Site-Directed Mutagenesis of Large DNA Palindromes: Construction and In Vitro Characterization of Herpes Simplex Virus Type 1 Mutants Containing Point Mutations That Eliminate the oriL or oriS Initiation Function

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Technical challenges associated with mutagenesis of the large oriL palindrome have hindered comparisons of the functional roles of the herpes simplex virus type 1 (HSV-1) origins of DNA replication, oriL and oriS, in viral replication and pathogenesis. To address this problem, we have developed a novel PCR-based strategy to introduce site-specific mutations into oriL and other large palindromes. Using this strategy, we generated three plasmids containing mutant forms of oriL, i.e., pDoriL-IL, pDoriL-IR, and pDoriL-ILR, containing point mutations in the left, right, and both copies, respectively, of the origin binding protein (OBP) binding site (site I) which eliminate OBP binding. In in vitro DNA replication assays, plasmids with mutations in only one arm of the palindrome supported origin-dependent DNA replication, whereas plasmids with symmetrical mutations in both arms of the palindrome were replication incompetent. An analysis of the cloned mutant plasmids used in replication assays revealed that a fraction of each plasmid mutated in only one arm of the palindrome had lost the site I mutation. In contrast, plasmids containing symmetrical mutations in both copies of site I retained both mutations. These observations demonstrate that the single site I mutations in pDoriL-IL and pDoriL-IR are unstable upon propagation in bacteria and suggest that functional forms of both the left and right copies of site I are required to initiate DNA replication at oriL. To examine the role of oriL and oriS site I in virus replication, we introduced the two site I mutations in pDoriL-ILR into HSV-1 DNA to yield the mutant virus DorIL-ILR and the same point mutations into the single site I sequence present in both copies of oriS to yield the mutant virus DorIS-I. In Vero cells and primary rat embryonic cortical neurons (PRN) infected with either mutant virus, viral DNA synthesis and viral replication were efficient, confirming that the two origins can substitute functionally for one another in vitro. Measurement of the levels of oriL and oriS flanking gene transcripts revealed a modest alteration in the kinetics of ICP8 transcript accumulation in DorIL-ILR-infected PRN, but not in Vero cells, implicating a cell-type-specific role for oriL in regulating ICP8 transcription.

Replication of the 152-kb double-stranded DNA (dsDNA) herpes simplex virus type 1 (HSV-1) genome is a pivotal event in the dual life cycle of the virus in that the commencement of viral DNA synthesis signals the commitment of the infected cell to the production of new infectious virus and, in most cases, cell lysis. In contrast, the absence of viral DNA synthesis in sensory neurons is a hallmark of latent infection and cell survival. The correlation between the levels of viral DNA synthesis and the establishment, maintenance, and reactivation of latency reflects the complex relationship between viral DNA synthesis and the regulation of viral gene expression. Specifically, pharmacological or genetic inhibition of viral DNA synthesis has been reported to result in marked decreases in immediate-early (IE) and early (E) transcript levels in primary sympathetic neurons but not in Vero cells (39). A similar response to the inhibition of viral DNA synthesis has been reported to occur in vivo during acute viral replication and reactivation from latency in mouse trigeminal ganglia (32, 33).

Viral DNA synthesis is also required for the efficient expression of HSV-1 late (L) genes in all cell types tested (28). Viral DNA synthesis initiates at defined sequences in viral genomes termed origins of replication. Viral origins are commonly composed of core palindromic sequences and auxiliary sequences (17). Core sequences are essential for origin function and typically contain a DNA-unwinding element, often consisting of AT-rich sequences, and an origin recognition element. Auxiliary sequences contain transcription factor binding sites which are commonly associated with promoter/regulatory elements of genes transcribed divergently from the core origin. Both core and auxiliary sequences serve as sites for the assembly of protein-DNA complexes that initiate the process of DNA synthesis. In addition, viral origins can also be involved in nuclear matrix attachment (1, 30, 37), mediated in part by AT-rich sequences located in the core origin (5). Moreover, because they are frequently located within the promoter/regulatory elements of flanking genes, origins are thought to be involved in the transcriptional regulation of these genes (24).

HSV-1 contains three origins of DNA replication, namely, one copy of oriL located in the center of the U₄ region of the genome and two copies of oriS located in the “c” repeats flanking the U₅ region of the genome (Fig. 1A). Both oriL and oriS reside within the promoter/enhancer regions of diver-
gently transcribed genes, contain palindromic sequences that serve as core elements, and share >90% sequence homology in these core elements (60). Specifically, the oriL core origin consists of a perfect 144-bp palindrome with a 20-bp AT-rich sequence at the apex (Fig. 1B) which is thought to unwind during the initiation of DNA synthesis. Contiguous with both the 5' and 3' ends of the AT-rich apex are consensus, functional glucocorticoid response element (GRE) half-sites (26). In contrast to oriL, the oriS core origin consists of a 90-bp sequence which includes a 45-bp imperfect palindrome, an
18-bp AT-rich apical sequence, and degenerate GRE half-sites immediately adjacent to the apex (Fig. 1B). The virus-encoded origin binding protein (OBP), six other viral E proteins (Fig. 1A), and an unknown number of cellular proteins are required to initiate DNA synthesis at all three viral origins (2, 10, 11, 38). As a consequence of the sequence homology of their respective palindromes, oriL and oriS share two types of OBP binding sites, sites I and III. OBP has a high affinity for site I, which is essential for origin-dependent DNA replication, and a lower affinity for site III, which is required for efficient initiation (27). A third OBP binding site, site II, present solely in oriS, is bound by OBP with an affinity intermediate between those of sites I and III. Although only 6 of the 71 nucleotides present in shared oriL and oriS sequences differ, 5 of these 6 nucleotides occur near the AT-rich apex, of which 4 are located within GRE half-sites (Fig. 1B). Thus, despite extensive DNA sequence homology in shared core sequences, a comparison of the complete nucleotide sequences of the oriL and oriS core elements demonstrates that the two origins differ significantly in their structure, sequence, and type and number of OBP binding sites, suggesting that the two origins perform distinct functions in the HSV-1 life cycle.

Functional characterizations of HSV-1 origins using genetic approaches have been carried out almost exclusively with oriS because (i) the oriS but not the oriL palindrome is stable during propagation in standard strains of Escherichia coli (45, 50, 59, 60), (ii) oriS but not oriL sequences are readily changed by site-directed mutagenesis, and (iii) oriS but not oriL is easily sequenced by standard automated DNA sequencing procedures. To date, the major limitation of studies with oriS has been the difficulties associated with the introduction of identical mutations into both copies of oriS in the viral genome. The only published virus lacking both copies of oriS described to date (R7711) (29) was constructed using a complex scheme involving insertions and deletions of origin sequences, resulting in a virus that lacks not only both origins but also three viral genes, UL23, US11, and US12. This virus also contains the ICP27 promoter in place of the ICP22 promoter and the simian virus 40 (SV40) promoter in place of one of the two ICP4 promoters (29). Although studies with this virus demonstrated that oriS is not essential for productive infection in vitro, the multiple alterations in R7711 render it unsuitable for further analyses of large DNA palindromes in other eukaryotic systems.

