

The Conserved Helicase Motifs of the Herpes Simplex Virus Type 1 Origin-Binding Protein UL9 Are Important for Function

RIK MARTINEZ, LEI SHAO, AND SANDRA K. WELLER*

*Department of Microbiology, The University of Connecticut Health Center,
Farmington, Connecticut 06030*

Received 5 May 1992/Accepted 3 July 1992

The UL9 gene of herpes simplex virus encodes a protein that specifically recognizes sequences within the viral origins of replication and exhibits helicase and DNA-dependent ATPase activities. The specific DNA binding domain of the UL9 protein was localized to the carboxy-terminal one-third of the molecule (H. M. Weir, J. M. Calder, and N. D. Stow, *Nucleic Acids Res.* 17:1409-1425, 1989). The N-terminal two-thirds of the UL9 gene contains six sequence motifs found in all members of a superfamily of DNA and RNA helicases, suggesting that this region may be important for helicase activity of UL9. In this report, we examined the functional significance of these six motifs for the UL9 protein through the introduction of site-specific mutations resulting in single amino acid substitutions of the most highly conserved residues within each motif. An *in vivo* complementation test was used to study the effect of each mutation on the function of the UL9 protein in viral DNA replication. In this assay, a mutant UL9 protein expressed from a transfected plasmid is used to complement a replication-deficient null mutant in the UL9 gene for the amplification of herpes simplex virus origin-containing plasmids. Mutations in five of the six conserved motifs inactivated the function of the UL9 protein in viral DNA replication, providing direct evidence for the importance of these conserved motifs. Insertion mutants resulting in the introduction of two alanines at 100-residue intervals in regions outside the conserved motifs were also constructed. Three of the insertion mutations were tolerated, whereas the other five abolished UL9 function. These data indicate that other regions of the protein, in addition to the helicase motifs, are important for function *in vivo*. Several mutations result in instability of the mutant products, presumably because of conformational changes in the protein. Taken together, these results suggest that UL9 is very sensitive to mutations with respect to both structure and function, perhaps reflecting its multifunctional character.

Initiation of DNA replication in many organisms utilizes a common mechanism involving the specific binding of an initiator protein to a site within the origin of replication. Since binding of an initiator protein represents the first committed step in initiation of DNA replication, this event clearly has important regulatory potential. In *Escherichia coli*, an initiator protein, *dnaA*, binds DNA and facilitates unwinding at the origin by recruiting a DNA helicase, *dnaB*, to the site (4). In other systems such as the eukaryotic virus simian virus 40, the initiator protein, large T antigen, not only binds the origin in a sequence-specific manner but also has intrinsic helicase activity and is capable of unwinding the duplex DNA in an ATP-dependent fashion (16, 17, 19, 64).

The herpesviruses provide a model for eukaryotic DNA replication in which many of the replication proteins are encoded by the virus. The 152-kb genome of herpes simplex virus type 1 (HSV-1) contains three *cis*-acting origins of DNA replication (63, 65, 77). Two complementary approaches have been used to identify *trans*-acting viral factors required for viral DNA synthesis. HSV-1 mutants representing seven distinct complementation groups which fail to induce viral DNA synthesis under nonpermissive growth conditions have been identified (6, 7, 9, 11, 27, 50, 51, 57, 58, 75, 76, 80) (for a review, see reference 74). These complementation groups represent seven viral genes which have been shown to be necessary and sufficient for origin-dependent plasmid amplification in a transient transfection assay

(78). Biochemical activities have now been assigned to the products of these genes: UL30 and UL42 encode the viral DNA polymerase and its accessory protein (26, 56-58); UL29 encodes the major DNA-binding protein, ICP8 (12, 76); and UL5, UL8, and UL52 genes encode three members of a protein complex with helicase-primase activities (13). UL9 encodes the HSV origin-specific DNA-binding protein (53).

Purified recombinant UL9 protein has the following properties: cooperative origin-specific DNA-binding activity, DNA-dependent nucleoside 5'-triphosphatase, DNA helicase on partially double-stranded substrates, and ability to form dimers in solution (5, 21, 22, 24, 39, 53). Although specific unwinding of HSV origin-containing plasmids by purified UL9 has not been seen (24), several observations of the interactions between UL9 and duplex DNA have been made. When UL9 protein binds to supercoiled plasmids containing *ori_S*, the structure in the AT region is distorted and the region becomes sensitive to agents such as KMnO₄ and dimethyl sulfate (38). Fierer and Challberg demonstrated that UL9 wraps supercoiled DNA in a sequence-independent fashion (24). Nucleoprotein complexes consisting of purified UL9 and origin-containing DNA have been visualized by electron microscopy and form inter- and intramolecular interactions, raising the possibility that multimers of UL9 protein are formed at the replication origin (59). However, despite these observations concerning *in vitro* interactions of UL9 with duplex DNA, it is still unclear how UL9 functions *in vivo* during infection. In infected cells, UL9 presumably binds at one of the three origins of HSV DNA

* Corresponding author.

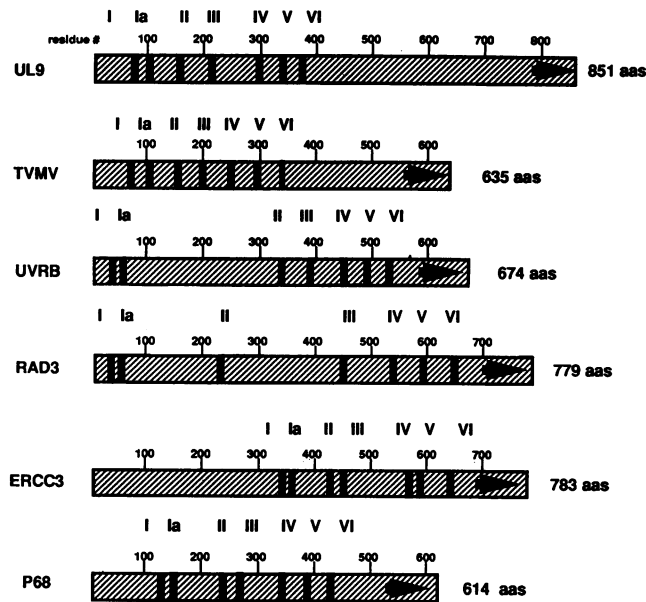


FIG. 1. Conserved motifs in a superfamily of helicases. The number of amino acid residues is listed above each bar. Amino acid sequences from several representatives of helicase superfamily 2 are shown, including HSV UL9; the CI protein of the potyvirus tobacco vein mottling virus (TVMV) (20); UvrB, an *E. coli* protein implicated in excision repair (32); RAD3, a yeast (*S. cerevisiae*) DNA repair protein (32); ERCC-3, a human protein implicated in excision repair and involved in the disorders xeroderma pigmentosum and Cockayne's syndrome (72); and p68, a human protein with RNA helicase activity (35). The seven conserved motifs are represented by dark bars (30, 31, 36). The motifs are numbered as indicated above the UL9 protein, and the order of the motifs in each protein is identical although the spacing varies within this superfamily.

replication and either acts alone to unwind the duplex or interacts with other components of the replication machinery to unwind the template at the origin. Understanding the precise role of UL9 in the initiation of viral DNA replication is important to our understanding of the mechanisms of viral DNA replication and to the development of an *in vitro* system for the study of HSV DNA replication.

