

Use of Transdominant Mutants of the Origin-Binding Protein (UL9) of Herpes Simplex Virus Type 1 To Define Functional Domains

AJAY K. MALIK AND SANDRA K. WELLER*

Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06030-3205

Received 17 May 1996/Accepted 23 July 1996

UL9, the origin-binding protein of herpes simplex virus type 1, contains six sequence motifs conserved in a large superfamily of RNA and DNA helicases. Single-amino-acid substitution mutations in these motifs inactivate UL9 function in vivo (R. Martinez, L. Shao, and S. K. Weller, *J. Virol.* 66:6735–6746, 1992). Overexpression of wild-type UL9 is inhibitory to plaque formation in a transfection assay which measures viral plaque formation by infectious herpes simplex virus type 1 DNA. Constructs containing mutations in motif I, II, or VI exhibit even stronger inhibitory effects in the same assay and thus can be considered strong transdominant inhibitors of plaque formation by the wild-type virus. The transdominant phenotype can be relieved by introducing a second mutation in the DNA-binding domain or by deleting the N-terminal 35 amino acids of the protein. The inhibitory effects of wild-type UL9 can also be partially relieved by deletion of amino acids 292 to 404. We propose that the N-terminal 35 amino acids of UL9 and residues 292 to 404 may define new functional domains of the UL9 protein.

Most replication systems utilize an initiator protein which specifically recognizes its cognate origin of replication. The DnaA protein of *Escherichia coli* and large T antigen of the DNA virus simian virus 40 (SV40) provide two paradigms for understanding the functions of initiator proteins. The DnaA protein initiates replication by binding cooperatively to the *E. coli* origin of replication, *oriC*; this results in the localized unwinding of the AT-rich repeats in the origin. This DnaA-*oriC* complex is recognized by the DnaB helicase which further unwinds DNA, thereby providing a template for priming and replication enzymes (reviewed in reference 20). Large T antigen binds the SV40 origin as a double hexamer around the origin DNA. The intrinsic helicase activity of large T antigen leads to origin unwinding in the presence of ATP. In the case of large T antigen, the origin-binding and helicase functions reside in a single polypeptide (reviewed in reference 11).

UL9 is the origin-binding protein of herpes simplex virus type 1 (HSV-1). Like large T antigen, UL9 has both intrinsic helicase and origin-specific DNA-binding activities (5, 12). The N-terminal domain has six canonical motifs shared with superfamily II of DNA and RNA helicases (Fig. 1) (15, 16, 19). In this superfamily of helicases, motifs I and II are believed to constitute an ATP-binding fold (48). The functions of the other motifs (III to VI) are not clear for UL9 or any of the other superfamily II helicases. Evidence that the helicase function of UL9 is important for HSV-1 DNA replication comes from genetic experiments. Site-directed mutations in each of these motifs abolish the ability of the UL9 protein to complement a UL9-null mutant virus in an in vivo replication complementation assay (32). Furthermore, the analysis of the binding of purified UL9 to origin DNA by electron microscopy suggests that UL9 may be able to carry out limited unwinding of duplex DNA (28).

The HSV-1 genome contains three origins of replication, *oriL* (52) and a diploid *oriS* (35, 43). The minimal HSV-1 origin consists of two UL9-binding sites flanking an AT-rich

region (46). The origin-specific DNA-binding domain has been localized to a 283-amino-acid region within the C-terminal one-third of UL9 (Fig. 1) (1, 8). Full-length UL9 is a dimer in solution (5, 12) and binds cooperatively to origin DNA (9, 17). The N-terminal two-thirds of UL9 is required for cooperative binding to origin DNA (10, 17). The domains of UL9 responsible for stable dimer formation and cooperativity have not been identified. Although the biochemical events leading to the duplex unwinding at an HSV-1 origin are not well understood, one model proposes that dimers of UL9 bind cooperatively to the origin, leading to the formation of higher-order structures. It is also possible that other viral and/or host proteins participate in origin unwinding in vivo (6, 7).

The study of transdominant mutants provides a genetic approach to map the functional domains of a protein (reviewed in reference 18). Isolation and characterization of transdominant mutants of several HSV-1 proteins, including the transactivators ICP0, ICP4, and VP16, have facilitated the identification of regions required for protein-protein interactions (homo- or heterodimerization) (14, 40, 49). Since UL9 is a multifunctional protein, it is particularly suitable for this type of analysis. Thus, it may be possible to isolate mutants of UL9 that are deficient in some but not all wild-type functions. The study of transdominant mutants of UL9 is complicated by the fact that overexpression of wild-type UL9 is inhibitory to HSV-1 infection. We previously reported that in cell lines containing high copy numbers of the UL9 gene (more than 20 copies per haploid genome), virus replication was inhibited, whereas inhibition was not observed in cells with 2 to 10 copies (29). Similar observations have also been made by Stow et al. (45). Inhibitory effects of UL9 have also been observed by Skaliter and Lehman in an in vitro DNA replication system using a partially purified protein fraction from insect cells infected with recombinant baculoviruses expressing HSV-1 essential replication proteins (41). This fraction supported HSV-1 origin-independent replication from a circular plasmid, pUC18, which does not contain an HSV-1 origin of replication (41). UL9 is not required for DNA replication in this assay; in fact, it is inhibitory if the plasmid template also contains an HSV-1 origin (41). The observations that UL9 is essential for

* Corresponding author. Mailing address: Department of Microbiology, MC3205, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-3205. Phone: (860) 679-2310. Fax: (860) 679-1239. Electronic mail address: weller@panda.uhc.edu.

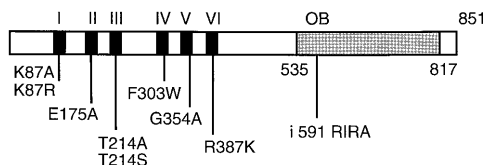


FIG. 1. Helicase motif and DNA-binding domain mutants of UL9 used in this study. The black boxes represent six helicase motifs in the N-terminal region. Substitution mutations in UL9 helicase motifs are shown below. The shaded region represents the 283-amino-acid origin DNA-binding domain of UL9 (1, 8). The four-amino-acid (RIRA) insertion in the origin-binding region (OB) is indicated.

DNA synthesis and can also be inhibitory if overexpressed may indicate that HSV-1 DNA replication occurs in two or more stages. Recent evidence from a study using temperature-sensitive mutations in the UL9 gene indicate that the thermosensitive function of the UL9 protein is essential at early but not late times during viral DNA synthesis (2). It is possible that origin-initiated DNA synthesis requiring UL9 gives way to a recombination-based mode of replication which is inhibited by UL9 (reviewed in reference 50).

Despite the fact that wild-type UL9 can inhibit productive infection by wild-type virus, several viral mutants exhibit an even greater inhibitory effect on viral plaque formation. For example, overexpression of the DNA-binding domain of UL9 results in inhibition of HSV-1 DNA replication (39, 44, 45). The mechanism of transdominance presumably involves the titration of one or more of the origins of replication in an inactive complex. Helicase motif mutants provide additional probes with which to study UL9 function, since they map outside the origin-specific DNA-binding domain. Constructs containing mutations in motif I, II, or VI are strong transdominant inhibitors of viral plaque formation. The transdominant phenotype can be relieved either by introduction of a mutation in the DNA-binding domain or by deletion of the N-terminal 35 amino acids of UL9. Possible models for the mechanism of transdominance will be considered.

(This work constitutes part of the Ph.D. thesis of A.K.M. to be submitted to The University of Connecticut in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Cells and viruses. Vero cells (obtained from the American Type Culture Collection) were maintained in culture as previously described (51). The KOS strain of HSV-1 was used as the wild-type virus. *hr94*, a UL9-null virus containing a *lacZ* insertion, was previously described (29). *hr94* was propagated in 2B-11 cells containing the wild-type version of the UL9 gene (29).