Unlike oriS, oriL is highly deletion-prone during the propagation of oriL-containing plasmids in standard strains of E. coli (45, 50, 59, 60). Intact copies of oriL are also far less amenable to DNA sequencing and site-directed mutagenesis (25, 26). Indeed, the only reported cloned copy of oriL containing specific point mutations required nearly 2 years to generate and sequence (26). For these reasons, considerably less is known about the functions of oriL or the roles of individual cis-acting elements within oriL core sequences in oriL function. Despite the difficulties associated with mutagenesis and the insertion of mutations into the HSV-1 genome, a definitive functional characterization of the roles of viral origins in the dual life cycle of HSV-1 will necessitate the isolation of viruses mutated in specific origin elements.

In this study, we describe a method of introducing site-specific mutations into oriL and the effects of mutations in OBP binding site I on oriL function. We show that single point mutations in site I that eliminate OBP binding in only one arm of the oriL palindrome are unstable upon propagation in bacteria, whereas these point mutations remain stable when introduced into both arms of the palindrome (thus conserving the palindromic sequence of the oriL core). Mutant viruses containing a single point mutation in both copies of site I in oriL or a single point mutation in site I sequences in both copies of oriS were generated, as were the corresponding resurrected viruses. In vitro experiments comparing these four viruses with wild-type virus for viral growth, DNA replication, and transcription of origin-flanking genes confirmed and extended the results of previous studies of HSV-1 origin function, and we recommend the use of the mutagenic procedure for genetic analyses of large DNA palindromes in other eukaryotic systems.
100 ng purified infectious KOS DNA, 0.25 µM of primer 237 or 238, 0.1 µM of primer LT/BstDwt, doril/BstDILT, RT/BstDwt, or doril/BstDIRT, 1X buffer F (Invitrogen), a 0.25 mM concentration of each deoxynucleoside triphosphate, 5% glycerol, and Taq polymerase (Promega, Madison, WI) or Herculase (Ftaibased blend of thermophilic polymerases; Stratagene, La Jolla, CA) per the manufacturer's instructions. PCRs were hot started by the addition of thermophilic polymerase at 90°C and then incubated for 20 thermal cycles at 95°C for 1 min, 65°C for 1 min, 30 s, and 72°C for 1 min. Taq polymerase- and Herculase-amplified PCR products were cloned into vector pCR2.1-TOPO (Invitrogen) (to generate pMutant_mut-left arm, pwild type-left arm, and pwild type-Herculase-amplified PCR products were cloned into vector pCR2.1-TOPO (Invitrogen) (to generate pMutant_mut-left arm, pwild type-left arm, and pwild type-right arm) and vector pCR-BLUNT II-TOPO (Invitrogen) (to generate pMutant_mut-right arm), respectively, per the manufacturer's instructions. Wild-type and mutant left and right arms were identified by restriction enzyme analysis and confirmed by DNA sequencing.

The construction of plasmids containing wild-type or mutant forms of complete oriL palindromes was performed as follows. (i) pDoril-I_L and poril (Fig. 2C) were generated by three-way ligation of an ~475-bp BstXI (located in the polylinker of the vector)-to-BsrDI fragment from pMutant_mut-left arm or pwild type-left arm, respectively, and an ~450-bp BsrDI-to-XbaI (located in the polylinker of the vector) fragment from pwild type-right arm into vector pCR2.1-TOPO digested with BstXI and XbaI. (ii) pDoril-I_R (Fig. 2C) was generated by three-way ligation of an ~410-bp EcoRI (located in the polylinker of the vector)-to-BsrDI fragment from pMutant_mut-right arm and an ~510-bp BsrDI-to-XbaI fragment (located in the polylinker of the vector) fragment from pwild type-right arm into vector pBlueScript SK(-) (Stratagene) digested with EcoRI and XbaI. (iii) pDoril-I_2 (Fig. 2C) was generated by three-way ligation of an ~510-bp XbaI (located in the polylinker of the vector)-to-BsrDI fragment from pMutant_mut-left arm and an ~410-bp EcoRI (located in the polylinker of the vector)-to-BsrDI fragment from pMutant_mut-right arm into vector pBlueScript SK(-) digested with EcoRI and XbaI.

Plasmid pDOL5.3 was generated as the backbone for introducing oriL mutations into the viral genome by marker transfer (48). It was constructed by three-way ligation of a 2.2-kb Ascl-to-BamHI fragment (nt 60,491 to 62,653; see Fig. 4C) from p8B-S (20), which contains a deletion in oriL core sequences, and a 3.2-kb BamHI-to-PstI fragment (nt 62,653 to 65,829; see Fig. 4C) from pBSpol2 (K. Bryant and D. Coen, unpublished results) into vector pWEB193 (New England BioLabs, Beverly, MA) digested with Ascl and PstI. Plasmids pDOL5.3 and pOL5.3, which were constructed to introduce Dorill-I_L and wild-type oriL, respectively, into the HSV-1 genome were generated by introducing an ~310-bp BsrGI-to-BamHI fragment (nt 62,342 to 62,653; see Fig. 4C) from pDoril-I_L or poril, respectively, into pDOL5.3.

(ii) oriL. Plasmid pBFHSH-mutR, used to generate the mutant virus Doril-I by marker transfer (48), was constructed as follows. The BglII FH fragment (nt 106,750 to 142,759) from a KOS genomic library (22) was digested with SacI and HindIII (nt 60,601 to 133,466; see Fig. 4C and E) to generate a 4.8-kb fragment which was cloned into vector pGEMZeroZf(+) (Promega, Madison, WI) to produce plasmid pBFHSH-100. This plasmid was digested with SpeI and the resulting 6.1-kb fragment was ligated to the 1.7-kb SpeI fragment (nt 127,730 to 133,402; see Fig. 4E) from pGoril-7mutR (10) to produce plasmid pBFHSH-mutR.