Protein sequence analysis indicates that UL9 is a member of a superfamily of proteins (SF2) which include at least 40 established or putative helicases of *E. coli*, yeasts, insects, mammals, poxviruses, and herpesviruses and three groups of positive-strand RNA viruses (31, 32, 37). Seven motifs which are shared among these proteins have been identified. Consistent with their ability to hydrolyze ATP, these proteins all contain two highly conserved motifs known to define a nucleotide-binding domain (motifs I and II in Fig. 1) (71). In addition, four other conserved motifs (III to VI) have been observed in both DNA and RNA helicases. The strong conservation of these six motifs in a large number of RNA and DNA helicases implies that these sequence elements may be important for helicase function; however, no direct evidence exists to demonstrate their functional significance. An additional region of sequence conservation (motif Ia) located between motifs I and II is less well conserved between members of the superfamily and will not be considered further in this paper (29, 37, 45). We report herein our attempts to determine which regions of the UL9 gene are required for its function. A series of mutations were intro-

duced into the UL9 gene in a eukaryotic expression plasmid. Each mutant plasmid was tested in a transient replication complementation assay designed to analyze the replication ability of mutant UL9 gene products. Our results indicate that at least five of the six conserved motifs are important to the function of the UL9 gene product in HSV DNA replication. In addition, deletion and small insertions were introduced into the UL9 gene to further map important functional domains. It is anticipated that this type of structure-function analysis will lead to the identification of protein domains that contribute to the various activities of the protein and will lead to a better understanding of the mechanism of action of this important protein.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, Md.) were propagated and maintained as described previously (76). The KOS strain of HSV-1 was used as the wild-type virus. The *lacZ* insertion mutant *hr94* which does not synthesize the UL9 gene product and the cell lines S22 and 2B-11 which support the growth of UL9 mutants are described elsewhere (6, 47).

Plasmids. p100-1 containing *ori_s* on a 100-bp *MspI* fragment was kindly provided by M. D. Challberg, National Institutes of Health, Bethesda, Md. (53). p6UL9 and p6UL9-119b, which contain the coding sequences of UL9 downstream from the ICP6 regulatory sequences, were constructed as described previously (47). p6UL9 is in the vector Bluescribe (Stratagene, San Diego, Calif.), and p6UL9-119b is in pUC119 (70); both vectors contain an origin of replication from the phage M13 and can thus be packaged as single-stranded DNA in the presence of helper phage M13K07 (60). Recombinant plasmids were propagated in UT481 by standard procedures (49).

Site-directed mutagenesis. Single-stranded DNA from p6UL9 or p6UL9-119b was generated following infection with the helper phage M13K07 by standard procedures (2). Site-directed mutagenesis was performed on single-stranded DNA in which uracil residues had been substituted by using the Mutagenesis kit (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions (40, 41). For some mutants, relevant small fragments were subcloned from p6UL9 into M13mp19 and site-directed mutagenesis was carried out on single strands propagated in *E. coli* CJ236 (*dut*-minus *ung*-minus). Mutations were confirmed as described below and cloned back into the p6UL9 or p6UL9-119b construct for testing. Mutagenic oligonucleotides were synthesized by using a Cyclone DNA synthesizer (Biosearch Inc., Burlington, Mass.) (Table 1). In addition to incorporating the desired base substitution, most oligonucleotides also contained mutations which generated a restriction enzyme site polymorphism without altering the predicted gene product, allowing the identification of mutants by restriction mapping. A deletion (Δ 130-851) removing the C-terminal 721 amino acid residues was constructed by complete digestion of p6UL9 with *SalI* and religation. This deletion removes 527 bp of the UL9 coding sequence and results in a frameshift following the novel junction. An in-frame deletion (Δ 597-703) removing 106 amino acid residues was constructed by using DNA from two mutants, p6UL9-6i and p6UL9-7i (described in Results), as starting material. p6UL9-6i and p6UL9-7i each contain a 6-bp insertion at positions 597 and 703, respectively, resulting in the introduction of a unique *NotI* site into each plasmid. Δ 597-703 was constructed by

TABLE 1. Oligonucleotides used to create motif and insertion mutants

Mutation	Motif no. or position ^a	Oligonucleotide sequence (5'-3') ^b	Restriction site change
Motif			
K87A	I	CGCGGTAGTT <u>GC</u> TCCCGACCCCATgGGCGCGGGACC	Creates <i>Nco</i> I
K87R	I	CGCGGTAGTT <u>CT</u> TCCCGACCCCATgGGCGCGGGACC	Creates <i>Nco</i> I
E175A	II	GGCCAGCGTgGACATAACC <u>CG</u> GTCCAGAACGAG	Abolishes <i>Hinc</i> II
T214S (III)	III	GCGTTGGCGGATGCGTCCATgCGGATGATCC	Abolishes <i>Nco</i> I
T214A (IIIa)	III	GCGTTGGCGG <u>CT</u> GCGTCCATgCGGATGATCC	Abolishes <i>Nco</i> I
A213S (IIIb)	III	TTGGCGGT <u>TA</u> GTCATgCGGATGATCC	Abolishes <i>Nco</i> I
F303W	IV	AGACCGTCGATGAC <u>CAAA</u> TGCAGAT	Abolishes <i>Sal</i> I
G354A	V	GATCGAAAGCTtAGGGCCACGGTTAC	Creates <i>Hind</i> III
R387K	VI	GGAGGGTGGGgACTTTTCCAGGGACT	Abolishes <i>Fsp</i> I
Insertion			
1i	i97AA	AGAGTGGATCGCGG <u>CGCG</u> CTTCCCGCAGCCA	Creates <i>Not</i> I
2i	i195AA	TAGCATTAAAGCGCGGCGCATCCACGCGGCC	Creates <i>Not</i> I
3i	i291AA	GCCAAGGCGCGCGGCGCGCTTCCAGCTCCCC	Creates <i>Not</i> I
4i	i404AA	CTCCGAGCGCGCGGCGCGCCCGGAGCCGTC	Creates <i>Not</i> I
5i	i520AA	GGCCCTGAGGGCGGCGCGCTCGAAATGTAC	Creates <i>Not</i> I
6i	i597AA	GCTGCGGGTGGCGGCGCGCATGGGAAACGCG	Creates <i>Not</i> I
7i	i703AA	GCGCTTGACGGCGGCGCGCTTCTCCGAGAG	Creates <i>Not</i> I
8i	i798AA	CTTGAAGCGCGCGGCGCGCCCGCAAACCTC	Creates <i>Not</i> I

^a For the insertion mutations, the Ala Ala insertion occurs after the indicated amino acid residue.

^b For motif mutations, the underline indicates the base change to create the amino acid change; lowercase letters indicate silent changes to create or abolish restriction sites. For insertion mutations, the underline indicates inserted Ala Ala, creating a unique *Not*I site.

digesting each plasmid with *Not*I and *Eco*RI and religating the appropriate fragments to generate a plasmid with a deletion between the *Not*I site at position 597 in p6UL9-6i and the *Not*I site at position 703 in p6UL9-7i. All the mutations were verified by DNA sequence analysis by using Sequenase and the supplier's instructions (United States Biochemical Corporation, Cleveland, Ohio). Double-stranded plasmid DNA was denatured prior to sequencing as described previously (10).

Transient replication complementation (TRC) assay. Vero cells were transfected with the *ori*_s-containing plasmid p100-1 by itself or cotransfected with wild-type or mutant p6UL9 by using a modification of the standard calcium phosphate coprecipitation procedure (33). Exponential-phase Vero cells (1.5×10^6) were transfected in suspension as described previously (81). At 24 h posttransfection, the medium was changed. At 30 h posttransfection, the cells were superinfected with KOS or *hr*94 at a multiplicity of infection of 10 PFU per cell and incubated at 34°C. At 48 h posttransfection, cells were harvested and total DNA was isolated as described previously (77). DNA (2 µg) was analyzed by digestion with *Eco*RI alone or in combination with *Dpn*I, followed by agarose gel electrophoresis and Southern blot hybridization. Bluescribe or pBR322 DNA was labeled with ³²P as described previously (23).

