lox*P-*Pac*I-*Bgl*II-3′. The unique *Bam*HI, *Ava*I, *Sma*I, *Sac*I and *Eco*RI polylinker sites of pBS64-BP were all destroyed by digestion with *Bam*HI and *Eco*RI, filling-in with Klenow and dNTP's (Sambrook *et al*, 1989), and self-ligation of the vector using T4 DNA ligase to create pBS64-BPΔEB. pPac was generated by converting the unique *Sac*I site in the polylinker of pBSIISK$^-$ to a *Pac*I site using *Pac*I linkers. A 13 kb joint-spanning *Eco*RI fragment of plasmid pRB380 (Mocarski and Roizman, 1982), containing the HSV-1 (F) L-S junction *a* sequence, was inserted into the *Eco*RI site of pPac to create pPac-*a*. A 1.3 kb *Pac*I-*Eco*RV fragment of pPac-*a*, containing the *a* sequence and several unique restriction sites derived from the pBSIISK$^-$ polylinker, was then subcloned into the *Pac*I/*Nae*I sites of pBS64-BPΔEB to generate pSR521, a *lox*P plasmid containing several unique restriction sites, including *Pac*I, and the HSV-1 (F) *a* sequence. The U_S6 gene, driven by its native promoter, was taken out on a 2.9 kb *Sac*I fragment from plasmid pSG364 (Goldin *et al*, 1981). a *Bam*HI J subclone of KOS, and subcloned into the *Sac*I site of pSP72-pA to generate pgD-pA. pSP72-pA had been previously derived from pSP72 (Promega, Madison, WI) by insertion of the 133 bp *Hpa*I-*Bam*HI fragment encoding SV40 late poly(A) signals (Fiers *et al*, 1978; Reddy *et al*, 1978) into the *Sma*I/*Bam*HI sites of pSP72. The chimeric 3.0 kb U_S6-poly(A) fragment was released from pgD-pA by a *Bgl*II-*Bam*HI digestion, and subcloned into the unique *Bam*HI site of pSR521 to generate pSR540. Subsequently, a 5.5 kb *Pst*I fragment from pIEp-lacZ, containing the E. *coli lac*Z gene under the transcriptional control of the human cytomegalovirus (HCMV) immediate-early promoter-enhancer (IEp) and followed by SV40 late poly(A) signals, was inserted into the unique *Pst*I site of pSR540 to create pSR580. An 820 bp *Xba*I-*Pac*I fragment, containing ori$_S$ and ICP4 upstream regulatory sequences extending to 27 bp downstream of the transcriptional initiation site and within the untranslated 5′-leader sequence of ICP4 (m.u. 0.863 on the HSV-1 genome), was inserted into the *Xba*I/*Pac*I sites of pSR580 to generate pSR581. Plasmid DNA was prepared from 250-ml overnight bacterial cultures, and purified on QIAGEN-tip 500 columns (QIAGEN, Chastsworth, CA).

Cells and viruses

Vero (African green monkey kidney), Neuro-2A (mouse neuroblastoma), and SK-N-SH (human neuroblastoma) cells were obtained from American Type Culture Collection (ATCC). E5, a Vero-derived cell line stably transfected with the HSV-1 (KOS) ICP4 gene driven by its native promoter (DeLuca and Schaffer, 1987), was obtained from Neal DeLuca, University of Pittsburgh. B103 is a rat neuroblastoma cell line (Stallcup and Cohn, 1976) obtained from Myron Levine, University of Michigan. All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with nonessential amino acids, antibiotics and 10% (v/v) fetal bovine serum (FBS) (Gibco, Long Island, NY). d120 is an ICP4 deletion mutant of HSV-1 (KOS) (DeLuca *et al*, 1985), and was obtained from Neal DeLuca. KLP virus was constructed by co-transfection of an *Xba*I/*Kpn*I double-digest of pICP4-lox-pac plasmid (Rasty and Glorioso, unpublished data) and d120 viral DNA onto Vero cells by the calcium phosphate precipitation method as described previously (Graham and Van der Eb,