(iii) Plasmids for riboprobes. Plasmids p22ss and p4Sma, constructed for the synthesis of riboprobes that hybridize to ICP22 and ICP4 transcripts, respectively, have been described previously (63). Plasmids pCMRF and pDNAoMRP were constructed by ligation of a 5.5-kb PstI fragment (nt 60,288 to 65,829) in the forward or reverse orientation, respectively, into PstI-digested pCMRF and pDNAoMRP. The polarities of the sense and antisense strands of DNA are indicated. Brackets indicate the locations of the site I OBPl binding site. The leftward arrow in the palindrome represents the PCR primer that will specify mutant “X” and is complementary to the sense DNA strand. The rightward arrow in the palindrome represents a wild-type PCR primer complementary to the antisense strand. Note the addition of a BsrDI restriction site to the 5’ termini of primers that anneal to core oriL sequences. (B) Diagram of the left arm (mutated in site I) and right arm (wild type) of the oriL palindrome generated by the PCR strategy diagrammed in panel A. The left arm of the oriL palindrome is shown in black, and the right arm is shown in gray. The sense and antisense DNA strands are indicated. The terminal hexameric BsrDI restriction sites are underlined, with the cleavage site indicated by black lines. (C) Wild-type and mutant forms of oriL generated using the PCR strategy outlined in panels A and B. Each diagram represents a dsDNA palindrome, with black indicating the left arm and gray indicating the right arm of the oriL palindrome. “X” indicates the locations of point mutations in site I. The mutations used in the present study, a C-to-A or G-to-T transversion, introduce a novel BstBI (B) restriction site into site I.
membranes (Micron Separations Inc., Westborough, MA). DNAs were UV irradiated, resulting fragments were separated in 0.8% agarose gels and transferred to nylon membranes. The BstEII fragment of HSV-1 DNA that contains the 5\\text{\textsuperscript{th}} to 65,382; see Fig. 4F) that contains oriL. Among several candidate Doris-I mutants, a single virus was plaque purified three times and used in functional studies. Using the procedure described above, a marker rescant of Doris-I-R, Doris-I-R, was also generated by replacing the sequences containing the two oriL point mutations with wild-type oriL sequences contained in a 2.4-kb BspDI-to-NheI fragment from pBR322. The BstBI fragment of HSV-1 DNA that contains oriS (nt 131,186 to 134,063) that contains oriS. The DNA of the plaque-purified virus was reanalyzed by Southern blot analysis, and the virus, Doris-I, was used to prepare a virus stock. A rescant of Doris-I-R, Doris-I-R, was generated by cotransfection of 293 cells with a virus stock, Doris-I-R, was used to prepare a virus stock, Doris-I-R, and infected at a multiplicity of infection of 10 PFU per cell in 1 ml of inoculum. After adsorption for 1 h at 37°C, inocula were replaced with fresh medium, and cells were incubated at 37°C for the indicated times. After infection, the DNA of the plaque-purified virus was reanalyzed by Southern blot analysis. This virus was used to generate a virus stock, Doris-I-R. Southern blot analysis. To confirm the genotypes of Doris-I-L, Doris-I-L, Doris-I-L, Doris-I-L-R, Doris-I-L-R, 8.0, 0.2% sodium dodecyl sulfate (SDS), and 400 mM 3-[\text{\textsuperscript{32}}P]-labeled random-primed probes specific for either the BstBI fragment of HSV-1 DNA that contains oriL (nt 61,191 to 65,382) or the BstEII fragment of HSV-1 DNA that contains oriS (nt 131,186 to 134,063). The blots were washed under the manufacturer's instructions, and the hybridized membrane was exposed on a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) overnight. In vitro origin-dependent DNA replication assays. In vitro oriL-dependent DNA replication assays were performed as described previously (25). Briefly, 8.0 x 10\textsuperscript{5} Vero cells were seeded in 60-mm plates. At 24 hours postplating, cells were transfected with 2 \mu g of plasmid containing either wild-type or mutant oriL, using Fugene 6 transfection reagent (Roche, Indianapolis, IN). At 18 to 24 h posttransfection, cells were infected with wild-type HSV-1 at a multiplicity of infection of 10 PFU per cell and were harvested 18 h later. After cells were infected, the titers of viral inocula were determined by standard plaque assays on Vero cell monolayers. Viral inocula did not vary more than twofold from one another. To quantify viral DNA at the same time points as those used for single-cycle growth curve experiments, infected cells were scraped into medium, pelleted by centrifugation (800 \times g for 10 min), and washed twice with cold phosphate-buffered saline. The cell pellet was frozen at −80°C and thawed, and total cellular DNA was extracted using a QIAmp DNA mini kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. Viral DNA for the standard curve was isolated from sucrose gradients, and the purity of the viral DNA was verified by BamHI restriction digestion. Samples for the standard curve for the DNA replication assay contained 0 to 6 x 10\textsuperscript{5} viral genomes per 3 \mu g of Vero cell DNA. DNA for the standard curve or 3 \mu g of DNA from each experimental sample was diluted to 400 \mu l with 0.4 N NaOH, heated to 70°C for 10 min, and placed on ice. The denatured samples were vacuum transferred to a nylon membrane (Scheicher & Schuell, Keene, NH), which was Southern blotted using 3\text{\textsuperscript{P}}-labeled random-primed probes specific for the EcoRI F. G, and H fragments of HSV-1 DNA (22). The hybridized membrane was exposed on a PhosphorImager cassette (Molecular Dynamics) overnight. Quantitation of the hybridization signal was performed using ImageQuant 3.3 software (Molecular Dynamics). The amount of DNA was calculated from the standard curve and plotted as the number of genomes per cell. Northern blot analysis. Three million Vero cells or 1 x 10\textsuperscript{6} PRN were plated in 100-mm dishes 18 h prior to infection. Cells were counted at 18 h postplating and infected at a multiplicity of infection of 10 PFU per cell in 1 ml of inoculum. After adsorption for 1 h at 37°C, inocula were replaced with fresh medium, and cells were incubated at 37°C for the indicated times. After infection, the DNA of the plaque-purified virus was reanalyzed by Southern blot analysis. This virus was used to generate a virus stock, Doris-I-R. RESULTS Construction of plasmids containing point mutations in oriL and oriS, site I, oriS. The working hypothesis of this study is that oriL and oriS have distinct roles in the HSV-1 life cycle. To test this hypothesis, viruses containing mutant copies of oriL or oriS defective in the capacity to initiate origin-dependent DNA replication were generated. We have shown previously that a C-to-A transversion in OBp binding site I in oriS (Fig. 1B and 4G) introduces a novel BstBI site and eliminates the need for viral oriS to stimulate viral DNA replication. An ORF-36 mutant was constructed by substituting the ORF-36 ORF with an ORF-36 ORF containing a novel BstBI site. This ORF-36 mutant was then used as a template for the construction of the mutation in ORF-36 ORF. Using the procedure described above, a marker rescant of Doris-I, Doris-I-R, Doris-I, or Doris-I-R per cell. After 1 h of adsorption at 37°C, the inoculum was removed, cells were washed twice with Hank’s balanced salt solution, and growth medium was added back to each culture. After infection, actual titers of viral inocula were determined by standard plaque assays on Vero cell monolayers. Viral inocula did not vary more than twofold from one another. For single-cycle growth curves, infected monolayers were frozen at −80°C at the indicated times postinfection (p.i.). Cultures were later thawed, cells were scraped into medium, and cell suspensions were transferred to tubes, sonicated, and clarified by centrifugation (800 \times g for 10 min). Infectious virus in supernatant fluids was quantified by standard plaque assays on Vero cell monolayers.
inates the ability of OBP and the C-terminal form of OBP, OBPC (a product of the UL8.5 gene) (3), to bind to site I in gel shift assays (10). When introduced into oriS to generate a mutant origin, oriS-mutR, this mutation also eliminates oriS-dependent DNA replication in transient DNA replication analysis (10).

(ii) oriL. Unlike oriS, oriL contains two sites I binding sites (Fig. 1B). When we began this study, it was unknown whether a point mutation in one or both copies of site I would be required to eliminate oriL-dependent DNA replication. Because of the difficulties associated with propagating and mutating the large deletion-prone oriL palindrome, we devised a strategy by which each arm of the palindrome can be cloned, mutated, amplified separately for the stable maintenance of oriL sequences in bacteria, and later reassembled into a complete copy of oriL. Using this strategy, we have introduced mutations into the left, right, and both copies of site I in oriL.