Transient complementation with plasmids. Vero cells were transfected with 2 µg of wild-type or mutant p6UL9 and 10 µg of salmon sperm DNA. At 30 h posttransfection, cells were superinfected with *hr*94 as described above for the TRC assay. At 48 h posttransfection, the cultures were harvested, frozen and thawed once (-80°C), sonicated, and clarified. Supernatants were assayed on permissive cells (either S22 or 2B11) for total yield and on Vero cells for recombinants. This assay has been called the transient complementation (TC) test.

Detection of UL9 proteins in viral infection and transient transfection by immunoblotting. To detect UL9 protein in cells transiently transfected with the expression clone p6UL9-119b, 1.5×10^6 Vero cells were transfected with 12

µg of the expression plasmid as described previously (28) and plated in a 60-mm tissue culture dish. Induction of the ICP6 promoter was achieved by superinfection at 30 h posttransfection with *hr*94. At 48 h posttransfection, the cells were washed in phosphate-buffered saline (PBS) and resuspended in 400 µl of PBS plus 200 µl of 3× lysis buffer (30 mM Tris-HCl [pH 8.0] 15 mM EDTA [pH 8.0], 3% sodium dodecyl sulfate [SDS]) and chilled on ice for 10 min. The cells were disrupted by sonication, and the proteins were precipitated with 4 volumes of acetone at -20°C for 30 min (69). Proteins were pelleted at 15,000 × g for 15 min at 4°C and resuspended in 160 µl of PBS and 40 µl of 5× loading dye (containing 0.0625 M Tris [pH 6.8], 2% SDS, 50% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue) (43). The samples were heated at 70°C for 15 min, boiled for 10 min, centrifuged to remove any insoluble debris, and subjected to electrophoresis on SDS-9% polyacrylamide gels. The bands were electrophoretically transferred overnight onto polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, Mass.) (in 25 mM Tris [pH 8.3], 192 mM glycine, and 20% methanol) (68). The filters were incubated in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 20% fetal calf serum and 1% bovine serum albumin for 30 min, washed in TBST, and incubated for 3 to 4 h with primary rabbit α-UL9 antibody (generously provided by M. D. Challberg) diluted 1:1,000 in TBST. α-UL9 is a rabbit polyclonal antiserum directed against the 10 C-terminal amino acid residues of UL9 (53). Following several washes in TBST, the blot was incubated for 1 h with secondary antibody (goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate; Protoblot system; Promega, Madison, Wis.). All incubations were done at room temperature with shaking. Alkaline phosphatase color development was carried out as described previously (80).

RESULTS

Functional assay for engineered mutants. Site-directed mutagenesis was used to determine the functional significance

of various regions in the UL9 gene. To facilitate the analysis of these engineered mutations, we used an assay in which mutant forms of the UL9 proteins could be tested directly from cloned expression plasmids. The assay is based on the demonstration that a plasmid bearing an HSV origin of replication (*ori_S* or *ori_L*) can be amplified in a transient transfection experiment if all the necessary *trans*-acting functions are provided. *trans*-acting replication functions can be supplied by superinfection with HSV (65, 66, 77) or by cotransfection with a set of seven cloned HSV genes (8, 78). Viral mutants in any one of the seven required replication proteins are incapable of amplifying an origin-containing plasmid. However, cotransfection with a plasmid expressing the wild-type version of the mutated gene product can complement the defect in the HSV mutant. Thus, the defect in two host range mutants of UL9, *hr27* and *hr156* (7), could be complemented by cotransfection with a plasmid expressing the intact UL9 gene (data not shown). This assay was previously described for use in the analysis of mutations in the HSV-1 UL5 gene and was termed the TRC assay (81).

In this study, a UL9 null mutant, *hr94*, was used as the superinfecting virus in the TRC assay. In *hr94* the UL9 gene has been interrupted with a *lacZ* insertion, and the virus is completely defective for viral growth and DNA synthesis (47). Since no UL9 protein was detected in *hr94*-infected cells, *hr94* is considered a null mutant (47). The use of *hr94* avoids any possible interference or complication from the mutant form of the UL9 protein specified by *hr27* or *hr156*, both of which synthesize stable forms of UL9 containing point mutations (47). The other component of the TRC assay is an expression plasmid for wild-type and mutant forms of UL9. The UL9 gene product in infected cells is expressed at low levels compared with other viral gene products, suggesting that the endogenous promoter of the UL9 gene may be relatively inefficient (54). To increase the expression of UL9, we chose to use the promoter region of ICP6 (the large subunit of the ribonucleotide reductase) because the ICP6 protein is an abundant protein in HSV-infected cells and the ICP6 promoter is inducible upon infection (81). p6UL9 was constructed such that the coding region of the UL9 gene was placed within the 5' untranslated region of the ICP6 gene (47). Expression of UL9 from p6UL9 can be induced in transient transfection experiments by providing transactivating proteins VP16 and/or ICP0 either by cotransfection or by superinfection with *hr94* (47).

A typical TRC assay is shown in Fig. 2. Vero cells or 2B-11 cells, containing the wild-type version of the UL9 gene, were transfected with p100-1, containing *ori_S*, and in some cases also with the UL9 expression clone p6UL9. At 30 h posttransfection, cells were superinfected with KOS or *hr94*. At 48 h posttransfection, total DNA was harvested and digested with *EcoRI* alone (lanes a) or in combination with *DpnI* (lanes b). *EcoRI* cleaves p100-1 once, generating a fragment of 2.8 kb. *DpnI* recognizes only those cleavage sites which have been methylated by the *dam* methylation system of *E. coli*. Thus, plasmid DNA which has been propagated in *E. coli* is sensitive to *DpnI* (Fig. 2, lane 5b), but DNA which has replicated in a mammalian cell is expected to be *DpnI* resistant. When Vero cells were transfected with p100-1 and superinfected with KOS, a *DpnI*-resistant band of 2.8 kb representing linearized p100-1 was observed, indicating that KOS can support the replication of this origin-containing plasmid (Fig. 2, lane 2b). The lack of a 2.8-kb *DpnI*-resistant band in cells transfected with p100-1 and superinfected with *hr94* indicates that this mutant cannot support the replication of p100-1 (Fig. 2, lane 1b). The *DpnI*

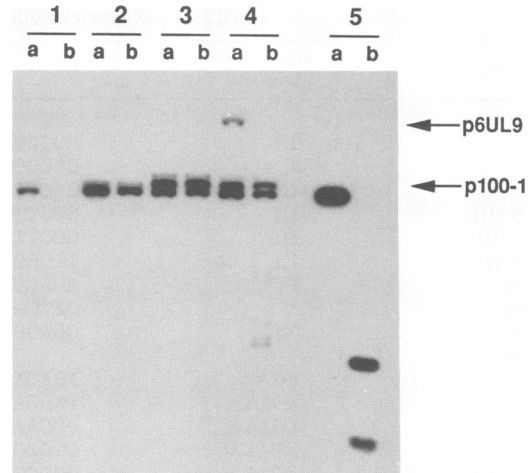


FIG. 2. TRC assay. The TRC assay was performed with either Vero or 2B-11 cells as described in Materials and Methods. At 48 h after transfection, total cellular DNA was isolated. DNA was digested with either *EcoRI* alone (lanes a) or *EcoRI* plus *DpnI* (lanes b); thus, every sample is represented by a pair of lanes (a and b). Lanes 1 to 4 contain 2 μ g of total cellular DNA harvested from the TRC assay as follows: 1, transfection of Vero cells with p100-1 and superinfection with *hr94*; 2, transfection of Vero cells with p100-1 and superinfection with KOS; 3, transfection of 2B-11 cells with p100-1 and superinfection with *hr94*; 4, transfection of Vero cells with p100-1 and p6UL9 and superinfection with *hr94*. Lane 5 contains 5 ng of p100-1 containing HSV-1 *ori_S* propagated in *E. coli*. After restriction enzyme digestion, the samples were subjected to 0.8% agarose gel electrophoresis and blotted onto GeneScreen Plus membrane. The blot was hybridized with ³²P-labeled Bluescribe plasmid. The positions of the linearized p100-1 and the 6.3-kb fragment from p6UL9 are indicated.