Plasmids. p6UL9-119b contains the UL9 gene expressed from the inducible ICP6 promoter of HSV-1 (29). Plasmids containing point mutations in the helicase motifs (p6UL9-K87A, p6UL9-K87R, p6UL9-E175A, p6UL9-T214S, p6UL9-T214A, p6UL9-F303W, p6UL9-G354A, and p6UL9-R387K) were previously described (Fig. 1) (32). p100-1 contains an HSV-1 origin of replication, *oriS*, on a 100-bp *MspI* fragment cloned into the *SmaI* site of the pUC19 vector (kindly provided by Mark Challeng, National Institutes of Health, Bethesda, Md. [36]). pVP16-9, which expresses the HSV-1 transactivator, VP16, from the murine sarcoma virus long terminal repeat promoter cloned into pTZ19R, was kindly provided by S. Weinheimer (Bristol-Myers Squibb, Wallingford, Conn.).

p6UL9-OB, containing an origin-binding insertion mutation (OB mutation) in the UL9 gene (Fig. 1), was created by linearizing p6UL9-119b (propagated in a *dm⁺ E. coli* host) with *SauI* and inserting a 12-mer *BamHI* linker (5'-CGC-GGA-TCC-GCC). This mutation creates an insertion of four amino acids (Arg-Ile-Arg-Ala [RIRA]) after Ala-591 in the UL9 protein sequence. Double mutants containing one motif mutation and the OB mutation (p6-K87A-OB, p6-K87R-OB, p6-E175A-OB, p6-F303W-OB, p6-G354A-OB, and p6-R387K-OB) were constructed by replacing the 805-bp *EcoNI-EagI* fragment from the UL9 gene in p6UL9-K87A, p6UL9-K87R, p6UL9-E175A, p6UL9-F303W, p6UL9-G354A, and p6UL9-R387K, respectively, with the corresponding fragment from p6UL9-OB, which contains the four-amino-acid RIRA insertion.

p6UL9-Δ1-35 expresses an N-terminal 35-amino-acid deletion mutation of the UL9 gene. It was constructed from p6UL9-119b by digestion with *XhoI*, which cleaves at a site between the AUG start codon and the ICP6 promoter (29), and

NheI, which cleaves at a position 96 nucleotides downstream of the AUG start codon. The resulting overhanging ends were ligated after treatment with the Klenow enzyme. The resultant plasmid, p6UL9-Δ1-35, is expected to start translation from an in-frame methionine at codon 36 of UL9. The Δ1-35 derivative of motif VI mutant, R387K, was constructed from plasmid p6UL9-R387K by a similar deletion strategy. Plasmids expressing internal deletions of UL9, p6UL9-Δ98-195, p6UL9-Δ196-291, p6UL9-Δ292-404, p6UL9-Δ405-520, p6UL9-Δ521-597, and p6UL9-Δ598-703, are described elsewhere (30).

p6UL9-Δ13-536, containing the N-terminal 12 amino acid residues, fused to the DNA-binding domain of UL9 (amino acids 537 to 851), was constructed by deleting the internal *BstYI-BamHI* fragment in the UL9 gene as follows. p6UL9-119b was digested with *HindIII* and *BamHI* to generate 2.2- and 4.3-kbp fragments. The 2.2-kbp fragment, containing the ICP6 promoter and the N-terminal two-thirds of UL9, was isolated and was further digested with *BstYI*, generating a 0.6-kbp fragment. This 0.6-kbp fragment containing the ICP6 promoter and the N-terminal 12 amino acids of UL9 was gel purified and ligated to the 4.3-kbp fragment obtained from the *HindIII* and *BamHI* digest (above) to construct plasmid p6UL9-Δ13-536.

A *BamHI* site at the 5' end of UL9 was introduced into p6UL9-119b by in vitro mutagenesis (22, 32). The mutagenic oligonucleotide corresponding to the non-coding strand was 5'-GAA-AGG-CAT-TTC-GGA-TCC-AAC-AGA-CGC-GG C-3'. The start codon and the *BamHI* site are underlined. The resulting plasmid, p6-NBam, also contains a second *BamHI* site within the UL9 gene at amino acid position 534. p6UL9-Δ1-534, containing the C-terminal 317 amino acids of UL9; was constructed from p6-NBam by introducing a *NcoI* linker containing a start codon as follows: p6-NBam was digested with *BamHI*, and the resulting 4.8-kbp fragment lacking the N-terminal 1 to 534 amino acids was isolated. Following treatment with Klenow enzyme, an *NcoI* linker (5'-AGC-CAT-GGC-T) was inserted. The expected protein contains Met and Ala, followed by amino acids 535 to 851.

All mutations were verified by the dideoxy-chain termination sequencing method, using a Sequenase kit from United States Biochemical, Cleveland, Ohio.

Viral and plasmid DNA isolation. Infectious HSV-1 viral DNA was made as previously described (53). Plasmids were purified by using an anion-exchange column (Qiagen, Chatsworth, Calif.) followed by phenol-chloroform extraction and ethanol precipitation according to standard protocols. Plasmid DNA was quantified by A_{260} . DNA dilutions were subjected to 1% agarose gel electrophoresis to verify DNA concentration and to rule out RNA contamination.

Plaque reduction assay. Efficiency of plaque formation was studied by cotransfecting 1.5×10^6 Vero cells with infectious viral DNA and a 40-fold molar excess of UL9 mutant-expressing plasmids (for example, 2 μ g of plasmid DNA was used for every 1 μ g of viral DNA). Generally 0.125 to 0.5 μ g of viral DNA was used, depending on the infectivity of a particular preparation. Cotransfections were performed as described previously (53), with some modifications. A DNA-CaPO₄ precipitate of viral and plasmid DNA was incubated with cells in suspension at 37°C for 30 min, with gentle shaking at 75 rpm. The transfected cells were plated on two 60-mm-diameter culture plates in 5 ml of regular culture media (Dulbecco modified Eagle medium containing 5% fetal bovine serum), and at 2.5 h postplating, monolayers were treated with 15% glycerol in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline for 2 min. Cells were allowed to recover in regular culture media for 3 to 5 h, at which point they were overlaid with approximately 5 ml of 2% (wt/vol) methylcellulose in culture medium. Four days later, plaques were stained by adding 4 ml of 1% neutral red in Tris-buffered saline. Each experiment was repeated three to five times. The mean number of plaques was calculated, and standard errors were determined by using KaleidaGraph software on a Macintosh computer.

Transient transfection-immunoblot assay. For immunoblot analysis of the UL9 mutants, 1.5×10^6 Vero cells were transfected with 0.5 to 12 μ g of plasmid as previously described (53). At 24 h posttransfection, cells were infected with *hr94* at a multiplicity of 10 PFU per cell, and 18 h thereafter, cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 200 μ l of PBS, and lysed by adding 100 μ l of buffer containing 30 mM Tris-HCl, 15 mM EDTA, and 3% sodium dodecyl sulfate (SDS). The DNA in the samples was sheared by sonication for 30 to 60 s at 4°C in a water bath sonicator. Total protein was precipitated by adding 5 volumes of acetone at -20°C. The acetone precipitates were pelleted, air dried, and resuspended in approximately 100 μ l of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. The samples were boiled, and 20 μ l was loaded per lane on an SDS-8% polyacrylamide minigel (Hoefer Scientific, San Francisco, Calif.). Proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford, Mass.) and incubated with a 1:5,000 dilution of R250, a rabbit antiserum directed against the C-terminal decapeptide of UL9. R250 was generously provided by Mark Challeng (37). Alkaline phosphatase-conjugated anti-rabbit immunoglobulin G was used for color development (Protoblot system obtained from Promega, Madison, Wis.).