The PCR-based strategy is carried out as follows (Fig. 2A). DNA fragments containing either the 5' 144 bp (left arm) or the 5' 144 bp (right arm) of the 144-bp oriL palindrome are generated by PCR. For this purpose, each arm is synthesized using an oligonucleotide primer pair (black and gray dashed arrows, Fig. 2A) that hybridizes to oriL flanking and core sequences. Mutations can be introduced into any nucleotide in either arm of oriL by PCR using mutagenic primers. An example of a mutation in the left arm of the oriL palindrome is shown as an “X” in Fig. 2A. In addition, the member of the oriL sequence-specific primer pair that hybridizes to core sequences is engineered with a BsrDI consensus restriction site attached to its 5' end, which introduces this site into the apex of the AT-rich region of each arm of the palindrome (shown in boxes in Fig. 2A and as open block letters in Fig. 2B). BsrDI cleaves DNA adjacent to its hexameric consensus recognition site, leaving a 2-nt overhang such that restriction digestion of plasmids containing either the oriL left or right arm with BsrDI removes the BsrDI site from the apical AT-rich sequences of oriL (Fig. 2B). When the two arms are ligated together through the 2-nt overhangs, the oriL apical sequence is restored to the wild type. Using this strategy, we introduced mutations into OBP binding site I in the left, right, or both arms of oriL. Mutations were introduced using palindrome-specific mutagenic primers that contain a single C-to-A (left arm) or G-to-T (right arm) transversion (see Fig. 4G). As noted above, these nucleotide substitutions not only eliminate OBP binding to site I DNA but also introduce a BstBI site into origin sequences at site I (10), which allows for identification of the mutations. In these experiments, primer pairs were annealed to purified, infectious viral DNA as the template, and PCR was allowed to proceed for no more than 20 cycles to minimize the amplification of spurious mutations. The PCR products, wild-type left arm, wild type-right arm, mutant site I-left arm, and mutant site I-right arm, were cloned into plasmids and stably maintained in DH5α cells. To ascertain whether the site I mutation was present, the cloned arms were screened by cleavage with BstBI and by DNA sequencing to ensure that no unwanted mutations had been introduced during PCR. Spurious mutations were frequently observed when Taq polymerase was used, whereas the use of a Pfu-based blend of thermophilic polymerases greatly reduced the frequency of these mutations. An unexpected consequence of the use of the Pfu-based polymerase blend, however, was the insertion of 1 to 3 Gs after a stretch of 11 Gs in the ICP8 5' untranslated region (UTR; nt 62142 to 62152) into most left-arm clones. Wild-type and mutant arms of oriL with the correct sequence were selected for further study and digested with BsrDI to liberate apical sequences. To assemble complete oriL palindromes, wild type-left arm and mutant site I left arm were ligated with wild type-right arm and mutant site I right arm in all combinations and propagated in SURE cells, which support the amplification of plasmids containing palindromic sequences with minimal deletion of palindromic sequences. In this way, four plasmids containing complete copies of oriL were generated and designated poriL (contains a wild-type oriL palindrome), pDoriL-I LR (plasmid defective in oriL site I left, which contains a single C-to-A transversion in the left copy of site I), pDoriL-I LR (contains a single G-to-T transversion in the right copy of site I), and pDoriL-I LR (contains a transversion in each copy of site I) (Fig. 2C and 4G).

Characterization of oriL-containing plasmids. (i) DNA sequence and restriction enzyme analysis. Although automated DNA sequencing of cloned copies of wild-type and mutant arms of oriL presented no problems, we were unable to determine the DNA sequence of site I in the intact oriL palindrome, presumably due to extensive secondary structure (60). Therefore, the presence or absence of the site I mutations in oriL was verified by restriction enzyme digestion. Two clones each of pDoriL-I L and pDoriL-I LR and three clones of pDoriL-I LR were digested with EcoRI to free the oriL-containing fragment from vector sequences or with a combination of EcoRI and BstBI, which, if the mutation were present in one or both copies of site I, would cleave the EcoRI fragment into two or three smaller fragments (Fig. 3A). EcoRI digestion of mutant plasmids liberated an ~860-bp fragment from all clones tested (designated BstBI in Fig. 3A). The digestion of pDoriL-I L and pDoriL-I LR with EcoRI and BstBI generated 422- and 440-bp “b” and “a” fragments, respectively, from BstBI cleavage of the 862-bp EcoRI fragment from pDoriL-I L and 397- and 482-bp “d” and “e” fragments, respectively, from BstBI cleavage of the 861-bp fragment from pDoriL-I LR. Notably, however, a portion of the ~860-bp fragments derived from all pDoriL-I L and pDoriL-I LR plasmids tested were resistant to BstBI cleavage (BstBI'), indicating the loss of the BstBI restriction site and therefore, likely, the site I mutation (Fig. 3A). In contrast to the pDoriL-I L and pDoriL-I LR clones, none of the three clones of pDoriL-I LR contained detectable amounts of BstBI-resistant EcoRI fragments in the double digestion lanes, although the expected 397- and 440-bp BstBI “d” and “a” fragments, respectively, were observed. The 42-bp BstBI “e” fragment containing oriL apical sequences was not resolved in these tests but was regularly observed in 6% acrylamide gels (data not shown). The fact that two of these clones each of pDoriL-I L and pDoriL-I LR, but none of the three clones of pDoriL-I LR contained plasmids that had lost the BstBI site suggests that a single point mutation in one copy of oriL site I is unstable during propagation in E. coli but that point mutations in both copies of site I are tolerated.

(ii) Transient origin-dependent DNA replication assays. The same mutant plasmid preparations shown in Fig. 3A were tested for the ability to direct origin-dependent DNA replication in transient DNA replication assays. Vero cell monolayers were transfected with each mutant plasmid; wild-type plasmid
(poriL) and the empty vector served as positive and negative controls, respectively. Following transfection, Vero cells were infected with 10 PFU/cell of wild-type HSV-1. Eighteen hours later, total cellular DNA was isolated, subjected to restriction enzyme digestion with DpnI to distinguish newly replicated plasmid DNA from input DNA, and analyzed by Southern blot hybridization using a probe complementary to vector sequences.

As expected, wild-type poriL, which was generated by the PCR strategy, replicated efficiently, and the empty vector did not (Fig. 3B). Both clones of pDoriL-I_R, replicated at reduced levels relative to poriL. pDoriL-I_R, clone 2 replicated at wild-type levels; however, replication of pDoriL-I_R, clone 3 and all clones of pDoriL-I_R, was not detected. Notably, with the exception of clone 3, which exhibited no detectable DNA replication but contained very small amounts of the BstBI fragment, the levels of replication of mutant plasmids (Fig. 3B) correlated with the amounts of BstBI-resistant EcoRI fragments in the double digestion lanes in Fig. 3A. This observation supports the hypothesis that wild-type site I sequences were restored in a subset of plasmids in clones 2, 3, 15, and 18, but not in clones 20, 21, and 22. These observations suggest that (i) a single point mutation in one copy of site I is sufficient to eliminate oriL-dependent DNA replication, but this replication is restored upon propagation in bacteria, whereas (ii) point mutations at corresponding sites in both copies of site I render the plasmid replication incompetent but stable upon propagation in bacteria.

Isolation of origin mutant viruses. (i) oriS. To assess the roles of oriL and oriS independently in the HSV-1 replication cycle, we introduced initiation-incompetent forms of each origin into the viral genome (Fig. 4). The oriS-mutR mutation initially described by Dabrowski et al. (10) was introduced into both copies of oriS (Fig. 4G). Specifically, a 4.9-kb SacI-to-HindIII fragment, which spans nt 128,601 to 132,660 and contains two-thirds of the ICP4 coding sequence, oriR, and one-half of the ICP22 coding sequence, was used to transfer the mutR mutation into the genome of n12, a replication-incompetent, ICP4 nonsense mutant virus (16), simultaneously rescuing the n12 mutation (Fig. 4D and E). Among several candidate recombinant viruses selected for the presence of BstBI sites in both copies of oriS and analyzed by Southern blotting as described below, one isolate designated DoriS-I (defective oriS site I) was selected for further study. A marker rescuant of DoriS-I, DoriS-I-R, was generated using standard marker rescue procedures and a 1.2-kb BstNI fragment spanning nt 131,462 to 132,660 as the rescuing fragment (Fig. 4F).