1973). Viral DNA from single plaques growing on Vero cells was isolated by a modified Hirt extraction protocol (Rasty *et al*, 1995) and analyzed by Southern blotting to confirm the presence of a *Pac*I site in each copy of the diploid ICP4 gene (Rasty and Glorioso, unpublished data). d311 blue plaques were isolated as described previously (Rasty *et al*, 1995), and progeny virions were subjected to three rounds of plaque purification by limiting dilution on 96-well plates.

Southern hybridization analysis of viral DNA
Following complete digestion by the appropriate restriction enzymes, extraction with phenol-chloroform, ethanol precipitation, 70% ethanol wash and resuspension in TE buffer (pH 8.0) (Sambrook *et al*, 1989), 5.0 μg of each viral DNA sample was fractionated by electrophoresis through 25 cm 0.8% agarose gels in Tris-borate-EDTA buffer (Sambrook *et al*, 1989) at 40 V overnight. DNA was then transferred overnight to duplicate sheets of Nytran Plus nylon membranes (Schleicher & Schuell, Keen, NH) according to a bidirectional alkaline transfer protocol (Sambrook *et al*, 1989). Viral DNA fragments to be used as hybridization probes were obtained by restriction digestion of appropriate plasmid subclones of the HSV-1 (KOS) genome. Subsequently, DNA fragments were gel purified using a GeneClean kit (Bio101, La Jolla, CA) and labelled with [α-^{32}P]dCTP to specific activities of greater than 10^9 cpm μg using a random-primed labelling kit (Boehringer Mannheim, Indianapolis, IN). Prehybridization of the Southern blots was carried out at 42°C for 2 to 4 h in a buffer containing 50% formamide-5 × Denhardt's-6 × SSC-1% SDS-250 μg/ml denatured salmon sperm DNA-25 mM sodium phosphate (pH 6.5) (Sambrook *et al*, 1989). Hybridization was performed at 42°C for 18 – 24 h in a buffer containing the same composition as that of the prehybridization buffer, with the exception that 1 × Denhardt's was used in the hybridization buffer and to which was added 10^6 cpm of ^{32}P-labelled, denatured probe per ml of solution. Following hybridization, blots were washed for 30 min in 2 × SSC-0.2% SDS at room temperature and for 1 h in 0.2 × SSC-0.2% SDS at 65°C. They were then exposed to Kodak X-OMAT AR film at −80°C for 12 to 40 h depending on the relative strength of the hybridization signal from the various probes.

DNA sequencing
Two *Dra*I subfragments of d311 viral DNA, 4.5 and 1.2 kb in size, were gel-purified following electrophoresis through a 0.8% agarose gel in Tris-acetate-EDTA buffer (Sambrook *et al*, 1989), and subcloned into the *Sma*I site of the cloning vector pBSIISK$^-$. The 1.2 kb *Dra*I fragment was subjected to a 15 min treatment with T4 DNA polymerase in the presence of 200 μM dNTP's (Sambrook *et al*, 1989) to flush the 3′-terminus of d311 DNA prior to subcloning. Recombinant plasmids from clones containing the 4.5 kb *Dra*I fragment as insert were subjected to Southern blot hybridization to a ^{32}P-labelled probe prepared from the 760 bp HCMV IEp to confirm that their insert was a d311-specific DNA fragment. Subsequently, double-stranded plasmid DNA prepared from two such clones which hybridized to the probe were subjected to automated cycle sequencing (Olesen *et al*, 1993) using a synthetic oligonucleotide primer containing the sequence 5′-GGTGATTTATACCATGCCAGCTGG-3′ [complementary to nucleotides 138069-138192 on the HSV-1 genome (McGeoch *et al*, 1985) upstream of the U_S6 promoter sequences]. Similarly, recombinant plasmids containing the 1.2 kb *Dra*I fragment as insert were identified by Southern blot analysis using a ^{32}P-labelled 318 bp HSV-1 (KOS) *a* sequence probe derived from plasmid pA23 (Smiley *et al*, 1990), and subjected to automated cycle sequencing (Olesen *et al*, 1993) using a synthetic oligonucleotide primer containing the sequence 5′-GCATTATCCTTATCCAAAACAGCC-3′ [complementary to nucleotides 2088-2112 on the SV40 genome (Fiers *et al*, 1978; Reddy *et al*, 1978) within the late poly(A) signal].