Immunofluorescence microscopy. UL9 was expressed from the ICP6 promoter by induction with VP16 (29). A total of 10^6 Vero cells were transfected with 8 μ g of wild-type or mutant versions of a UL9 expression plasmid and 1 μ g of VP16 plasmid. Transfections were performed by the CaPO₄ precipitation method as previously described (53). At 24 h posttransfection, the cells grown on coverslips were washed with PBS and fixed in methanol-acetone (1:1) for 30 min at -20°C. Cells were permeabilized with 1% Triton X-100 in PBS for 5 min at room temperature and incubated in 3% normal goat serum (NGS) in PBS for 30 min at room temperature. The primary monoclonal antibody, 17B, recognizes an

epitope in the N-terminal 33 amino acids of UL9 (30). 17B was used without dilution. The rabbit antiserum, R250, was used at 1:50 dilution. The secondary antibodies (goat anti-mouse or anti-rabbit rhodamine conjugates; Organon Teknika [Cappel], Durham, N.C.) were used at a 1:200 dilution in 3% NGS. Twenty microliters of each antibody was added to each coverslip for 30 min. Coverslips were washed three to five times with 1% NGS and mounted in glycerol-gelatin mounting solution (Sigma, St. Louis, Mo.) containing 2.5% DABCO (1,4-diazabicyclo [2.2.2] octane) to retard bleaching. Immunofluorescence images were collected on a Zeiss Axiovert 100TV microscope equipped with a Plan-Neofluar 63X 1.25-numerical aperture oil immersion lens and charge-coupled device camera and controlled by the Innovations software ISEE. The images were arranged and labeled by using Adobe Photoshop software on a Silicon Graphics workstation.

In vivo transient replication complementation assay. The in vivo transient replication complementation assay was performed as previously described (32). Briefly, 1.5×10^6 Vero cells were transfected with 0.2 μ g of oriS-containing plasmid (p100-1), 1 μ g of wild-type or mutant UL9-expressing plasmid, and 11 μ g of carrier DNA (sonicated salmon sperm DNA). At 24 h posttransfection, the cultures were infected with the UL9-null virus, *hr94*, at a multiplicity of 10 PFU per cell. At 18 h postinfection, total cell DNA was prepared, analyzed by digestion with *EcoRI* alone or in combination with *DpnI*, and then subjected to agarose gel electrophoresis and Southern blotting on a GeneScreenPlus membrane (DuPont-NEN, Boston, Mass.). The blots were hybridized with 32 P-labeled pBluescript plasmid (Stratagene, La Jolla, Calif.). The replication of oriS-containing plasmid was assessed by resistance to *DpnI* digestion.

RESULTS

Transdominance of motif mutant versions of UL9. Single-amino-acid substitutions in all six helicase motifs (Fig. 1) abolish the ability of UL9 to complement *hr94* in the in vivo complementation assay (32). The motif V mutant protein was not detected by transient transfection-immunoblot assay, and thus no conclusion could be drawn about the specific requirement for motif V (32). To determine whether mutant UL9 protein expressed from a plasmid could inhibit viral plaque formation, it was first necessary to confirm previous observations that wild-type UL9 can be inhibitory (29, 45). Vero cells were cotransfected with viral DNA and various amounts of the wild-type UL9 expression plasmid. In the experiments shown in Fig. 2A, cotransfection of a 20- or 40-fold molar excess of UL9 plasmid resulted in a 30 to 50% reduction in the number of plaques observed; cotransfection with a 60- or 80-fold molar excess of UL9 plasmid resulted in 65 to 80% reduction. These results confirm and extend the observation that overexpression of UL9 is inhibitory to viral plaque formation.

To ascertain whether an inhibitory effect even greater than that obtained with wild-type UL9 could be obtained by the motif mutants and to establish optimal conditions, the motif VI mutation, R387K, was chosen. A plasmid containing the R387K mutation was cotransfected with infectious wild-type HSV-1 DNA at three different molar ratios (10-, 20-, and 40-fold plasmid excess). As shown in Fig. 2A, a 40-fold molar excess of the motif VI mutant, R387K, resulted in a greater than 90% reduction in plaque formation. At this ratio, wild-type UL9 generally results in a 40 to 60% inhibition of plaque formation. In all subsequent experiments, a 40-fold molar excess of plasmid DNA was used.

The entire set of helicase motif mutants were tested at a 40-fold plasmid molar excess in the plaque reduction assay. As shown in Fig. 2B, the two motif III mutants, T214S and T214A, and the motif IV mutant, F303W, showed an inhibition of plaque formation comparable with that of wild-type UL9. The motif V mutant, G354A, apparently potentiates plaque formation (see Discussion). On the other hand, mutations in motif I, II, or VI inhibited viral plaque formation by approximately 90% (K87A, K87R, E175A, and R387K in Fig. 2B). Thus, it appears that mutations in motif I, II, or VI are strongly transdominant in this assay.

Transdominance of motif mutants is not due to differences in protein expression levels. To determine whether the greater

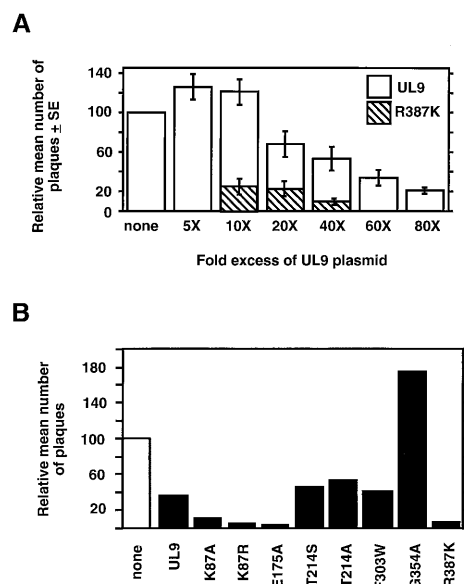


FIG. 2. Effects of plasmids expressing wild-type UL9 or mutants on viral plaque formation by infectious viral DNA. (A) Vero cells were cotransfected with infectious viral DNA and a 5- to 80-fold molar excess of the plasmid expressing wild-type UL9 or R387K as described in Materials and Methods. A histogram depicts the results of four experiments. For comparison, the no-plasmid control was normalized to a value of 100 PFU, and experimental values were adjusted accordingly. The relative mean numbers of plaques \pm standard errors (SE) are shown. The actual numbers of viral plaques obtained with viral DNA alone (no-plasmid control) were 55 (experiment 1), 65 (experiment 2), 130 (experiment 3), and 59 (experiment 4). These numbers reflect variability of infectivity between different batches of viral DNA and variability in transfections. (B) Plaque reduction assay with plasmids expressing helicase motif mutants of UL9. Vero cells were cotransfected with infectious viral DNA and a 40-fold molar excess of the plasmid expressing the indicated protein. A histogram depicting normalized relative mean numbers of plaques from two experiments is shown. The actual numbers of viral plaques obtained with viral DNA alone (no-plasmid control) were 295 (experiment 1), and 182 (experiment 2).

than wild-type inhibition seen with the R387K mutant in Fig. 2A could be explained by differences in protein expression levels, a transient transfection-immunoblot experiment was performed. Vero cells were transfected with 0.5 to 12 μ g of wild-type UL9 or R387K expression plasmid, and expression was induced by *hr94* infection. Total cell protein extracts were resolved by SDS-PAGE, immunoblotted, and analyzed by using the UL9-specific rabbit antiserum, R250. As shown in Fig. 3A, wild-type UL9 and R387K express comparable amounts of protein at each of the plasmid concentrations used. This result suggests that differences in protein expression levels are not a significant factor in the greater than wild-type inhibition seen with the R387K mutant. Similarly, to rule out the possibility that differences in protein expression levels were responsible for the observed transdominant effect of other motif mutants, a transient transfection-immunoblot experiment was performed as described above except that cells were transfected with 8 μ g of expression plasmid. As shown in Fig. 3B, mutants with mutations in motifs III and V (T214S, T214A, and G354A) exhibited decreased levels of protein (Fig. 3B, lanes 5, 6, and 8). Mutations in motif III were not studied further. Given the potentiating effects of transfection with a plasmid bearing a motif V mutation, it was of interest to further examine the expression of this mutant protein. Experiments with an N-terminus-specific monoclonal antibody, 17B, and another polyclonal antibody, RH7, raised against the C-terminal 317 amino acids also failed to detect a protein expressed from the motif V mutant plasmid (data not shown) (see Discussion). On