digestion fragments representing input plasmid are not seen in this exposure but are seen in longer exposures (data not shown). If a functional UL9 product is supplied either by cotransfection with the UL9 expression clone p6UL9 (lane 4a and b) or by transfecting 2B11 cells which contain a wild-type version of the UL9 gene (lane 3a and b), the defect in *hr94* is complemented. The 6.5-kb band seen with *EcoRI* digestion (lane 4a) represents input p6UL9 plasmid. This plasmid lacks an HSV origin and cannot replicate in Vero cells; therefore, it is sensitive to *DpnI* digestion (lane 4b). The 2.8-kb *DpnI*-resistant bands marked p100-1 in lanes 3b and 4b which are positive for plasmid amplification migrate as a doublet. This doublet is routinely observed when *hr94* is complemented by a functional UL9 gene product expressed either from the cotransfected plasmid p6UL9 (lane 4b) or from the integrated copies of p6UL9 resident in 2B-11 cells (lane 3b); it is not seen in KOS-infected cells (lane 2b). We speculate that this may be due to an alteration in the conformation of replicating plasmids in the presence of an excess of UL9 protein. In summary, the results presented in Fig. 2 indicate that the TRC assay can be used to test the function of UL9 protein expressed from the expression plasmid p6UL9. This assay provides a convenient method for testing the function of UL9 protein expressed from plasmids bearing wild-type and mutant versions of the UL9 gene.

At least five of the six conserved helicase motifs in UL9 are important for function. In this report, the functional significance of the six conserved putative helicase motifs in UL9

was examined by the introduction of engineered mutations into the most conserved residue(s) in each motif.

Motifs I and II. Oligonucleotide mutagenesis was performed on single-stranded DNA from p6UL9 as described in Materials and Methods. Table 1 lists the oligonucleotides used to generate each mutation. Motifs I and II are very well conserved among enzymes which utilize ATP and/or GTP (71) (see Table 2). Motif I (also called the "A" site) containing a sequence (G/AX_nGKS/T) contains a flexible loop between a beta strand and an alpha helix and is believed to be involved in the binding of the PP_i moiety of nucleoside triphosphate (NTP). Through conformational changes induced by ATP binding, this loop would control access to the nucleotide-binding domain (34). The conserved lysine residue is thought to interact directly with one of the phosphate groups of the ATP molecule and therefore be essential for ATPase activity. This lysine is invariant among all members of superfamily 2 (Table 2). To determine the importance of this lysine in UL9, two separate mutations were made at the residue: a conservative change to arginine (K87R) and a change to the small nonpolar amino acid alanine (K87A). Both mutations were constructed to create an *Nco*I site without altering the UL9 sequence (Table 1). The motif I mutations were tested for their ability to complement *hr94* in the TRC assay as described above. As shown in Fig. 3, wild-type UL9 (p6UL9) can complement *hr94* in its ability to support the replication of p100-1 in Vero cells (lane marked p6UL9); however, mutant K87R and K87A fail to do so (lanes as marked). The small *Dpn*I-sensitive fragments (lanes b in Fig. 3) represent input DNA. The loss of UL9 function in K87R and K87A demonstrates the importance of the conserved lysine in motif I.

Motif II contains one or two negatively charged residue(s) (D or DE) at the COOH terminus of a beta strand; the negatively charged residue(s) is believed to interact with the Mg²⁺ associated with the β or γ phosphate of a purine nucleotide substrate (motif II) (25, 42). In molecules whose structure is known, motif II is believed to be close to the glycine-rich flexible loop (motif I) and to form the homing pocket for the hydrophobic adenine moiety of the ATP molecule (3). UL9 and all members of superfamily 2 contain two charged residues, an aspartic acid followed by a glutamic acid (DE) in this motif (Table 2). We targeted this motif by replacing the glutamic acid residue at position 175 with an alanine (E175A), leaving only one charged amino acid residue instead of two (Table 2). The plasmid carrying mutant E175A was unable to complement *hr94* in the replication assay (Fig. 3).

Motifs III to VI. The four other motifs conserved among all members of this superfamily of helicases have no known function at present. Our goal was to assess the functional significance of these conserved residues; thus, we have introduced single amino acid alterations into the most conserved residues in each. Thus, mutations T214S (motif III), F303W (motif IV), G354A (motif V), and R387K (motif VI) were introduced into p6UL9 (Table 2). In each case, a restriction site was either created or abolished (Table 1). Figure 4 shows that mutants in motifs IV, V, and VI are unable to complement *hr94* in the plasmid amplification assay, while the T-to-S mutation in motif III resulted in levels of replication which were compromised compared with those seen with wild-type p6UL9. The partial ability of T214S to complement is perhaps not surprising since one other member of SF2, a vaccinia virus gene product, has a serine in this position (32). To further investigate the importance of motif III, two additional mutations were con-

structed: one in which the threonine at position 214 was changed to an alanine (T214A, also referred to as IIIa) and one in which an alanine at position 213 was changed to a serine (A213S, also referred to as IIIb). Figure 5 shows that, as seen above, T214S is partially compromised for complementation as is A213S; however, mutant T214A totally abolishes plasmid amplification in this assay. The partial replication activity of two of the mutations in motif III (T214S and A213S) were of interest since these represent the first mutations to give a partial phenotype in this type of assay. Since the TRC assay is not quantitative, we used a TC assay to test the ability of the plasmids containing motif mutations to complement *hr94* for virus production; in this assay, yields of *hr94* were measured by plaque assay. Table 3 indicates that the results of this assay are quite consistent with the results of the TRC assay. The complementation index (CI) with no UL9 plasmid is expected to be 1. Plasmid p6UL9-119b bearing wild-type UL9 is capable of complementing *hr94* (CI = 23). Mutant plasmids which fail to complement *hr94* for plasmid amplification (K87A, K87R, E175A, T214A, F303W, G354A, and R387K) give values very close to 1.0 in the TC assay. The two mutants which partially complemented *hr94* in the TRC assay (T214S and A213S) generated intermediate but low CIs. These results indicate not only that results from the TRC and TC assays are consistent, but also that all three mutations in motif III impair the function of UL9 to various degrees of severity.