Assay for kinetics of virus penetration
To compare the rate of entry of d311 into Vero cells to that of KOS, a low pH inactivation assay was used (Highlander *et al*, 1987; Huang, 1964). A 0.5 ml inoculum containing 400 PFU of either virus was used to overlay confluent monolayers of Vero cells in six-well plates. Virus adsorption was allowed to proceed for 2 h at 4°C with gentle rocking of the plates every 15 min. Monolayers were then washed three times with phosphate buffered saline (PBS)-$MgCl_2$, overlaid with 0.5 ml of DMEM-10% FBS, and shifted to 37°C to allow virus penetration. At 30 min intervals following the temperature shift, media was aspirated and cells were treated for 1 min with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0). The monolayers were then washed twice with PBS-$MgCl_2$ and overlaid with growth medium containing 1% methylcellulose. The fraction of intracellular virus at a given time was calculated as the per cent of viral PFU surviving citrate treatment, where 100% is defined as the number of plaques formed on a monolayer treated with BPS-$MgCl_2$ only.

Single-step growth curves of viral replication
Vero, E5, B103 and Neuro-2A cells were seeded in 35-mm dishes. Confluent cell monolayers were infected with either KOS or d311 using 5 PFU per cell for 2 h at 37°C, after which the monolayers were rinsed once with PBS-$MgCl_2$, treated for 1 min with 1 ml of citrate buffer (pH 3.0) to inactivate unpenetrated viruses, washed twice with PBS-$MgCl_2$, overlaid with 3.0 ml of DMEM-10% FBS and incubated

at 37°C. At various times post-infection plates were removed, media and cells were collected separately and frozen at −80°C. Time-point samples were later thawed, infected cells were subjected to two more cycles of freeze-thaw, sonicated, and all samples were plaques on Vero cell monolayers under standard HSV plaque assay conditions. After 72 h for KOS or 96 h for d311 infections monolayers were fixed and stained with crystal violet-methanol and plaqes were counted.

In vivo *studies of viral invasiveness*
5 μl containing 1.5×10^6 pfu of KOS or d311 virus was injected stereotactically into the hippocampus of Sprague Dawley rats (225–250 *g*) using a pulled glass microelectrode according to the coordinates of the Watson and Paxinos atlas. At 2 or 7 days post-inoculation the rats were sacrificed by intracardiac perfusion with 4% paraformaldehyde. Brains were removed and post-fixed overnight in the same fixative, cryoprotected in 30% sucrose in PBS for 48 h, and 40 μm sections were cut on a sliding microtome. The expression of β-galactosidase from the *lac*Z transgene in the d311 recombinant was assayed by X-gal staining (Fink *et al*, 1992), and the presence of virus determined by immunocytochemical staining using a polyclonal rabbit anti-HSV antibody (1 : 2000, Accurate Chemicals, Westbury, NY) and a biotin-labelled secondary antibody detected with spreptavidin-conjugated horseradish peroxidase and diaminobenzidine (Vector Laboratories, Burlingame, CA). The extent of damage to the injected hippocampus was evaluated by Nissl staining.

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References

Adler R, Glorioso JC, Cossman J and Levine M (1978). Possible role of Fc receptors on cells infected and transformed by herpesvirus: escape from immune cytolysis. *Infection & Immunity* **21**:(2): 442–447.