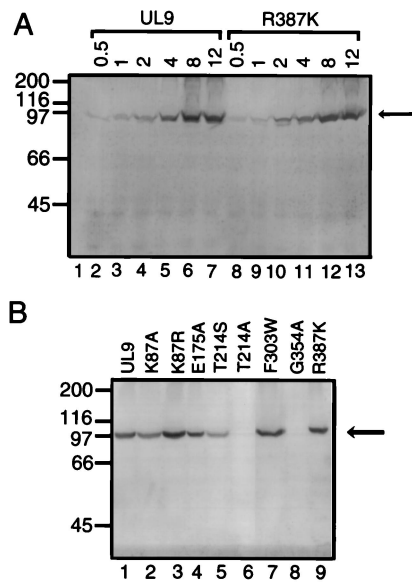


FIG. 3. Immunoblot of extracts from cells transfected with wild-type UL9 and motif mutant versions of UL9. Cells were transfected with an expression plasmid, and protein expression was induced by infection with the *hr94* virus as described in Materials and Methods. Total cell extracts were resolved by SDS-PAGE and analyzed by immunoblotting using R250, a C-terminal 10-amino-acid-specific rabbit antiserum. Positions of the molecular mass markers (200, 116, 97, 66, and 45 kDa; obtained from Bio-Rad, Hercules, Calif.) are indicated on the left. The arrow on the right indicates the position of wild-type UL9 and mutants. (A) Vero cells were transfected with 0.5, 1, 2, 4, 8, or 12 μ g of the wild-type or R387K plasmid as indicated at the top (lanes 2 to 13). Lane 1 shows extracts from untransfected cells which were infected with *hr94*. (B) Vero cells were transfected with 8 μ g of the wild-type or mutant expression plasmids as indicated. Similar protein expression results were obtained when Vero cells were transfected with 4 μ g of plasmid and also when immunoblots were probed with the RH7 rabbit antiserum (not shown). The RH7 antiserum was raised against a glutathione *S*-transferase N-terminal fusion form of the DNA-binding domain (residues 535 to 851) of UL9 (47).

the other hand, cells transfected with plasmids expressing mutations in motifs I, II, IV, or VI accumulate levels of protein comparable to the expression from the wild-type UL9 plasmid. Similar results were obtained when cells were transfected with 4 μ g of expression plasmid (data not shown). Thus, the strongly transdominant effect of mutations in motif I, II, or VI cannot be explained by higher than expected levels of protein expression compared with wild-type UL9.

Transdominance of motif mutants can be partially relieved by the introduction of a mutation in the DNA-binding domain. Stow et al. previously showed that inhibition of viral plaque formation by overexpression of wild-type UL9 can be relieved by the introduction of a mutation (i591RIRA) in the origin-binding domain of UL9 (45). This insertion mutation was previously shown to disrupt origin-specific DNA binding (1, 8). In this report, we constructed an identical RIRA insertion mutant (designated the OB mutation) (Fig. 1). To determine whether the introduction of the OB mutation could relieve the inhibitory effects of UL9 in our assay system, plasmids expressing wild-type UL9 and the OB mutant form of UL9 were used in a plaque reduction assay. Wild-type UL9 reduced plaque formation from a normalized value of 100 PFU in the absence of any cotransfected plasmid to 40 ± 6 PFUs ($n = 9$). When the OB mutation was used, a normalized value of 108 ± 11 PFU ($n = 9$) was obtained. Thus, introduction of the OB mutation was able to relieve the inhibitory effect of the wild-type UL9. This finding suggests that the inhibitory effect of overexpressed

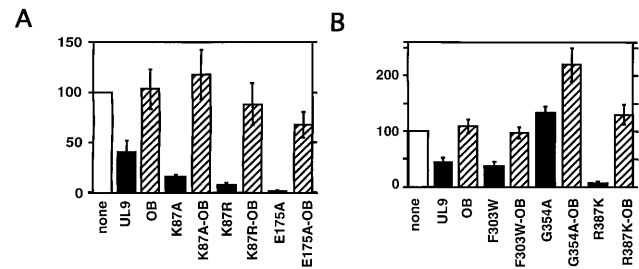


FIG. 4. Plaque reduction assay with plasmids expressing motif mutants containing an additional mutation in the DNA-binding domain of UL9 on viral plaque formation. The histogram plots show data obtained from four (A) and five (B) experiments. Vero cells were cotransfected with infectious viral DNA and a 40-fold molar excess of the plasmid expressing the indicated protein as described in Materials and Methods. The number of viral plaques in each experiment was normalized with respect to viral DNA alone (no-plasmid control), which was set at 100. (A) The actual numbers of viral plaques obtained with viral DNA alone (no-plasmid control) were 95 (experiment 1), 165 (experiment 2), 234 (experiment 3), and 94 (experiment 4). (B) The actual numbers of viral plaques obtained with viral DNA alone (no-plasmid control) were 76 (experiment 1), 78 (experiment 2), 230 (experiment 3), 174 (experiment 4), and 82 (experiment 5).

wild-type UL9 is likely to be mediated through origin DNA binding (see Discussion).

It is clear that overexpression of wild-type UL9 inhibits viral plaque formation and that mutations in motifs I, II, or VI exert even stronger inhibitory effects. It is possible that the motif mutants inhibit plaque formation by the same mechanism as the wild type but more strongly because of the nonfunctional nature of the protein containing the motif mutation. Thus, we were interested in whether the addition of an OB mutation to a UL9 gene bearing a mutation in motif I, II, or VI would relieve the strong transdominance effect. Expression plasmids containing double mutations, one motif mutation as well as the OB mutation, were constructed. The abilities of the double-mutant constructs to inhibit viral plaque formation were measured and compared with those of the motif mutants. As shown in Fig. 4, the introduction of the OB mutation into the motif mutant background partially or fully relieved inhibition by the transdominant motif mutants. Wild-type UL9 and the OB mutant are shown for comparison. The addition of the OB mutation to the motif V mutant even further potentiates plaque formation (see Discussion). In summary, these results suggest that the motif mutants K87A, K87R, E175A, and R387K may sequester origin DNA in an inactive complex, and thus introduction of an OB mutation can partially relieve the observed transdominance.

The stability and protein expression levels of double-mutant proteins were assessed in a transient transfection-immunoblot assay as described above. All motif-OB double mutants except the G354A-OB mutant express proteins of the expected sizes and at levels which are comparable to those of wild-type UL9 and the OB mutant (Fig. 5A). The instability of the G354A-OB protein is not surprising, since the G354A mutation itself was previously shown to be unstable (Fig. 3B, lane 8) (32).