The amino acid substitutions which result in loss of function of UL9 replication activity could, in theory, be due to two different types of changes: a change in protein conformation or a change in a residue that is involved in function. Changes in structure often affect the stability of proteins (55). To assess whether mutations resulting in loss of function induce gross loss of stability, UL9 protein in cells transiently transfected with wild-type and mutant versions of UL9 was examined. Transfected cells were induced by infection with *hr94*, and extracts were subjected to SDS polyacrylamide gel electrophoresis and immunoblotting as described in Materials and Methods. Transfection with p6UL9 followed by superinfection with *hr94* gives higher yields of protein than cells infected with wild-type virus KOS (Fig. 6). Considering that transfections usually result in less than 5% of the cells taking up DNA and infections can result in 100% infection, it is clear that the ICP6 promoter is efficient. Figure 6 also demonstrates that the motif mutations shown, with the exception of G354A in motif V, result in levels of UL9 comparable to those in p6UL9, indicating that no loss of stability can be observed this way. Of the mutations in motif III, T214S, which results in partial activity in both the TRC and TC assays, is shown in Fig. 6 to induce approximately wild-type levels of UL9 protein; mutant T214A, which abolished activity in the TRC and TC assays, was not able to induce a stable protein, as judged by the absence of detectable UL9 protein, and mutant A213S, which also results in partial activity in both assays, was able to induce wild-type levels of UL9 (data not shown). The absence of detectable UL9 protein in T214A (motif III) and G354A (motif V) indicates that these mutations very likely confer conformational changes on the protein which result in instability.

Insertion and deletion mutations. Although the existence of conserved sequence motifs provides a useful target for mutagenesis, it is also of interest to determine whether other regions of the gene are essential for its function. To this end, a series of linker insertion mutations were introduced into the gene roughly every 100 residues along the UL9 gene.

TABLE 2. Site-directed mutagenesis of the UL9 gene

Gene product ^a	Motif I			Motif II			Motif III			Motif IV			Motif V			Motif VI		
	Position ^b	Sequence	Position	Sequence	Position	Sequence	Position	Sequence	Position	Sequence	Position	Sequence	Position	Sequence	Position	Sequence		
TMV	81	ILMGAVSGSGKSTGLP	164	FDRYQFIIFDFHFVLD	199	IKVSATPPGREGD	248	DNILVYVASYNEVDQLSKM	294	GSSIKKHFIVATNIIIE-NGVTL	342	FISLGERIQRFQRFVGRNK						
UvrB	35	TLLGVTGSGKFTFII	328	LPADGLELVDFESHVTI	389	IYVSATPPGNYLEK	446	ERVLVTLTKRMAEDLTFEY	492	LRLGFEDVLVGINLLF-EGIDM	528	LRSESLIQTIGRAARN						
RAD3 ^c	38	ILEMPSTGKTVSLL	225	VSKDSIVIFDEAHNID	456	LIITSGTISPLDMY	534	DGVVTFPPEYLYMESIVSM	588	ACSNRGAILLVBARQ-EGIDF	655	FDAMREAAQCLGRVLRGK						
ERCC-3	336	VIVLPCGAGKSLVGV	431	TQEWGLMILDEVEHTIP	463	LGLTATLVREDDKI	575	NKPYIYGPPTSQGERMQLIQ	597	KHNPKIINTIFESKVGID-TSFKL	629	GGSRFQEAQRLGRVLRGK						
p68	134	VGVAQTGSGKTLSTYL	238	LRRTYLVLDDEADRML	275	LMSWATWPEVRQ	341	NKTIYVFEIERRCDELTRK	387	FKHGKAPILLIATDVAS-RGLDV	418	FNSSEDIYHRIQRTARSTK						
UL9 ^d	77	VVRAPMGGKFTALI	164	LNNYDVLVLDDEVMSL	208	LAMDATANAQLVD	297	DNICIFSSIVSFAIVARF	337	TTWGGQYRVVYITVVVT-VGLSF	374	GPDMSVSVYQSLGRVETLRK						
Conserved residue ^e	+	G GKT +	+	++++DE H D	+	+TAT 0	+	F B O 0	+	0 0 +++ T0+ G O+	+	0 0 Q +GR R						
Targeted residues		S				AT		Y T		S B		H						
Mutated to		K		E			F			G		R						
		A		A		SS		W		A		K						
		R				A												
Mutant name	K87A		E178A		T214S, T214A	F303W		G364A				R387K						
	K87B				A213B													

^a TMV stands for the CI protein of the potyvirus tobacco vein mottling virus (20); UvrB is an *E. coli* protein implicated in excision repair; RAD3 is a yeast (*Saccharomyces cerevisiae*) DNA repair protein (32); ERCC-3 is a human protein implicated in excision repair and involved in the disorders xeroderma pigmentosum and Cockayne's syndrome (72); p68 is a human protein with RNA helicase activity (35). Most conserved residues are taken from the work of Gorbalenya et al. (32).

^b Numbers refer to the position of each motif within each gene.

^c Underlined residues in RAD3 indicate the positions of mutations which are either moderately or severely UV sensitive (52, 62).

^d Underlined residues in motif IV correspond to alterations observed in *hr27* and *hr156*, two previously described UL9 mutants. Both mutants contain two base changes resulting in the alteration of phenylalanine 309 to tyrosine and glutamic acid 311 to glycine (48).

^e +, hydrophobic residues (I, L, V, M, F, Y, and W); 0 represents charged or polar residues (S, T, D, E, N, Q, K, and R). Conserved segments are numbered I to VI according to the method of Gorbalenya et al. (32).

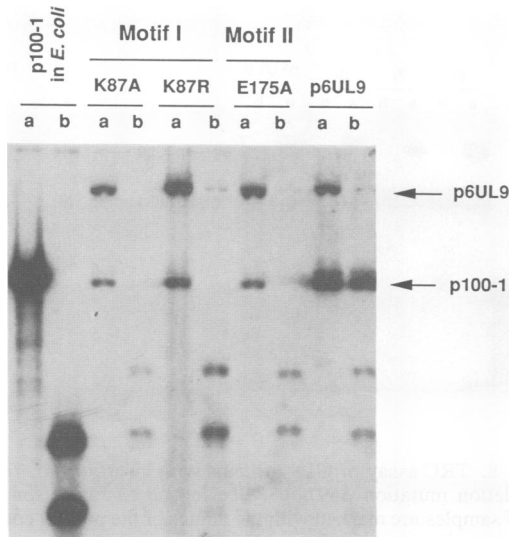


FIG. 3. TRC assay of UL9 proteins with site-directed mutations in motifs I and II. The TRC assay was performed with Vero cells as described in Materials and Methods and in the legend to Fig. 2. In each sample, p100-1 was used in transfection either alone or in combination with p6UL9 containing wild-type UL9 (lane marked p6UL9) or various mutant constructs of p6UL9 as marked. *hr94* was used for superinfection.

Specific oligonucleotides were synthesized for each insertion mutation designed to introduce a unique *NotI* site and to result in the insertion of two alanine residues (Table 1). Two alanines were chosen in an attempt to introduce fairly subtle alterations which would not be expected to grossly alter the structure of the UL9 protein. These linker insertion mutations have generated variable results in the TRC assay (Fig. 7 and 8). Three insertions (1i, 6i, and 7i) are tolerated, as

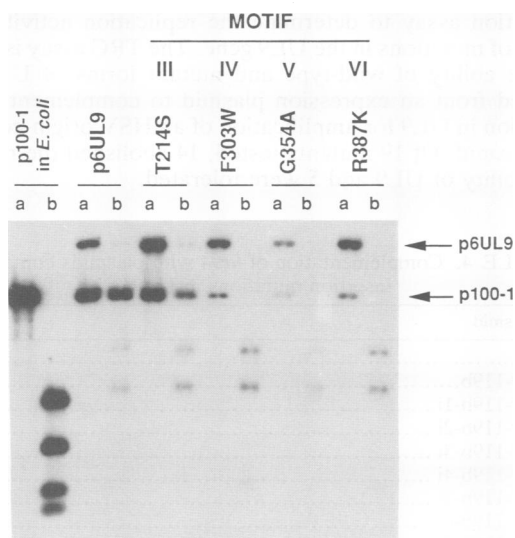


FIG. 4. TRC assay of UL9 proteins with site-directed mutations in motifs III, IV, V, and VI. See the legend to Fig. 2 for the description of the assay. In each sample, p100-1 was used in transfection either alone or in combination with p6UL9 containing wild-type UL9 (lane marked p6UL9) or various mutant constructs of p6UL9 as marked. *hr94* was used for superinfection.