(ii) oriL. The replication incompetence and genetic stability of pDoriL-I_R plasmids and the genetic instability of the pDoriL-I_L and pDoriL-I_R plasmids led us to introduce the DoriL-I_R origin (Fig. 4G) into the HSV-1 genome. To generate a virus containing the DoriL-I_R mutations, a fragment containing these mutations was transferred from pDoriL-I_R to a 5.3-kb Ascl-to-PstI fragment (pDOL5.3) spanning nt 60,491 to 65,829 (Fig. 4E). The Ascl-to-PstI fragment contains one-half of the ICP8 open reading frame (ORF), oriL, and three-quarters of the DNA polymerase (pol) ORF (Fig. 4D and E). This fragment was isolated and cotransfected with infectious wild-type viral DNA using the standard marker transfer procedure (48). Because a procedure to identify the desired recombinant was not available, individual plaque isolates were screened for the presence of BstBI sites in a BstBI fragment that contains oriL (nt 61,191 to 65,382; Fig. 4C and H). Five of 202 (2.5%) plaques on Vero cell monolayers were positive for the BstBI site in oriL, by Southern blot analysis. Among several candidate viruses, a single virus, designated DoriL-I_R, was plaque purified four times and used in functional studies. Additionally, a marker rescuant of DoriL-I_R, DoriL-I_R-R, was generated by replacing the sequences con-

FIG. 3. Restriction enzyme analysis and in vitro DNA replication assays with plasmids containing wild-type and mutant forms of oriL. (A) Restriction enzyme analysis of clonal isolates of oriL mutant plasmid DNA. The three mutant forms of oriL generated using the strategy outlined in Fig. 2 are diagrammed at the top of panel A. Each line represents a dsDNA oriL palindrome. “X” indicates the locations of point mutations in site I that introduce unique BstBI restriction sites. Lowercase letters in the gel photo indicate BstBI restriction fragments whose map locations are shown in the upper diagrams. Note that the small BstBI “c” fragment between the two BstBI sites in pDoriL-I_R is not detectable in the agarose gel photograph. The numbers of individual clonal isolates of each oriL type are shown under the diagrams. Plasmid DNA was cleaved with EcoRI, which liberates oriL-containing sequences from the vector (designated BstBI), or with EcoRI and BstBI, which discriminate intact oriL sequences from mutant oriL fragments (designated BstBI), BI, BstBI; RI, EcoRI. (B) In vitro DNA replication assay with the empty vector, vectors containing wild-type oriL, and clonal isolates of mutated forms of oriL. Vero cell monolayers were transfected with 2 μg each of the indicated plasmids. Twenty-four hours later, cells were infected at a multiplicity of 10 PFU/cell with wild-type HSV-1 strain KOS. Eighteen hours later, cells were harvested and total cellular DNA was isolated. The DNAs were digested with HindIII to linearize the plasmid and with DpnI to discriminate newly replicated plasmid DNA from input plasmid DNA. The digested DNAs were analyzed by Southern blotting using a 32P-labeled probe specific for vector sequences.
FIG. 4. Construction of viruses containing point mutations in oriL and oriS site I and their rescuants. (A) Diagram of the HSV-1 genome showing the UL and US regions, each of which is flanked by the inverted repeat sequences ab and b' a' or a' c' and ca, respectively, shown as dark gray boxes. The locations of oriL and both copies of oriS are indicated with white ovals. (B) Scales in kilobases of the regions of the HSV-1 genome containing oriL and oriS. (Only one of the two copies of oriS is shown in panels B to F) (C) Restriction maps of the oriL- and oriS-containing regions of the genome shown in B. A, Ascl; Bh, BamHI; Bp, BspDI; Br, BsrGI; BI, BstBI; BE, BstEI; BN, BstNI; H, HindIII; N, NheI; P, PstI; S, SalI; SH, SphiI; X, XhoI. (D) Locations of oriL, oriS, and origin-flanking gene transcripts within the two regions shown in B and C. Arrows indicate the locations and directions of transcription of origin-flanking genes. Light gray boxes indicate ORFs. X, nonsense mutation within the ICP4 ORF in virus n12 (16) used for marker rescue/transfer to generate DoriS. (E) Genomic fragments used for marker transfer. The shaded oval and starred BstBI (BI) sites denote the locations of the point mutations in oriL and oriS. Numbers are map units in bp in HSV strain 17 DNA at which specific restriction sites are located. (F) Wild-type genomic fragments used to rescue the mutations in DoriL-ILR and DoriS-I. Nucleotide numbers are as described in panel E. (G) Individual nucleotides mutated in DoriL-ILR and DoriS-I are circled. Bold nucleotides indicate nucleotides substituted in DoriL-ILR and DoriS-I. Nucleotide numbers, which define the core oriL and oriS origins, are shown at the base of each palindrome. (H) Southern blot analysis of wild-type, DoriL-ILR, and DoriL-ILR-R DNA (left panel) and wild-type, DoriS-I, and DoriS-I-R DNA (right panel) digested with the restriction enzymes noted beneath each blot. Total cellular DNA from Vero cells infected at a multiplicity of 5 PFU/cell was analyzed using either a 32P-labeled PCR fragment using primers 237 and 238 (nt 62,025 to 62,865; left panel) or a BstEII fragment (nt 131186 to 134063; right panel) as a probe. Molecular weight markers in kb are indicated on the left.
taining the two oriL point mutations with wild-type oriL sequences using standard marker rescue procedures with a 2.4-kb BspDI-to-NheI fragment spanning nt 61393 to 63781 as the rescuing fragment (Fig. 4F). Thirteen of 180 (7.2%) plaques on Vero cell monolayers lacked the BstBI site in oriL.

The genotypes of mutant and rescuant viruses were verified by Southern blot analysis (Fig. 4H). Total cellular DNAs from Vero cell monolayers infected with KOS, DoriL-ILR, DoriL-ILR-R, DoriS-I, and DoriS-I-R were digested with BstBI (to detect mutations in oriL) or SacI and BstBI (to detect mutations in oriS). DNA fragments were separated in an agarose gel, transferred to a membrane, and hybridized to 32P-labeled HSV-1 DNA fragments specific for either oriL (left panel, Fig. 4H) or oriS (right panel, Fig. 4H) flanking sequences. Digestion of KOS and DoriL-ILR-R DNA with BstBI produced the expected 4.2-kb fragment, whereas digestion of DoriL-ILR DNA with BstBI produced two fragments, of 2.9 and 1.3 kb. The third predicted apical fragment, of 42 bp, was not detected in these agarose gels. Digestion of KOS and DoriS-I-R DNA with BstBI and SacI produced two predicted fragments, of 8.9 and 4.2 kb, corresponding to oriS-containing fragments from the internal and terminal short repeat sequences, respectively. DoriS-I DNA digested with BstBI and SacI produced three fragments, of 6.0, 2.9, and 1.3 kb. Collectively, these results demonstrate the presence of the site I mutations in oriL and oriS in DoriL-ILR-R and DoriS-I-R, respectively, and the absence of these mutations in the marker-rescued viruses.