Balan P, Davis-Poynter N, Bell S, Atkinson H, Browne H and Minson T (1994). An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *Journal of General Virology* **75**(Pt 6): 1245–1258.

Carter KL and Roizman B (1996). The promoter and transcriptional unit of a novel herpes simplex virus 1 alpha gene are contained in, and encode a protein in frame with, the open reading frame of the alpha 22 gene. *Journal of Virology* **70**(1): 172–178.

Chou J and Roizman B (1986). The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *Journal of Virology* **57**(2): 629–637.

Davison AJ and Wilkie NM (1983). Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. *Journal of General Virology* **64**(Pt 1): 1–18.

Deb S and Doelberg M (1988). A 67-base-pair segment from the Ori-S region of herpes simplex virus type 1 encodes origin function. *Journal of Virology* **62**(7): 2516–2519.

DeLuca NA, McCarthy AM and Schaffer PA (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *Journal of Virology* **56**(2): 558–570.

DeLuca NA and Schaffer PA (1987). Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. *Nucleic Acids Research* **15**(11): 4491–4511.

Dingwell KS, Brunetti CR, Hendricks RL, Tang Q, Tang M, Rainbow AJ and Johnson DC (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *Journal of Virology* **68**(2): 834–845.

Dingwell KS, Doering LC and Johnson DC (1995). Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. *Journal of Virology* **69**(11): 7087–7098.

Fiers W, Contreras R, Haegemann G, Rogiers R, Van de Voorde A, Van Heuverswyn H, Van Herreweghe J, Volckaert G and Ysebaert M (1978). Complete nucleotide sequence of SV40 DNA. *Nature* **273**: 113–120.

Fink DJ, Sternberg LR, Weber PC, Mata M, Goins WF and Glorioso JC (1992). In vivo expression of beta-galactosidase in hippocampal neurons by HSV-mediated gene transfer. *Human Gene Therapy* **3**(1): 11–19.

Frame MC, McGeoch DJ, Rixon FJ, Orr AC and Marsden HS (1986). The 10K virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. *Virology* **150**(2): 321–332.

Fruh K, Ahn K, Djaballah H, Sempe P, van Endert PM, Tampe R, Peterson PA and Yang Y (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* **375**(6530): 415–418.

Goldin AL, Sandri-Goldin RM, Levine M and Glorioso JC (1981). Cloning of herpes simplex virus type 1 sequences representing the whole genome. *Journal of Virology* **38**(1): 50–58.

Hayward GS, Jacob RJ, Wadsworth SC and Roizman B (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. *Proceedings of the National Academy of Sciences of the United States of America* **72**(11)**:** 4243–4247.

Highlander SL, Sutherland SL, Gage PJ, Johnson DC, Levine M and Glorioso JC (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. *Journal of Virology* **61**(11)**:** 3356–3364.

Hill A, Jugovic P, York I, Russ G, Bennink J, Yewdell J, Ploegh H and Johnson D (1995). Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**(6530)**:** 411–415.

Huang AS and Wagner RR (1964). Penetration of herpes simplex virus into human epidermoid cells. *Proc Soc Exp Biol Med* **116:** 863–869.

Igarashi K, Fawl R, Roller RJ and Roizman B (1993). Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *Journal of Virology* **67**(4)**:** 2123–2132.

Jenkins FJ, Casadaban MJ and Roizman B (1985). Application of the mini-Mu-phage for target-sequence-specific insertional mutagenesis of the herpes simplex virus genome. *Proceedings of the National Academy of Sciences of the United States of America* **82**(14)**:** 4773–4777.

Johnson DC, Frame MC, Ligas MW, Cross AM and Stow ND (1988). Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *Journal of Virology* **62**(4)**:** 1347–1354.

Lagunoff M, Randall G and Roizman B (1996). Phenotypic properties of herpes simplex virus 1 containing a depressed open reading frame P gene. *J Virol* **70:** 1810–1817.