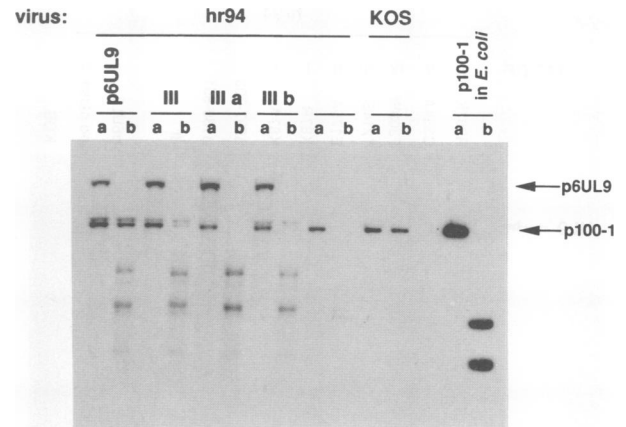


FIG. 5. TRC assay of UL9 proteins with additional mutations in motif III. See legend to Fig. 2 for details of the assay. In each sample, p100-1 was used in transfection either alone or in combination with p6UL9 containing wild-type UL9 (lane marked p6UL9) or various mutant constructs of p6UL9. From the left, pairs under the heading *hr94* contain wild-type, mutant III (T214S), mutant IIIa (T214A), mutant IIIb (A213S), and no UL9 expression plasmid, all superinfected with *hr94* as indicated. The pair under the heading KOS represents cells transfected with p100-1 alone and superinfected with KOS, and the last pair on the right represents p100-1 grown in *E. coli*.

judged by the appearance of wild-type levels of *DpnI*-resistant p100-1. Five insertions (2i, 3i, 4i, 5i, and 8i) result in little or no *DpnI*-resistant DNA at the position of p100-1. Faint bands at the position of *DpnI*-resistant p100-1 in 2i, 3i, 4i, and 5i (Fig. 7) are believed to represent partial digestion products, since they are not seen in duplicate experiments. To confirm that these mutations resulted in loss of function, the TC assay described above was performed. Table 4 demonstrates that the wild type and insertions 1i, 6i and 7i were able to complement the yields of *hr94* efficiently; the other five insertions were unable to do so, thereby confirming the results of the TRC assay. Since the insertion mutations are outside the conserved helicase motifs, these data indicate that other regions of the protein are also important. The N-terminal portion of the gene containing the conserved

TABLE 3. Complementation of *hr94* with plasmids containing motif mutations in UL9^a

Plasmid	CI ^b
None	1.0
p6UL9-119b	23.0
K87A	1.0
K87R	1.0
E175A	1.1
T214S (III)	2.9
T214A (IIIa)	1.1
A213S (IIIb)	4.5
F303W	0.8
G354A	1.3
R387K	0.9

^a Vero cells were transfected with the indicated plasmid and then superinfected with the UL9 *lacZ* insertion mutant *hr94*. The progeny of the transfected-infected cells was assayed for PFUs on S22 or 2B-11 cells. This experiment was repeated three times, and similar results were obtained each time.

^b CI for *hr94* is measured as the PFUs of *hr94* from cultures transfected with the indicated plasmid/PFUs of *hr94* from mock-transfected cultures.

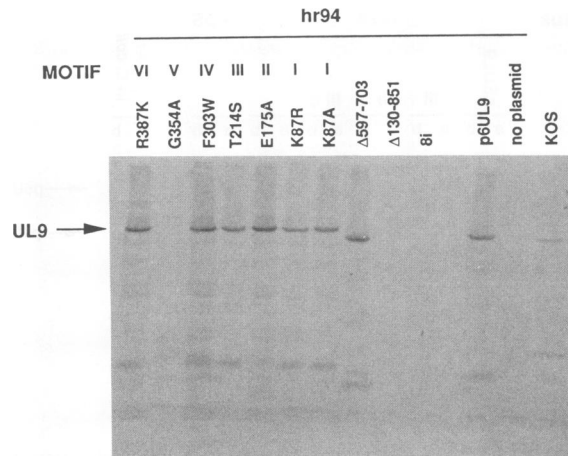


FIG. 6. Detection of wild-type and mutant UL9 proteins in transient transfection by immunoblot with α -UL9. Wild-type or mutant versions of p6UL9 (as marked) (12 μ g) were used to transfect Vero cells which were then superinfected with *hr94*. From left to right, mutations in motifs VI, V, IV, III, and II; two motif I mutations; two deletions (Δ 597-703 and Δ 130-851); insertion 8i; wild-type p6UL9; and no plasmid are shown. In the lane labeled no plasmid, 12 μ g of salmon sperm DNA was used. In the far right lane, KOS-infected cell extracts are shown for comparison. Transfected cells were processed for immunoblot as described in Materials and Methods.

helicase motifs appears to be slightly more sensitive to insertions than the C terminus. Two deletions removing 721 and 106 residues (Δ 130-851 and Δ 597-703, respectively) which also abolish the replication ability of these plasmids (Fig. 7 and 8, lanes as marked) were also constructed.

To assess whether the insertion and deletion mutations described above alter the conformation of the UL9 protein drastically enough to affect stability, Vero cells were transiently transfected with each construct and superinfected with *hr94* to induce the ICP6 promoter as described above. Extracts were subjected to electrophoresis and immunoblotting as described in Materials and Methods. Figures 6 and 9 show that all insertion mutations, with the exception of 8i,

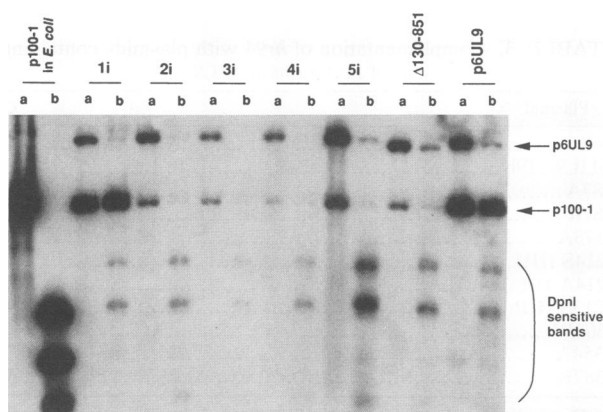


FIG. 7. TRC assay of UL9 proteins with insertions 1i, 2i, 3i, 4i, and 5i and deletion mutation Δ 130-851. See legend to Fig. 2 for details. Pairs of samples are marked with the names of the p6UL9 constructs used in transient transfections. Small fragments resulting from *DpnI* digestion are marked.