Effects of mutations in oriL and oriS on the parameters of viral replication in vitro. (i) Single-cycle growth curves. HSV-1 replicates in cells of nonneural and neural lineage in vivo. To examine the effects of the oriL and oriS mutations on viral replication in these two cell types in vitro, single-cycle growth experiments were performed in Vero cells and PRN at a multiplicity of 2.5 PFU per cell.

Although the five viruses tested replicated with similar kinetics in Vero cells, DoriL-ILR and DoriS-I replicated slightly but reproducibly less efficiently than the wild-type and rescuant viruses (left panel, Fig. 5A). Notably, similar differences between mutant viruses and the wild type were observed in Vero cells infected at a multiplicity of 0.1 PFU per cell, indicating that the multiplicity of infection does not contribute to the phenotypes of DoriL-ILR and DoriS-I (data not shown). The levels and kinetics of replication of the origin mutant viruses were nearly identical to those of the wild-type and rescuant viruses in PRN (right panel, Fig. 5A). Notably, however, viral yields never exceeded the input levels of virus (which were equal to Vero cell inocula), and peak titers were approximately 2 orders of magnitude lower in PRN than in Vero cells. This is reflective of the fact that initial levels of viral genomes in neurons were 1 log lower than those observed in Vero cells.

We conclude from these tests that relative to wild-type virus, the effects of the point mutations in site I in DoriL-ILR and DoriS-I on viral replication and viral DNA synthesis were negligible in both cell types in vitro, which (i) confirmed previous observations that the two types of origins substitute functionally for one another during viral replication in vitro in cells of nonneural lineage (29, 45) and (ii) extended those observations to include primary neurons.

(ii) Viral DNA synthesis. Because DoriL-ILR lacks a single functional origin and DoriS-I lacks two functional origins, we asked whether this would translate into reduced viral DNA synthesis during infection. The kinetics and efficiency of viral DNA synthesis were measured in Vero cells and PRN in the same experiments whose results are shown in Fig. 5A. As shown in Fig. 5B, no major differences in the kinetics or levels of viral DNA synthesis by wild-type, mutant, or rescuant viruses were observed by slot blot hybridization of total infected cell DNA for either cell type tested. Although the kinetics of viral DNA synthesis were similar in Vero cells and PRN, peak levels were an order of magnitude lower in PRN than in Vero cells. This is reflective of the fact that initial levels of viral genomes in neurons were 1 log lower than those observed in Vero cells.

We conclude from these tests that relative to wild-type virus, the effects of the point mutations in site I in DoriL-ILR and DoriS-I on viral replication and viral DNA synthesis were negligible in both cell types in vitro, which (i) confirmed previous observations that the two types of origins substitute functionally for one another during viral replication in vitro in cells of nonneural lineage (29, 45) and (ii) extended those observations to include primary neurons.

(iii) Transcription of origin-flanking genes. oriL and oriS lie within the promoter/regulatory regions of divergently transcribed essential E (encoding ICP8 and Pol; oriL) and IE genes (encoding ICP22 or ICP47 and ICP4; oriS). To examine the effects of the site I mutations in the two viruses on the trans-
scription of origin-flanking genes, Northern blot analysis was performed on total cellular RNA from Vero cell or PRN monolayers infected at a multiplicity of 10 PFU/cell using probes for ICP8 and pol transcripts in KOS- and DoriL-ILR-infected cells and for ICP4 and ICP22 transcripts in KOS- and DoriS-I-infected cells. Transcript levels were normalized to the cellular 18S rRNA level, which has been shown to be stable during the 12-h test period used for these studies (40). Each experiment was standardized such that the maximum level of transcripts, plotted as arbitrary units, equaled 12. Representative Northern blots and the average levels (± standard deviations [SD]) of transcripts from three to five experiments are shown graphically in Fig. 6.

An examination of the levels of E transcripts divergently transcribed from oriL revealed that ICP8 and pol transcripts in both wild type- and DoriL-ILR-infected Vero cells were detectable between 1 and 3 h p.i., peaked at 6 h p.i., and declined through 12 h p.i., although ICP8 transcripts declined somewhat more rapidly than pol transcripts (Fig. 6A). Notably, there were no significant differences between wild type- and DoriL-ILR-infected cells in the levels of ICP8 (P = 0.13 to 0.69; two-sided t test) and pol (P = 0.06 to 0.56) transcripts at any of the times tested. The absence of significant differences in the levels of ICP8 and pol transcripts in wild type- and DoriL-ILR-infected Vero cells indicates that neither OBP binding to oriL site I nor the DNA initiation function of oriL per se is required to achieve wild-type levels of transcription of oriL-flanking genes in vitro (Fig. 6A).

The quantitation of IE transcripts divergently transcribed from oriS revealed that in wild type-infected Vero cells, ICP4 transcripts were detectable by 1 h p.i., accumulated rapidly between 1 and 3 h p.i., peaked at 6 h p.i., and declined gradually thereafter (Fig. 6B). In DoriS-I-infected Vero cells, ICP4 transcripts were detectable by 1 h p.i., accumulated rapidly between 1 and 3 h p.i., at which time they peaked, and declined thereafter. Although there was a trend toward reduced levels (1.2- ± 0.2- to 1.6- ± 0.2-fold) of ICP4 transcripts in DoriS-I-infected relative to wild type-infected Vero cells between 6 and 12 h p.i., the differences were not statistically significant (P = 0.13 to 0.18) due to the variation in transcript levels from experiment to experiment. The levels of ICP22 transcripts in wild type- and DoriS-I-infected Vero cells were detectable by 1 h p.i., accumulated most rapidly between 3 and 6 h p.i., peaked at 6 h p.i., and declined thereafter (Fig. 6B). As for ICP8 and pol transcripts, which flank oriS, the absence of significant differences in the levels of ICP4 (P = 0.13 to 0.66) and ICP22 (P > 0.06 to 0.69) transcripts between DoriS-I- and wild type-infected Vero cells indicates that neither OBP binding to site I in oriS nor the DNA initiation function of oriS per se is required to achieve wild-type levels of transcription of oriS-flanking genes in Vero cells.

An examination of the kinetics of ICP8 transcript accumulation in wild type-infected PRN (Fig. 6C) revealed that ICP8 transcripts were detectable between 1 and 3 h p.i., peaked by 6 h p.i., and declined more slowly than ICP8 transcripts in wild type-infected Vero cells. In contrast, in DoriL-ILR-infected PRN, the pattern of ICP8 transcript accumulation was altered. ICP8 transcripts were detectable between 1 and 3 h p.i., peaked at 6 h, and declined more rapidly thereafter than in wild type-infected PRN. The levels of ICP8 transcripts were on average 2- ± 0.5-fold greater than those in wild type-infected cells at 3 h p.i. and 1.4- ± 0.2- and 1.7- ± 2-fold lower than wild-type levels at 9 and 12 h p.i., respectively. These modest, albeit reproducible, differences in the levels of ICP8 transcripts at 3, 9, and 12 h p.i. in DoriL-ILR-infected PRN were statistically significant (P = 0.015 to 0.03). Notably, for rescuant (DoriL-ILR-R)-infected PRN (Fig. 6C), the pattern of ICP8 transcript accumulation resembled that of wild type-infected PRN (P = 0.2 to 0.95), indicating that the site I mutations in oriL were responsible for the differences in the patterns of ICP8 transcript accumulation observed.