Transdominance of motif mutants is not due to defective nuclear localization. It is possible that factors other than binding of the origin DNA into an inactive complex also contribute to the transdominance of the motif mutants. For instance, it is possible that the strongly transdominant mutants of UL9 inhibit the nuclear localization of the wild-type protein. A mutation (K128N) in the nuclear localization signal of SV40 large T antigen inhibits the transport of wild-type large T antigen into the nucleus and is a transdominant inhibitor of SV40 replication (24, 25). To test this possibility, cells were cotransfected with plasmids expressing either the wild-type or motif

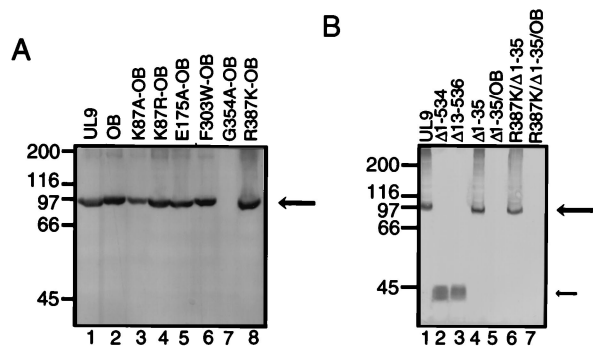


FIG. 5. Immunoblot of extracts from cells transfected with wild-type UL9 and mutant versions of UL9. Vero cells were transfected with 8 μ g of the wild-type or mutant expression plasmids, and protein expression was induced by infection with the *hr94* virus as described in Materials and Methods. The expressed proteins were resolved by SDS-PAGE and analyzed by immunoblot analysis using the R250 rabbit antiserum. Positions of the molecular mass markers (200, 116, 97, 66, and 45 kDa) are indicated on the left. The large arrow on the right indicates the position of UL9 and full-length mutants. The small arrow in panel B indicates the position of DNA-binding domain versions of UL9. Similar protein expression results were obtained when Vero cells were transfected with 4 μ g of plasmid and also when immunoblots were probed with the RH7 rabbit antiserum (not shown). A Vero cells were transfected with wild-type UL9 (lane 1) and variants of UL9 containing the OB mutation in addition to various motif mutations as indicated (lanes 2 to 8). (B) Vero cells were transfected with wild-type UL9 (lane 1) and various deletion mutants of UL9 as indicated (lanes 2 to 7).

mutant proteins from the ICP6 promoter and a VP16 expression clone to activate the ICP6 promoter. Nuclear localization was determined by an indirect immunofluorescence assay using an N-terminus-specific monoclonal antibody, 17B (30). Wild-type UL9 localizes to the nucleus (Fig. 6B). Similar results were obtained when UL9 was expressed from the cytomegalovirus immediate-early promoter or from the ICP6 promoter induced with ICP0 (30). Thus, as previously reported (29), no

other viral proteins are required for the nuclear localization of UL9. Motif mutants K87R, E175A, F303W, and R387K also localized efficiently to the nucleus (Fig. 6C to F). In summary, it does not appear that inefficient nuclear localization can be responsible for the strong inhibitory effects of mutations in motif I, II, or VI on viral plaque formation.

Use of deletions of UL9 to define important domains. Another mechanism by which the transdominant motif mutants may exert their inhibitory effects may be through protein-protein interactions, either by the formation of mixed dimers or higher oligomers or by sequestration of other viral and/or cellular protein(s). If protein-protein interactions play a role in inhibition of plaque formation, a deletion or mutation of an interaction domain may partially relieve the inhibitory effect. The next set of experiments were performed to determine whether an interaction domain could be identified by this strategy.

We constructed six internal deletion mutants of UL9 (Δ 98-195, Δ 196-291, Δ 292-404, Δ 405-520, Δ 521-597, and Δ 598-703) (30). These deletions were tested in the plaque reduction assay as described above (data not shown). Δ 598-703 relieved the inhibition seen with the wild-type UL9 almost completely (normalized value of 97 ± 8 PFU from three experiments). This result confirms that mutations in the DNA-binding domain relieve inhibition. The only other deletion which substantially relieved inhibition was Δ 292-404, which deletes motifs IV, V, and VI (normalized value of 75 ± 6 PFU from three experiments); all other deletions inhibited plaque formation comparably to the wild type. Δ 292-404 produces mutant UL9 protein in levels comparable to wild-type levels and also localizes to the nucleus (30). Further experiments will be necessary to explain the mechanism responsible for the partial relief of transdominance by Δ 292-404 and to determine if the relief is a result of subtle conformational changes.

Stow and colleagues reported that the C-terminal DNA-binding domain of UL9 when expressed alone inhibits HSV-1

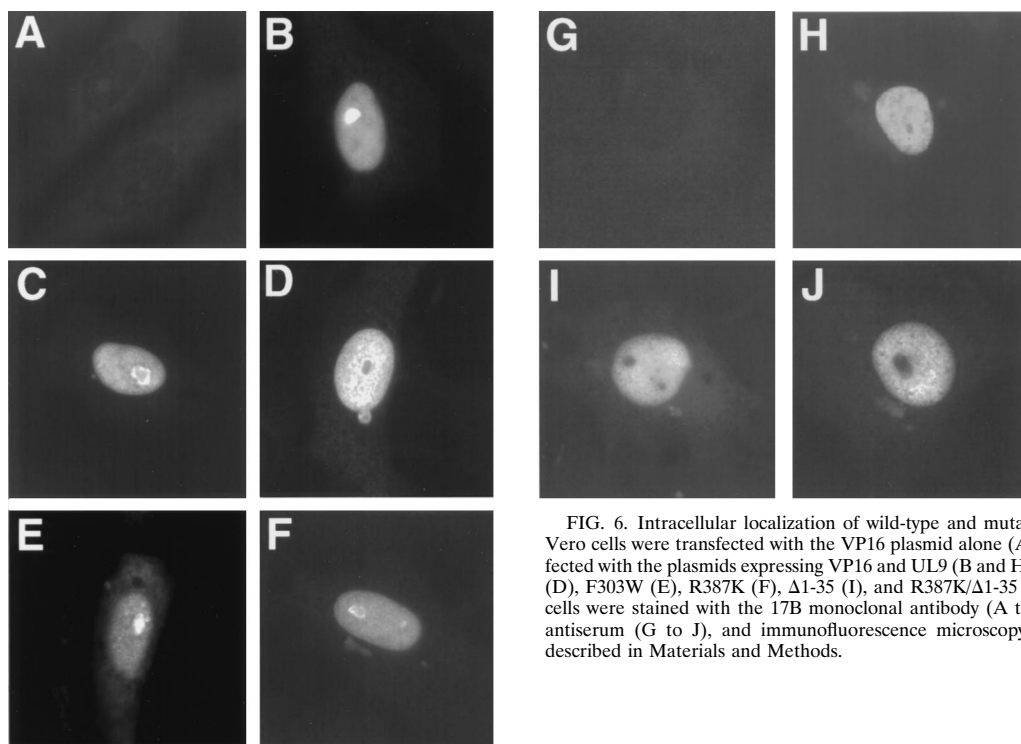


FIG. 6. Intracellular localization of wild-type and mutant versions of UL9. Vero cells were transfected with the VP16 plasmid alone (A and G) or cotransfected with the plasmids expressing VP16 and UL9 (B and H), K87R (C), E175A (D), F303W (E), R387K (F), Δ 1-35 (I), and R387K/ Δ 1-35 (J). The transfected cells were stained with the 17B monoclonal antibody (A to F) or R250 rabbit antiserum (G to J), and immunofluorescence microscopy was performed as described in Materials and Methods.