Lagunoff M and Roizman B (1994). Expression of a herpes simplex virus 1 open reading frame antisense to the γ34.5 gene and transcribed by an RNA 3′ coterminal with the unspliced latency-associated transcript. *J Virol* **68:** 6021–6028.

Lagunoff M and Roizman B (1995). The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus 1 genome. *J Virol* **69:** 3615–3623.

Lehner T, Wilton JM and Shillitoe EJ (1975). Immunological basis for latency, recurrences and putative oncogenicity of herpes simplex virus. *Lancet* **2**(7924)**:** 60–62.

Ligas MW and Johnson DC (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *Journal of Virology* **62**(5)**:** 1486–1494.

Locker H and Frenkel N (1979). BamI, KpnI, and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. *Journal of Virology* **32**(2)**:** 429–441.

Longnecker R, Chatterjee S, Whitley RJ and Roizman B (1987). Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. *Proceedings of the National Academy of Sciences of the United States of America* **84**(12)**:** 4303–4307.

Longnecker R and Roizman B (1986). Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene. *Journal of Virology* **58**(2)**:** 583–591.

Longnecker R and Roizman B (1987). Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. *Science* **236**(4801)**:** 573–576.

Mavromara-Nazos P, Ackermann M and Roizman B (1986). Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the alpha 47 gene. *Journal of Virology* **60**(2)**:** 807–812.

McGeoch DJ, Cunningham C, McIntyre G and Dolan A (1991). Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. *J Gen Virol* **72:** 3057–3075.

McGeoch DJ, Dolan A, Donald S and Rixon FJ (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *Journal of Molecular Biology* **181**(1)**:** 1–13.

Meignier B, Longnecker R, Mavromara-Nazos P, Sears AE and Roizman B (1988). Virulence of and establishment of latency by genetically engineered deletion of mutants of herpes simplex virus 1. *Virology* **162**(1)**:** 251–254.

Mocarski ES, Post LE and Roizman B (1980). Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. *Cell* **22**(1 pt 1)**:** 243–255.

Mocarski ES and Roizman B (1981). Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. *Proceedings of the National Academy of Sciences of the United States of America* **78**(11)**:** 7047–7051.

Mocarski ES and Roizman B (1982a). Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral a sequences and linked to an origin of viral DNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **79**(18)**:** 5626–5630.

Mocarski ES and Roizman B (1982b). Structure and role of the herpes simplex virus DNA termini in inversion, circulation and generation of virion DNA. *Cell* **31**(1)**:** 89–97.

Morse LS, Pereira L, Roizman B and Schaffer PA (1978). Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. *Journal of Virology* **26**(2)**:** 389–410.

Neidhardt H, Schroder CH and Kaerner HC (1987). Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. *Journal of Virology* **61**(2)**:** 600–603.

Olesen CE, Martin CS and Bronstein I (1993). Chemiluminescent DNA sequencing with multiplex labeling. *Biotechniques* **15**(3)**:** 480–485.

Poffenberger KL and Roizman B (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. *Journal of Virology* **53**(2)**:** 587–595.

Poffenberger KL, Tabares E and Roizman B (1983). Characterization of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. *Proceedings of the National Academy of Sciences of the United States of America* **80**(9)**:** 2690–2694.

Post LE and Roizman B (1981). A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**(1)**:** 227–232.

Purves FC and Roizman B (1992). The U_L13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein alpha 22. *Proceedings of the National Academy of Sciences of the United States of America* **89**(16)**:** 7310–7314.

Purves FC, Spector D and Roizman B (1991). The herpes simplex virus 1 protein kinase encoded by the U_S3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene. *Journal of Virology* **65**(11)**:** 5757–5764.

Rasty S, Goins WF and Glorioso JC (1995). Site-specific integration of multigenic Shuttle plasmids into the herpes simplex type 1 genome using a cell-free Cre-lox recombination system. *Methods in Molecular Genetics* **7:** 114–130.