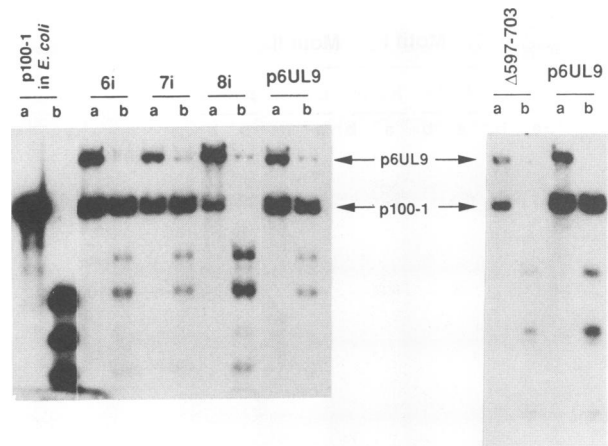


FIG. 8. TRC assay of UL9 proteins with insertions 6i, 7i, and 8i and deletion mutation Δ 597-703. See legend to Fig. 2 for details. Pairs of samples are marked with the names of the p6UL9 constructs used in transient transfections.

were capable of producing a stable protein. Deletion Δ 597-703 produced a version slightly smaller than full length as expected from the size of the deletion. No band is seen in Δ 130-851 as expected since the predicted UL9 gene product would not react with the antibody directed to the C terminus (Fig. 6). In summary, these results suggest that the loss of function seen in most of the mutant constructs is most likely due to changes in residues involved in UL9 function rather than disruptions which cause instability; however, the mutations T214A and G354A (in motifs III and V, respectively) and the insertion at 8i all apparently alter the stability of the resulting UL9 gene product.

DISCUSSION

In this report, we describe the use of an in vivo complementation assay to determine the replication activity of a series of mutations in the UL9 gene. The TRC assay is based on the ability of wild-type and mutant forms of UL9 expressed from an expression plasmid to complement a null mutation in UL9 for amplification of an HSV origin-containing plasmid. Of 19 mutants tested, 14 abolished the replication ability of UL9 and 5 were tolerated.

TABLE 4. Complementation of *hr94* with plasmids containing insertion mutations in UL9^a

Plasmid	CI ^b
None	1.0
p6UL9-119b	106
p6UL9-119b-1i	18
p6UL9-119b-2i	0.6
p6UL9-119b-3i	0.9
p6UL9-119b-4i	1.7
p6UL9-119b-5i	0.5
p6UL9-119b-6i	131
p6UL9-119b-7i	88
p6UL9-119b-8i	1.3

^a Vero cells were transfected with the indicated plasmid and then superinfected with the UL9 *lacZ* insertion mutant *hr94*. The progeny of the transfected-infected cells was assayed for PFU on S22 or 2B-11 cells.

^b CI for *hr94* is measured as PFUs of *hr94* from cultures transfected with the indicated plasmid/PFUs of *hr94* from mock-transfected cultured.

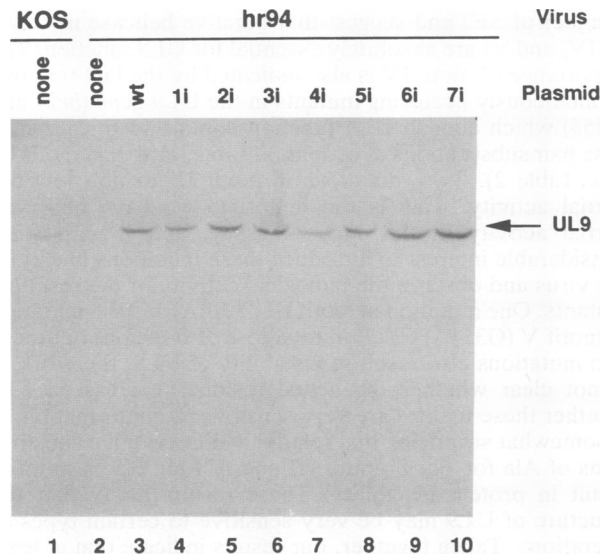


FIG. 9. Detection of wild-type and mutant UL9 proteins in transient transfection by immunoblot with α -UL9. Wild-type and mutant versions of UL9 are shown as described in the legend to Fig. 6. KOS-infected cell extract is shown in lane 1. From left to right (lanes 2 to 10, respectively) are extracts from cells transfected with the indicated plasmid and superinfected with *hr94*: no plasmid, wild-type pUL9 (wt), 1i, 2i, 3i, 4i, 5i, 6i, and 7i.

Five of six conserved helicase domains as well as other regions of the UL9 gene are important for function. The predicted protein sequence indicates that UL9 contains a set of motifs conserved among a large number of known and putative helicases from *E. coli*, yeasts, eukaryotes, and viruses. These six motifs are located within the first 400 amino acid residues of the UL9 gene (Fig. 1). Certain residues within these motifs are well conserved in all members of SF2 (Table 2); however, the sequence similarity is even more striking within other UL9 homologs within the herpesvirus family. Table 5 shows the sequences of the conserved motifs in varicella-zoster virus gene 51 (15) and in the human herpesvirus type 6 gene CH6R (14). In this paper, we have addressed the functional significance of these conserved regions in the UL9 gene by introducing single amino acid substitutions into the most conserved residues of each motif. The results suggest that at least five of the six conserved motifs are important to the function of the UL9 protein in viral DNA replication (see below).

The results of insertion and deletion mutagenesis indicate that several other regions outside the conserved helicase motifs are also required for replication activity. Insertions 2i, 3i, 4i, 5i, and 8i all abolished the ability of UL9 to function in

the TRC assay. Although 8i is probably nonfunctional because of instability of the mutant protein, it is unclear why the other four mutants have abolished activity. None of these mutations coincide exactly with the helicase motifs themselves, although 2i and 3i are nearby motifs II and IV, respectively. It is possible that the spacing between the various helicase motifs in UL9 is sensitive to insertion because of rigid requirements in the surrounding secondary and tertiary structures. Another possibility is that the regions outside the motifs which are sensitive to insertion represent functional regions for other activities displayed by UL9, such as protein-protein interaction, nuclear localization, or a previously unidentified function of UL9. UL9 is known to contain a putative leucine zipper, an amino acid sequence motif common to several DNA-binding proteins and believed to signal a region of protein-protein interactions (44). The leucine zipper overlaps motif II in the putative helicase domain. Preliminary results with mutations with alterations in the leucines of the leucine zipper suggest that these leucines are essential for UL9 function (61). Whether the leucine zipper represents a site for dimerization or for interactions with other members of the replication complex remains to be elucidated. Weir et al. demonstrated that the carboxy-terminal 317 amino acids contain all the information necessary for origin-specific DNA binding (73). The DNA-binding region was subsequently narrowed to the carboxy-terminal 269 amino acids (residues 564 to 832) (18). Insertion mutations 6i and 7i at residues 597 and 703, respectively, were tolerated for UL9 function, but the deletion spanning this region (Δ 597-703) is not tolerated. Since Δ 597-703 specifies a stable protein, we conclude that an essential region is located within the region defined by residues 597 and 703. We speculate that within this region are signals which could be involved in specific DNA binding. Further biochemical characterization of existing mutants and isolation of additional mutations across the UL9 gene will be required for a more complete understanding of the domain structure of the multifunctional UL9 protein.