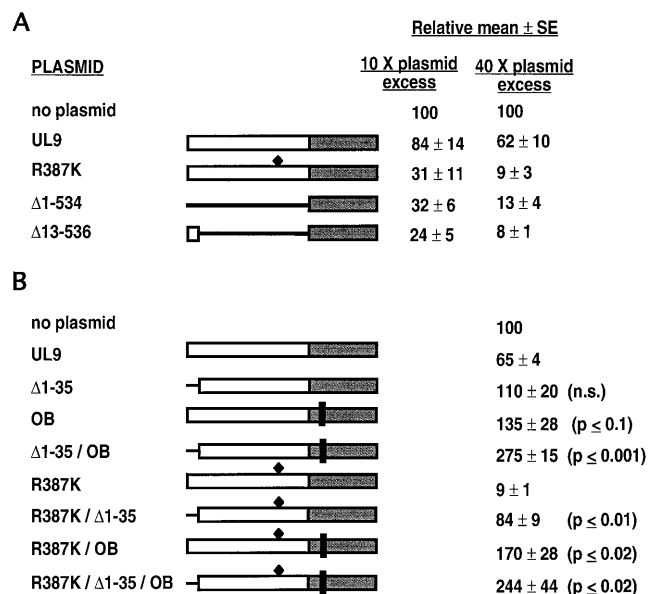


FIG. 7. Plaque reduction assay using the two versions of the DNA-binding domain and the N-terminal deletion mutations. (A) Inhibitory effects of plasmids expressing two versions of the DNA-binding domain of UL9 (Δ 1-534 and Δ 13-536). The deleted regions are shown as lines. The R387K mutation is shown by a black diamond. The plaque reduction experiments were performed at 10- and 40-fold molar plasmid excess ratios as described in Materials and Methods. The relative mean numbers of plaques \pm standard errors (SE) from five experiments are shown. (B) Effect of deletion of the N-terminal 35 amino acids on the inhibitory effect of wild-type UL9 and the transdominance effect of the R387K mutant. The N-terminal deletion is shown as a line, and the vertical black bars represent the OB mutation. The R387K mutation is shown by a black diamond. The plaque reduction experiments were performed at 40-fold molar plasmid excess. The relative mean numbers of plaques \pm standard errors from four experiments are shown. The statistical difference between the mutants was determined by the unpaired Student *t* test, and the results are indicated on the right. Δ 1-35, OB, and Δ 1-35/OB mutants were compared with wild-type UL9. R387K/ Δ 1-35, R387K/OB, and R387K/ Δ 1-35/OB mutants were compared with R387K. The difference between R387K/ Δ 1-35 and R387K/OB was $P \leq 0.05$. n.s., not significant.

replication (45). The plasmid used by these authors expresses the N-terminal 12 amino acids in addition to the 315-amino-acid C-terminal DNA-binding domain. To address the potential contribution of the N-terminal 12 amino acids to transdominance, we constructed plasmids Δ 13-536 and Δ 1-534, expressing the C-terminal DNA-binding domain with and without, respectively, the N-terminal 12-amino-acid region. The two mutants express comparable levels of protein in a transient transfection-immunoblot assay (Fig. 5B, lanes 2 and 3). Wild-type UL9, mutant R387K, and the two forms of the C-terminal DNA-binding domain were compared in the plaque reduction assay under the conditions of 10- or 40-fold molar plasmid excess. As shown in Fig. 7A, like R387K, both Δ 1-534 and Δ 13-536 were transdominant at both 10- and 40-fold molar ratios. The apparent differences between Δ 1-534 and Δ 13-536 were not statistically significant for either the 10- or the 40-fold molar ratios; nevertheless, there is a trend toward a higher transdominance effect with Δ 13-536.

To ascertain whether the N-terminal region plays a role in the context of the full-length UL9 protein, we constructed versions of the wild type and R387K with an N-terminal 35-amino-acid deletion, Δ 1-35 and R387K/ Δ 1-35, respectively (Fig. 7B). Both constructs expressed protein in amounts comparable to those of wild-type UL9 in a transient transfection-immunoblot assay (Fig. 5B, lanes 1, 4, and 6). An immunofluorescence assay was used to demonstrate that these two

mutants also localized to the nucleus (Fig. 6I and J). Figure 6G shows the background staining of cells transfected with VP16 alone, and Fig. 6H shows cells transfected with VP16 and wild-type UL9. In the plaque reduction assay, Δ 1-35 appears to be able to relieve the inhibitory effect of wild-type UL9; however, this relief is not statistically significant (compare UL9 and Δ 1-35 in Fig. 7B). On the other hand, this 35-residue deletion has a more dramatic effect on alleviating the transdominance of the motif VI mutant. R387K/ Δ 1-35 showed only a 15% inhibition of viral plaque formation, compared with a 90% inhibition seen with the R387K mutant (R387K and R387K/ Δ 1-35 in Fig. 7B). The difference between R387K and R387K/ Δ 1-35 was statistically significant with $P \leq 0.01$ (Fig. 7B). These results indicate that the N-terminal 35 amino acids contribute to the transdominance effect of the motif VI mutant.

To determine the combined effect of the OB and Δ 1-35 mutations on the inhibitory effect of UL9 or the R387K mutant, we tested plasmids with an additional OB mutation in Δ 1-35 or R387K/ Δ 1-35. As shown in Fig. 7B, these constructs further relieved the transdominance inhibition (mutants Δ 1-35/OB and R387K/ Δ 1-35/OB in Fig. 7B). However, neither the Δ 1-35/OB nor the R387K/ Δ 1-35/OB mutant could be detected by a transient transfection-immunoblot assay (Fig. 5B, lanes 5 and 7). Because of the apparent instability of these OB mutation-containing versions of Δ 1-35 and R387K/ Δ 1-35, no conclusions can be drawn about whether the OB and Δ 1-35 mutations are synergistic.

The N-terminal 35-amino-acid deletion (Δ 1-35) was also tested for function in the *in vivo* complementation assay that measures replication, of plasmid pOriS which is assessed by resistance to *Dpn*I digestion. In this assay, KOS infection results in an amplification of plasmid pOriS (data not shown) whereas no pOriS replication is seen in cells infected with *hr94*, a UL9-null virus (Fig. 8, lanes 3 and 4). The position of unit-length replicated *Dpn*I-resistant pOriS is marked with an arrow. Wild-type UL9 expressed from a plasmid complements *hr94* for the replication of pOriS (Fig. 8, lanes 5 and 6), whereas the OB mutant (lanes 7 and 8), the Δ 1-35 deletion (lanes 9 and 10), and the Δ 1-35/OB double mutant (lanes 11 and 12) were unable to complement *hr94*. These observations also support the notion that the N-terminal 35 amino acids may define an important domain of UL9 (see Discussion); nevertheless, further experiments will be required to rule out the possibility that this region may be required for proper conformation.

DISCUSSION

The inhibition of plaque formation seen by overexpression of wild-type UL9 in the plaque reduction assay is consistent with previous observations (29, 41, 45). In this report, we confirm the observations that inhibition by wild-type UL9 can be relieved by the introduction of an OB mutation. This result indicates that binding of DNA is required for the inhibitory effects of UL9. This interpretation is consistent with the observations that UL9 is inhibitory in an *in vitro* DNA replication system when a plasmid containing an HSV-1 origin of replication is present (41). In this report, we also demonstrate that several of the helicase motif mutants exert even stronger inhibitory effects than wild-type UL9 and that this transdominance can be relieved either by the introduction of an OB mutation or by deletion of the N-terminal 35 amino acids of UL9. In addition, the inhibitory effects of wild-type UL9 can be partially relieved by deletion of amino acids 292 to 404.