Reddy VB, Thimmappaya B, Dhar R, Subramanian KN, Zain BS, Pan J, Ghosh PK, Celma ML and Weissman SM (1978). The genome of simian virus 40. *Science* **200:** 494–502.

Rice SA, Long MC, Lam V, Schaffer PA and Spencer CA (1995). Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. *Journal of Virology* **69**(9)**:** 5550–5559.

Roller RJ and Roizman B (1990). The herpes simplex virus Us11 open reading frame encodes a sequence-specific RNA-binding protein. *Journal of Virology* **64**(7)**:** 3463–3470.

Roller RJ and Roizman B (1992). The herpes simplex virus 1 RNA binding protein US11 is a virion component and associates with ribosomal 60S subunits. *Journal of Virology* **66**(6)**:** 3624–3632.

Sambrook J Fritsch EF and Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*.

Sauer B, Whealy M, Robbins A and Enquist L (1987). Site-specific insertion of DNA into a pseudorabies virus vector. *Proceedings of the National Academy of Sciences of the United States of America* **84**(24)**:** 9108–9112.

Sears AE, Halliburton IW, Meignier B, Silver S and Roizman B (1985). Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. *Journal of Virology* **55**(2)**:** 338–346.

Sheldrick P and Berthelot N (1975). Inverted repetitions in the chromosome of herpes simplex virus. *Cold Spring Harbor Symposia on Quantitative Biology* **39**(Pt 2)**:** 667–678.

Smiley JR, Duncan J and Howes M (1990). Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. *Journal of Virology* **64**(10)**:** 5036–5050.

Smiley JR, Fong BS and Leung WC (1981). Construction of a double-jointed herpes simplex viral DNA molecule: inverted repeats are required for segment inversion, and direct repeats promote deletions. *Virology* **113**(1)**:** 345–362.

Stallcup WB and Cohn M (1976). Correlation of surface antigens and cell type in cloned cell lines from the rat central nervous system. *Experimental Cell Research* **98**(2)**:** 285–297.

Stow ND (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *EMBO Journal* **1**(7)**:** 863–867.

Stow ND and McMonagle EC (1983). Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130**(2)**:** 427–438.

Umene K (1986). Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the herpes simplex virus type 1 genome. *Journal of General Virology* **67**(Pt 6)**:** 1037–1048.

Varmuza SL and Smiley JR (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**(3)**:** 793–802.

Vlazny DA and Frenkel N (1981). Replication of herpes simplex virus DNA: localization of replication recognition signals within defective virus genomes. *Proceedings of the National Academy of Sciences of the United States of America* **78**(2)**:** 742–746.

Vlazny DA, Kwong A and Frenkel N (1982). Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* **79**(5)**:** 1423–1427.

Wadsworth S, Jacob RJ and Roizman B (1975). Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitons. *Journal of Virology* **15**(6)**:** 1487–1497.

Wagner MJ and Summers WC (1978). Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. *Journal of Virology* **27**(2)**:** 374–387.

Ward PL and Roizman B (1994). Herpes simplex genes: the blueprint of a successful human pathogen [published erratum appears in Trends Genet 1994 Oct;10(10):380]. *Trends in Genetics* **10**(8)**:** 267–274.

Weber PC, Challberg MD, Nelson NJ, Levine M and Glorioso JC (1988). Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. *Cell* **54**(3)**:** 369–381.

Weber PC, Levine M and Glorioso JC (1987). Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. *Science* **236**(4801)**:** 576–579.

Weller SK, Spadaro A, Schaffer JE, Murray AW, Maxam AM and Schaffer PA (1985). Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Molecular & Cellular Biology* **5**(5)**:** 930–942.

York IA, Roop C, Andrews DW, Riddell SR, Graham FL and Johnson DC (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* **77**(4)**:** 525–535.