Pol transcripts in wild type-infected PRN were detectable between 1 and 3 h p.i., peaked at 6 h p.i., exhibited minor reductions between 6 and 9 h p.i., and then continued to accumulate through 12 h p.i. (Fig. 6C). Thus, the patterns of pol transcript accumulation differed in PRN and Vero cells. In DoriL-ILR-infected PRN, pol transcripts were detectable between 1 and 3 h p.i., peaked at 6 h p.i., and declined slightly thereafter. Although the differences in the levels of pol transcripts between wild type- and DoriL-ILR-infected PRN at 3, 9, and 12 h p.i. (1.7- ± 0.3-, 1.2- ± 0.1-, and 1.4- ± 0.3-fold, respectively) were reminiscent of the differences observed for ICP8 transcript accumulation in infected PRN, the differences were not statistically significant (P = 0.10 to 0.69) due to the variation in transcript levels from experiment to experiment. Thus, these results indicate that neither OBP binding to site I in oriL nor the DNA initiation function of oriL per se is required to achieve wild-type levels of pol transcripts (Fig. 6C).

In wild type- and DoriS-I-infected PRN, both ICP4 and ICP22 transcripts were detectable by 1 h p.i. and, like pol transcripts in PRN, continued to accumulate throughout the experiment (Fig. 6D). Thus, the regulation of the accumulation of ICP4 and ICP22 transcripts differs in PRN and Vero cells. In addition, the fact that there were no statistically significant differences in the levels of ICP4 (P = 0.19 to 0.67) and ICP22 (P = 0.36 to 0.88) transcripts in wild type- and DoriS-I-infected PRN indicates that neither OBP binding to site I in oriS nor the DNA initiation function of oriS per se is required to achieve wild-type levels of transcription of oriS-flanking genes in PRN (Fig. 6D).

Collectively, Northern blot analysis demonstrated that the patterns of origin-flanking gene transcript accumulation differ...
in Vero cells and PRN. Moreover, because no differences were observed in the levels of ICP8 transcripts in Vero cells infected with the wild type and DoriL-ILR but an alteration in the kinetics of ICP8 transcript accumulation was evident in PRN infected with DoriL-ILR, but not with DoriL-ILR-R, either the absence of OBP binding to site I in oriL or the lack of oriL-dependent DNA replication alters the kinetics of ICP8 transcript accumulation specifically in neurons.

**DISCUSSION**

Efforts to investigate the individual roles of oriL and oriS in the dual life cycle of HSV-1 have been hampered by the inability to directly compare the phenotypes of viruses containing mutant forms of the two origins. This is due in large part to difficulties associated with introducing mutations into the large, deletion-prone palindrome of oriL as well as into both copies of oriS in the viral genome. To overcome these problems, we developed methods for (i) the deletion-free propagation of oriL in *E. coli*, (ii) the introduction of mutations into the large oriL palindrome, and (iii) the introduction of mutations into both copies of oriL in the viral genome. Insertion of a single point mutation into site I in either the left or right arm of the oriL palindrome resulted in the loss of the mutation during propagation of the oriL-containing plasmid in *E. coli*, resulting in phenotypically wild-type forms of oriL. A loss of mutations was not observed when single point mutations were introduced into site I in both arms of the palindrome, thus preserving its perfect symmetry. This form of oriL was replication incompetent but stable upon cloning.

The mutant viruses DoriL-IL and DoriS-I, containing site I mutations in oriL and both copies of oriS, respectively, were able to direct the synthesis of viral DNA and to replicate in Vero cells and PRN efficiently. Although no differences in the levels of origin-flanking gene transcripts were evident in Vero cells infected with mutant and wild-type viruses, the pattern of ICP8 transcript accumulation in DoriL-IL-infected PRN was altered relative to that in wild-type virus-infected PRN.

**Introduction and stability of point mutations in oriL.** The difficulties associated with cloning oriL in *E. coli* in an undelated form have impaired studies of this origin. Therefore, most studies of HSV-1 origin function have been conducted with the more stable oriS (25, 34, 45, 50, 51, 60). An intact copy of oriL was first propagated in yeast using a yeast cloning vector (60), and more recently, using *E. coli* SURE cells, which contain mutations in genes required for recombination and SOS repair (25). Although the propensity for the deletion of oriL sequences is greatly reduced in SURE cells relative to other strains of *E. coli*, deletions do occur with a measurable frequency. The frequency with which oriL palindromic sequences are lost can be greatly reduced by incubation of SURE cells at 30°C rather than 37°C and by limiting growth in broth cultures to only 12 to 14 h (25, 34; unpublished observations). Routine screening of colonies and broth cultures for the presence of plasmids containing deleted forms of oriL by PCR greatly facilitates the selective propagation of only undeleted plasmids (see Materials and Methods). Because the deletion of oriL sequences occurs even in SURE cells, the protocol developed to introduce point mutations into oriL involves cloning and mutagenesis of the left and right arms of the oriL palindrome separately. Using this approach, deletions in individual arms of the palindrome were not observed, even in standard cloning strains of *E. coli* (e.g., DH5α).

An interesting observation made during the construction of pDoriL-IL and pDoriL-I_R was the loss of the single site I mutations during plasmid propagation in SURE cells, resulting in a restoration of oriL function. Because oriL is composed of two 72-bp perfect inverted repeats, the introduction of a single point mutation into one repeat generates an imperfect palindrome. Imperfect palindromes are known to be hot spots for simultaneous frameshift and base substitution mutations in both prokaryotes (47, 56) and eukaryotes (46), resulting in the restoration of a perfect palindrome. A hairpin-template mutational mechanism (46) is likely the predominant mechanism by which mutant origins in pDoriL-IL and pDoriL-I_R are restored to the wild type. Whether this occurs during plasmid replication or possibly mismatch repair (14, 46) is not known.

Four of four oriL plasmid isolates with point mutations in only one copy of site I contained a heterogeneous population of wild-type and mutant palindromes. In in vitro DNA replication assays, the levels of replicating plasmids were proportional to the amount of wild-type oriL-containing plasmid in each preparation, suggesting that one functional copy of site I is insufficient for oriL-dependent DNA replication. The observations that oriL appears to require two intact site I binding sites, whereas oriS requires only one, and that OBP has a greater affinity for sites I and III of oriL than for sites I and III of oriS (25), together with the structural and nucleotide sequence differences between the two origins, support the hypothesis that DNA synthesis is initiated differently from oriL and oriS.

The results of this study and the findings of others support the hypothesis that oriL forms a complex secondary structure, possibly a cruciform structure. More specifically, (i) the DNA sequence of site I can be readily determined in individual cloned arms of oriL, but not in the complete palindrome; (ii) single point mutations in oriL are unstable in *E. coli*, but two mutations which preserve the perfect palindrome are stable; and (iii) deletions in palindromic sequences are stimulated by and occur within short direct repeats (4, 9, 12, 43, 44, 61) brought together during the formation of cruciform structures (21, 55). oriL is highly deletion-prone in *E. coli*, and deletions that either shorten or eliminate the palindrome occur in short direct repeat sequences within and adjacent to the oriL palindrome (45, 60). Finally, the majority of nucleotide differences between HSV-1 and HSV-2 which occur within the 144-bp palindrome occur in pairs at the same site in both arms of the palindrome, thus preserving intrastrand base pairing and suggesting a strong selective pressure to preserve palindromy (34).