Four mutants stimulated viral plaque formation by 50 to 175%: G354A (a motif V mutant), G354A-OB, Δ 1-35/OB, and R387K/ Δ 1-35/OB. We were unable to detect these four proteins by immunoblot analysis of transiently transfected cells

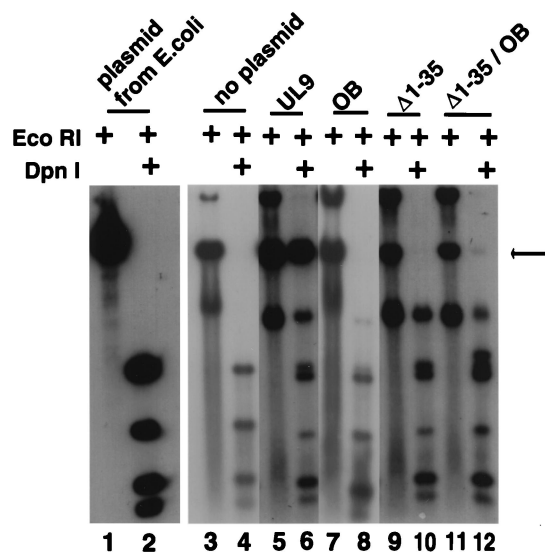


FIG. 8. In vivo transient replication complementation assay. The in vivo complementation assay was performed as described in Materials and Methods. Lanes 1 and 2 contain plasmid pOriS propagated in *E. coli*. The arrow indicates the position of the *DpnI*-resistant DNA corresponding to a monomer of pOriS. Lanes 3 and 4 contain cellular DNA isolated from cells transfected with pOriS alone and infected with *hr94*. Lanes 5 to 12 were transfected with the plasmids indicated at the top along with pOriS and infected with *hr94*.

with three different antisera: R250 against the C-terminal 10 amino acids, another polyclonal antibody, RH7, raised against the C-terminal 317 amino acids, and the N-terminus-specific monoclonal antibody, 17B. Despite the inability to detect these mutant proteins with three different antisera, it remains possible that a specific degradation intermediate of these mutants interacts with wild-type UL9 molecules and target them for degradation. A decreased level of UL9 in the later phase of replication may allow for increased replication and plaque formation in our assay.

Mutants containing mutations in motif I, II, or VI display a transdominant phenotype. Another mutation in motif I, K87E, was previously shown to inhibit viral plaque formation (45). The fact that the transdominance of mutants in motif I, II, or VI could be relieved by introducing a DNA-binding mutation lends support for the model that transdominance occurs by binding origin DNA into an inactive complex. The observation that only mutants in motif I, II, or VI are strongly transdominant is of interest. Motifs I and II are believed to be involved in ATP binding and hydrolysis, since they contain the established ATP-binding consensus sequences (32, 48). The role of motif VI in UL9 function is not known. A mutation in the corresponding motif in eIF-4A, a helicase superfamily II member, is deficient in ATPase binding and/or hydrolysis (38). Therefore, by analogy, it is possible that motif VI of UL9 also contributes to ATP binding and/or hydrolysis. If motifs I, II, and VI are involved in ATP binding, it is possible that mutations in these motifs alter the DNA-binding affinities of these mutants, and this may partially explain the strongly transdominant phenotype.

UL9 is capable of several protein-protein interactions, one or more of which may contribute to the transdominance of UL9 by mediating interactions through the N-terminal domain. The known UL9-UL9 interactions are the dimerization (5, 12) and ability to bind cooperatively to origin DNA (9, 17). Although the domains of UL9 responsible for dimer formation and cooperativity have not been identified, both interactions require the presence of the N-terminal two-thirds of UL9 (534

amino acids) (10, 13, 17, 42). UL9 interactions with other proteins have also been reported. The C-terminal 27 amino acids have been shown to interact with the HSV-1 single-stranded DNA-binding protein, ICP8 (3, 4). The N-terminal 534 amino acids of UL9 interact with UL8, a member of the heterotrimeric helicase-primase complex (34), and UL9 has recently been reported to interact with the 180-kDa catalytic subunit of cellular polymerase α -primase (26). With the exception of ICP8, the domains for other protein-protein interactions have not been finely mapped.

The N-terminal domain of UL9 contains four leucine residues spaced seven residues apart (33) and is similar to leucine zipper motif often associated with protein-protein interactions (21, 23). This putative leucine zipper (residues 147 to 171) has been postulated to play a role in dimerization or cooperative binding to the origin (8, 17, 32); however, a closer look at the overall composition of the zipper casts some doubt on this hypothesis. Most leucine zippers exist in an amphipathic α helix in which hydrophobic residues occupy positions on one face of the helix (positions a and d). In contrast, this proposed motif in UL9 contains a proline in the b position of the helix, which is not entirely consistent with α -helix formation (although in rare instances, prolines may be tolerated [27]), and a glycine in the a position, which should significantly decrease the hydrophobic nature of this face of the putative zipper. Hazuda et al. constructed a four-amino-acid insertion mutation in this motif and tested for cooperative binding to a radiolabeled origin DNA in an electrophoretic mobility shift assay (17). These authors concluded that the putative leucine zipper is responsible for cooperative binding of UL9. However, they have not ruled out the possibility that the insertion can cause conformational alterations in the protein.

We constructed two mutants (L12 and L34) in which the leucine zipper region was disrupted by the substitution of leucine residues in positions 1 and 2 and positions 3 and 4, respectively) with valine residues (31). If the putative leucine zipper plays a role in protein-protein interactions contributing to plaque reduction, mutations which disrupt the leucine zipper may relieve inhibition of wild-type plaque formation. However, disruption of the putative leucine zipper leads to a highly transdominant phenotype instead of relieving the inhibition (31). It is possible that the leucine zipper mutants exert their transdominant effects by binding origin DNA in an inactive complex. The L12 and L34 mutants were expressed in *E. coli* or translated in vitro and were tested for dimerization in a velocity sedimentation assay; both putative leucine zipper mutant proteins sedimented as dimers (31). This finding implies that the putative leucine zipper is not likely to specify a dimerization domain of UL9. Further experiments will be required to determine whether this region contributes to cooperativity.

The deletion of the N-terminal 35 amino acid residues was able to relieve the transdominance of the motif VI mutant, and deletion of residues 292 to 404 was able to partially relieve inhibition by wild-type UL9. It is possible that these regions play roles in protein-protein interactions, either UL9-UL9 interactions or interactions of UL9 and another viral or cellular protein(s). The abilities of various forms of UL9 to form dimers, bind origin DNA cooperatively, and interact with other proteins are currently being examined in order to distinguish between these possibilities.

ACKNOWLEDGMENTS

We gratefully acknowledge Mark Challberg for the R250 antiserum, p100-1, and discussions regarding the manuscript. We also thank Dan Tenney and Robert Hamatake for RH7 antiserum and Steve Wein-

heimer for plasmid pVP16-9. We also are also grateful to members of our laboratory for critically reviewing the manuscript.

This investigation was supported by Public Health Service grant A121747 from the National Institutes of Health. S.K.W. was the recipient of an American Heart Association-Genentech Established Investigator Award during the initial stages of this work.

REFERENCES

1. **Arbuckle, M. I., and N. D. Stow.** 1993. A mutational analysis of the DNA-binding domain of the herpes simplex virus type 1 UL9 protein. *J. Gen. Virol.* **74**:1349-1355.
2. **Blumel, J., and B. Matz.** 1995. Thermosensitive UL9 gene function is required for early stages of herpes simplex virus type 1 DNA synthesis. *J. Gen. Virol.* **76**:3119-3124.
3. **Boehmer, P. E., M. C. Craigie, N. D. Stow, and I. R. Lehman.** 1994. Association of origin binding protein and single strand DNA-binding protein, ICP8, during herpes simplex virus type 1 DNA replication in vivo. *J. Biol. Chem.* **269**:29329-29334.
4. **Boehmer, P. E., and I. R. Lehman.** 1993. Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8. *Proc. Natl. Acad. Sci. USA* **90**:8444-8448.
5. **Bruckner, R. C., J. J. Crute, M. S. Dodson, and I. R. Lehman.** 1991. The herpes simplex virus 1 origin binding protein: a DNA helicase. *J. Biol. Chem.* **266**:2669-2674.
6. **Dabrowski, C. E., P. J. Carmillo, and P. A. Schaffer.** 1994. Cellular protein interactions with herpes simplex virus type 1 oriS. *Mol. Cell. Biol.* **14**:2545-2555.
7. **Dabrowski, C. E., and P. A. Schaffer.** 1991. Herpes simplex virus type 1 origin-specific binding protein: oriS-binding properties and effects of cellular proteins. *J. Virol.* **65**:3140-3150.
8. **Deb, S., and S. P. Deb.** 1991. A 269 amino acid segment with a pseudo leucine zipper and a helix turn helix motif codes for the sequence specific DNA binding domain of herpes simplex virus type 1 origin binding protein. *J. Virol.* **65**:2829-2838.
9. **Elias, P., C. M. Gustafsson, and O. Hammarsten.** 1990. The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication oriS. *J. Biol. Chem.* **265**:17167-17163.
10. **Elias, P., C. M. Gustafsson, O. Hammarsten, and N. D. Stow.** 1992. Structural elements required for the cooperative binding of the herpes simplex virus origin binding protein to oriS reside in the N-terminal part of the protein. *J. Biol. Chem.* **267**:17424-17429.
11. **Fanning, E., and R. Knippers.** 1992. Structure and function of simian virus 40 large tumor antigen. *Annu. Rev. Biochem.* **61**:55-85.
12. **Fierer, D. A., and M. D. Challberg.** 1992. Purification and characterization of UL9, the herpes simplex virus 1 origin binding protein. *J. Virol.* **66**:3986-3995.
13. **Fierer, D. S., and M. D. Challberg.** 1995. The stoichiometry of binding of the herpes simplex virus type 1 origin binding protein, UL9, to oriS. *J. Biol. Chem.* **270**:7330-7334.
14. **Friedman, A. D., S. J. Triezenberg, and S. L. McKnight.** 1988. Expression of a truncated viral *trans*-activator selectively impedes lytic infection by its cognate virus. *Nature (London)* **335**:452-454.
15. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1988. A novel superfamily of nucleoside triphosphate-binding motif containing proteins which are probably involved in duplex unwinding in DNA and RNA replication and recombination. *FEBS Lett.* **235**:16-24.
16. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* **17**:4713-4730.
17. **Hazuda, D. J., H. C. Perry, and W. L. McClements.** 1992. Cooperative interactions between replication origin-bound molecules of herpes simplex virus origin-binding protein are mediated via the amino terminus of the protein. *J. Biol. Chem.* **267**:14309-14315.
18. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. *Nature (London)* **329**:219-222.
19. **Hodgman, T. C.** 1988. A new superfamily of replicative proteins. *Nature (London)* **333**:22-23.
20. **Kornberg, A., and T. A. Baker.** 1992. DNA replication, 2nd ed. W. H. Freeman & Co., San Francisco.
21. **Kouzarides, T., and E. Ziff.** 1988. The role of the leucine zipper in the fos-jun interaction. *Nature (London)* **336**:646-651.
22. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488-492.
23. **Landschultz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**:1759-1764.
24. **Lanford, R. E., and J. S. Butel.** 1980. Inhibition of nuclear migration of wild-type SV40 tumor antigen by a transport-defective mutant of SV40-adenovirus 7 hybrid virus. *Virology* **105**:303-313.
25. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**:801-813.
26. **Lee, S. S.-K., Q. Dong, T. S.-F. Wang, and I. R. Lehman.** 1995. Interaction of herpes simplex virus 1 origin-binding protein with DNA polymerase alpha. *Proc. Natl. Acad. Sci. USA* **92**:7882-7886.
27. **MacArthur, M. W., and J. M. Thornton.** 1991. Influence of proline residues on protein conformation. *J. Mol. Biol.* **218**:397-412.
28. **Makhov, A. M., P. E. Boehmer, I. R. Lehman, and J. K. Griffith.** 1996. The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms unwound stem-loop structures. *EMBO J.* **15**:1742-1750.
29. **Malik, A. K., R. Martinez, L. Muncy, E. P. Carmichael, and S. K. Weller.** 1992. Genetic analysis of the herpes simplex virus type 1 UL9 gene: isolation of a LacZ insertion mutant and expression in eukaryotic cells. *Virology* **190**:702-715.
30. **Malik, A. K., L. Shao, J. D. Shanley, and S. K. Weller.** Intracellular localization of the herpes simplex virus type-1 origin binding protein, UL9. *Virology*, in press.
31. **Malik, A. K., and S. K. Weller.** Unpublished data.
32. **Martinez, R., L. Shao, and S. K. Weller.** 1992. The conserved helicase motifs of the herpes simplex virus type 1 origin-binding protein UL9 are important for function. *J. Virol.* **66**:6735-6746.
33. **McGeoch, D. J., M. A. Dalrymple, A. Dolan, D. McNab, L. J. Perry, P. Taylor, and M. D. Challberg.** 1988. Structures of herpes simplex virus type 1 genes required for replication of virus DNA. *J. Virol.* **62**:444-453.
34. **McLean, G. W., A. P. Abbotts, M. E. Parry, H. S. Marsden, and N. D. Stow.** 1994. The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein. *J. Gen. Virol.* **75**:2699-2706.
35. **Mocarski, E. S., and B. Roizman.** 1982. Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral sequences and linked to an origin of viral DNA replication. *Proc. Natl. Acad. Sci. USA* **79**:5626-5630.
36. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. *Proc. Natl. Acad. Sci. USA* **85**:5414-5418.
37. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1989. Herpes simplex virus type 1 gene products required for DNA replication: identification and overexpression. *J. Virol.* **63**:196-204.
38. **Pause, A., and N. Sonenberg.** 1992. Mutational analysis of a DEAD box RNA helicase: the mammalian translational initiation factor eIF-4A. *EMBO J.* **11**:2643-2654.
39. **Perry, H. C., D. J. Hazuda, and W. L. McClements.** 1993. The DNA binding domain of herpes simplex virus type 1 origin binding protein is a transdominant inhibitor of virus replication. *Virology* **193**:73-79.
40. **Shepard, A. A., P. Tolentino, and N. A. DeLuca.** 1990. *trans*-dominant inhibition of herpes simplex virus transcriptional regulatory protein ICP4 by heterodimer formation. *J. Virol.* **64**:3916-3926.
41. **Skaliter, R., and I. R. Lehman.** 1994. Rolling circle DNA replication in vitro by a complex of herpes simplex virus type 1-encoded enzymes. *Proc. Natl. Acad. Sci. USA* **91**:10665-10669.
42. **Stabell, E. C., and P. D. Olivo.** 1993. A truncated herpes simplex virus origin binding protein which contains the carboxy terminal origin binding domain binds to the origin of replication but does not alter its conformation. *Nucleic Acids Res.* **21**:5203-5211.
43. **Stow, N. D.** 1982. Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *EMBO J.* **1**:863-867.
44. **Stow, N. D.** 1992. Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses. *J. Gen. Virol.* **73**:313-321.
45. **Stow, N. D., O. Hammarsten, M. I. Arbuckle, and P. Elias.** 1993. Inhibition of herpes simplex virus type 1 DNA replication by mutant forms of the origin-binding protein. *Virology* **196**:413-418.
46. **Stow, N. D., and E. C. McMonagle.** 1983. Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130**:427-438.
47. **Tenney, D., and R. Hamatake.** Unpublished data.
48. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**:945-951.
49. **Weber, P. C., and B. Wigdahl.** 1992. Identification of dominant-negative mutants of the herpes simplex virus type 1 immediate-early protein ICP0. *J. Virol.* **66**:2261-2267.
50. **Weller, S. K.** 1995. Herpes simplex virus DNA replication and genome maturation, p. 189-213. *In* G. M. Cooper, R. G. Temin, and B. Sugden (ed.), *The DNA provirus: Howard Temin's scientific legacy*. American Society for Microbiology, Washington, D.C.
51. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. *J. Virol.* **45**:354-366.
52. **Weller, S. K., A. Spadaro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer.** 1985. Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol. Cell. Biol.* **5**:930-942.
53. **Zhu, L., and S. K. Weller.** 1988. UL5, a protein required for HSV DNA synthesis: genetic analysis, overexpression in *Escherichia coli*, and generation of polyclonal antibodies. *Virology* **166**:366-378.