**Effects of origin mutations on parameters of viral replication in Vero cells and PRN.** The effects of site I mutations in oriL and oriS that eliminate the origin initiation function in origin-dependent DNA replication were tested in Vero cells and PRN, which represent two principal cell types (epithelial cells and neurons) infected in vivo. PRN were chosen because they are readily available in large numbers, contain few (<5%) contaminating, nonneuronal cells, and are one of the principle cell types infected in the mouse model of HSV-1 infection (6, 36, 52).

Growth curves for Vero cells revealed a reproducible three-
to fourfold reduction in the levels of infectious DoriL-I<sub>L,R</sub> and DoriS-I virus produced at 18 h p.i. relative to wild-type virus. This small reduction in viral yields is similar to that observed with viral mutants deleted for oriL (45) or both copies of oriS (29). The fact that DoriL-I<sub>L,R</sub> and DoriS-I behaved similarly to published origin deletion mutants suggests that (i) the site I mutations, like larger deletion mutations, eliminate the initiation function of oriL and oriS; (ii) the origin initiation function is the predominant function of oriL and oriS core sequences; and (iii) the origins can substitute for one another in vitro.

In growth curve analyses conducted with PRN, we observed no differences in the levels of infectious virus produced in wild type-, DoriL-I<sub>L,R</sub>-infected, and DoriS-I-infected cells. This finding was unexpected because in related studies of DoriL-I<sub>L,R</sub> and DoriS-I replication in nerve growth factor-differentiated PC12 cells, we observed a 10-fold reduction in the levels of mutant relative to wild-type virus (data not shown). Although one possible explanation for the absence of a replication phenotype in PRN may be due to the “stimulated” physiology of freshly cultured PRN, the fact that growth curves for wild-type and mutant viruses in PRN cultured for 2 weeks prior to infection also exhibited no differences suggests that this is not the case (data not shown). The reason for the absence of a replication phenotype in PRN is unclear, but the results demonstrate that the initiation function of either oriL or oriS individually is not required for efficient replication in ex vivo cultured PRN, indicating that the two origins can substitute for one another in PRN as in other cell types.

An examination of viral DNA synthesis in Vero cells with the oriS deletion virus R7711 (29) revealed little difference in the level or kinetics of viral DNA accumulation compared to that of the wild-type virus. Although that study demonstrated that oriL alone was sufficient for wild-type levels of viral DNA in HSV-1-infected Vero cells, it was not known whether oriS was sufficient for efficient viral DNA synthesis or whether either type of origin functions in a cell type-specific manner. The results of the present study demonstrate that two copies of intact oriS are sufficient to achieve wild-type levels and kinetics of viral DNA accumulation. In addition, oriL and oriS appear to function efficiently in a cell type-independent manner in vitro, suggesting a lack of preference of one type of origin over another in cultured epithelial or central nervous system neurons. Notably, for these tests, the timing of initiation, kinetics, and magnitude of viral DNA synthesis were similar in Vero cells and neurons, although the initial levels of genomes in neurons relative to those in Vero cells were an order of magnitude lower, suggesting that the mechanisms of viral DNA synthesis in the two cell types are similar and that the level or efficiency of viral DNA synthesis does not account for the nearly 3 orders of magnitude reduction in the amount of infectious virus produced in wild type-infected PRN versus Vero cells.

Given that all three HSV-1 origins lie within the promoter/regulatory regions of essential IE and E genes, we examined the effect of site I mutations on the accumulation of origin-flanking gene transcripts by Northern blot analysis. A comparison of the levels of viral transcripts in Vero cells and PRN showed a marked difference in the kinetics of transcript accumulation for pol, ICP4, and ICP22 (but not ICP8). Indeed, the continued accumulation of these three transcripts through 12 h p.i., together with reduced yields of viral DNA and infectious virus in PRN relative to Vero cells, reflects a fundamental difference in the strategy of HSV-1 replication in neurons versus that in epithelial cells. In addition, these tests showed that in Vero cells, intact copies of site I were not required for wild-type accumulation of oriL or oriS flanking gene transcripts. In PRN, however, this was not the case. Although the levels of ICP4 and ICP22 transcripts were unchanged in DoriS-I-infected PRN and the levels of pol transcripts were unaffected in DoriL-I<sub>L,R</sub>-infected PRN, the kinetics of ICP8 transcript accumulation were altered relative to wild type- and DoriL-I<sub>L,R</sub>-R-infected PRN, indicating that intact copies of site I in oriL are required to establish wild-type kinetics of ICP8 transcript accumulation. This observation is in contrast to the findings of Summers and Leib, who suggested that viral origins play no role in regulating flanking gene expression (53). In their study, the effects of deletions of core origin sequences on origin-flanking promoters fused to luciferase genes were measured as a function of reporter gene activity. Assuming that the mutations in oriL affect ICP8 promoter activity, the experimental design of Summers and Leib may not have been sufficiently sensitive to detect the modest alterations in ICP8 expression observed in this study because they measured promoter activity indirectly. In the present study, promoter activity was measured directly by examining the steady-state levels of viral transcripts, which reflect the combined rates of synthesis and degradation. An additional explanation for the differences between the two studies is that in contrast to the case for this study, origin-flanking gene expression was never examined in cell cultures consisting of primary neurons free of contaminating nonneuronal cells. In that study, flanking gene expression was assayed in dissociated trigeminal ganglion cultures, which are mixed cultures consisting of neurons and abundant nonneuronal support cells, including Schwann cells and fibroblasts. Thus, a neuron-specific origin-flanking gene transcription phenotype may have been suppressed because of efficient gene expression in nonneuronal cells. In contrast, the results of this study indicate that oriL is involved in regulating flanking gene expression in neurons. Although the alteration in the kinetics of ICP8 transcript accumulation had no effect on the efficiency of viral replication in cortical neurons, the results of these tests demonstrate that sequences in oriL affect flanking gene transcription.

Two hypotheses may explain how the site I mutations in DoriL-I<sub>L,R</sub> alter the kinetics of ICP8 transcript accumulation in neurons. First, based on the close proximity of oriL to the start of ICP8 transcription, the initiation of DNA synthesis may influence the timing and efficiency of ICP8 transcription, and second, OBP and perhaps OBPC (3) may be involved in the transcriptional regulation of ICP8. The latter hypothesis is attractive given that several viral origin binding proteins, including the EBNA1 and BZLF1 proteins of Epstein-Barr virus (8, 35) and T antigen of SV40 (54), have been shown to regulate transcription. Tests are currently under way to discern the mechanism by which the two site I mutations in DoriL-I<sub>L,R</sub> alter the levels of ICP8 transcripts in neurons.

**Why does HSV-1 contain two types of origins?** The results of this in vitro study support the prevailing hypothesis that oriS and oriL can substitute functionally for one another. Why then, are these two types of origins highly conserved in HSV-1? In
vitro tests appear to be insufficient to answer this question. The availability of Doril-LR and Doris-I, containing similar mutations in oriL and oriS, respectively, should facilitate a direct comparison of the roles of oriL and oriS in vivo assays. Indeed, preliminary findings in mice demonstrate functional differences between the two origins in relation to acute-phase replication, viral pathogenesis, and the kinetics and efficiency of reactivation from latency.

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