Relationship of UL9 to other superfamilies of helicases. UL9 and other members of SF2 are related to but distinct from another superfamily of putative helicases (superfamily 1) characterized by a different set of prokaryotic, eukaryotic, and viral helicases (including HSV-1 UL5 and *E. coli* genes *uvrD*, *rep*, *recB*, and *recD*) (36, 37, 81). The conserved helicase motifs in UL5 are dispersed throughout the entire gene (31, 81), whereas in UL9 they are confined to the first 400 amino acids (see Fig. 1). Many members of SF2 appear to be multidomain proteins in which the putative helicase motifs are confined to the N-terminal portion of the molecule. For instance, in many RNA viruses such as the potex-, tobamo-, flavi-, pesti-, alpha-, and potyviruses, the SF2 family members are multidomain proteins in which the

TABLE 5. Alignment of putative UL9 homologs in HSV-1, varicella-zoster virus, and human herpesvirus type 6

Gene	Motif I	Motif II	Motif III	Motif IV	Motif V	Motif VI
HSV UL9	VVRAPMGSQKTTAL	LNNYDVLVLDEVMSTL	IAMDATANAQLVD	NICIFSSSTVSTAE	VVIYTTVVTVGLSFD	GPDMSVSVYQSLGRVRLRK
VZV 51	VVRAPMGSQKTTAL	IDSYDVLILDEVMSVI	IAMDATVNSQFID	NICIFSSSTLSFSE	VLVYTTVVTVGLSFD	GPDMSVSVYQSLGRVRLLL
HHV-6 CH6R	LVRAPMGSQKTTAL	TENYDVLILDEIMSI	IAMDATLREHVVE	KLGLFCSTVLAAE	VVIYTSVTVGLSFE	GPDMSVSVYQSLGRVRRVID
Consensus sequence	*VRA* MGSQKTTAL	*Y DVL* LDE *MS	* IAMDA T	**h**	**C* F*ST**	*E V**YT* VVTVGLSF * GPDMSVSV*QS*GRVR *

* Alignment of the HSV-1 origin-binding protein UL9, the varicella-zoster virus origin-binding protein gene 51 (VZV 51) (15) and the putative human herpesvirus type 6 (HHV-6) origin-binding protein encoded by the open reading frame CH6R (14). Motifs are those predicted by Gorbalenya et al. (31). h represents hydrophobic residues (FYIVLM); * refers to residues shared by two of the three genes.

conserved region of the putative helicases is localized to the N terminus and the C-terminal portion contains regions conserved among polymerase molecules (32). The demonstration that at least five of the six putative helicase domains located within the N-terminal 400 residues of UL9 are important for UL9 function in replication of origin-containing plasmids strongly suggests that this region is involved in UL9 helicase activity. Whether this region makes up a discrete domain which is sufficient for helicase activity remains to be determined. As described above, the DNA-binding region has been located to the carboxy-terminal 269 amino acids (residues 564 to 832) (18). Taken together, these results suggest that UL9 may contain at least two functional domains, a helicase domain at the N terminus and a DNA-binding domain at the C terminus.

Roles of motifs I to VI. For some time, motifs I and II have been recognized as important for NTP binding on the basis of not only nuclear magnetic resonance and X-ray diffraction data (25, 42) but also the results of site-directed mutagenesis aimed at one or more of the conserved residues. For instance, mutational analysis of motif I in other ATP-binding proteins showing ATPase activity indicates that substitutions at the conserved lysine residue generally destroy the ATPase activity and in some but not all cases leave ATP binding intact, depending on the nature of the changed residue (3). For instance, mutation of the invariant lysine to arginine in motif I of the yeast excision repair protein RAD3 (a member of SF2) resulted in a protein which could still bind ATP but was defective in hydrolysis (67). Some motif I mutations appear to affect binding of NTPs per se; for instance, a threonine-to-serine replacement in motif I of the HSV thymidine kinase exhibited an altered K_m for thymidine and ATP (46). Thus, motif I has been strongly implicated in binding and hydrolysis of NTPs in several known NTP-binding proteins. Our studies are in agreement with those previous studies in that the replacement of the invariant lysine in motif I of UL9 with either an arginine or an alanine totally abolishes the ability of the mutant form of UL9 to carry out *in vivo* complementation of HSV DNA replication.

Motif II has been less well studied; however, in two studies, substitution of the invariant Asp residue in both the *E. coli* F1-ATPase and a thermophilic ATPase resulted in a greater-than-90% loss of ATPase activity (1, 79). In RAD3, a Glu-to-Lys substitution within motif II resulted in extreme UV sensitivity and an increased rate of spontaneous mutation, indicating that this motif is important for excision repair and perhaps postreplicative mismatch correction (62). In UL9, motif II contains a DE pair and substitution of the Glu by an Ala (E175A) was sufficient to abolish UL9 function in the *in vivo* assay, confirming the importance of this motif.

The importance of motifs III to VI within SF2 has received little attention to date, and few mutations have been reported. To our knowledge, the only other member of SF2 in which mutations in motifs III to VI have been reported is RAD3. Among eleven RAD3 mutants whose mutations have been mapped which either severely or moderately affect UV sensitivity, the positions of all eleven correspond exactly to one of the conserved motifs as described by Gorbalenya et al. (32) (two in motif I, two in motif II, three in motif III, two in motif IV, two in motif V, and one in motif VI); the positions of the UV-sensitive mutations are indicated in Table 2 as underlined residues (52, 62). Several of these are also associated with an increased spontaneous mutation rate. These results indicate the functional importance of the conserved helicase motifs of SF2. The mutations presented in this report represent the second mutational analysis of a

member of SF2 and suggest that putative helicase motifs I, II, IV, and VI are absolutely essential for UL9 function. The importance of motif IV is also indicated by the fact that two spontaneously occurring mutants in the UL9 gene (*hr27* and *hr156*) which abolish UL9 function contain identical single base pair substitutions at codons 309 and 311 within motif IV (see Table 2). Two mutations in motif III exhibit low but partial activity. This is the first time we have observed partial activity in this type of assay, and it will be of considerable interest to introduce these mutations back into the virus and observe the biological activity of the resulting mutants. One mutation in motif III (T214A) and the mutation in motif V (G354A) result in total loss of function, but these two mutations also result in instability of UL9; therefore, it is not clear whether the actual residues are essential or whether these regions are very sensitive to conformation. It is somewhat surprising that relatively conservative substitutions of Ala for Thr in motif III and Ala for Gly in motif V result in protein instability. These results imply that the structure of UL9 may be very sensitive to certain types of alterations. Taken together, our results indicate that at least five of the six helicase motifs are important for UL9 function. Thus, although unwinding of duplex DNA sequences containing the HSV origin of replication has not been demonstrated with purified UL9 protein (24), the genetic data in this report indicate that, in fact, the helicase motifs are important for its function. It is possible that other viral or cellular factors will be required in order to observe duplex unwinding by UL9.

Our goal was to determine the roles of the residues in motifs I to VI in the overall function of UL9. In addition to an NTP-binding fold, it is anticipated that a helicase might contain several other functional regions, including one which contacts the nucleic acid substrate. Motif VI is made up of several basic residues, and it is tempting to speculate that this region may be involved in either nuclear localization or nonspecific DNA binding or both. UL9 has been expressed in a functional form in *E. coli* (48), and the mutations described in this report will be transferred to the *E. coli* expression system and tested for various biochemical activities, including NTP binding, DNA-dependent NTPase, nuclear localization, ability to dimerize, specific and nonspecific DNA-binding, and helicase activities. In this report, we have initiated a structure-function analysis of the UL9 gene and laid the groundwork for a much more detailed analysis of functional and biological aspects of UL9, which will lead to the identification of domains responsible for its various activities. It is anticipated that the isolation of mutants which can separate these functional domains will facilitate efforts to elucidate the precise role of the UL9 protein in the initiation of DNA replication.

ACKNOWLEDGMENTS

We are grateful to M. Challberg for providing origin-containing plasmids and antisera to UL9. We gratefully acknowledge contributions to this project at its initial stages from E. Carmichael. We also thank E. Carmichael and D. Dorsky for helpful comments on the manuscript.

This investigation was supported by Public Health Service grant AI21747 and American Heart Association grant-in-aid 880731. S.K.W. is the recipient of an American Heart Association-Genentech Established Investigator Award.

REFERENCES

1. Al-Shawi, M. K., D. Parsonage, and A. E. Senior. 1988. Directed mutagenesis of the strongly conserved aspartate 242 in the

- β -subunit of Escherichia coli proton-ATPase. *J. Biol. Chem.* **263**:19633–19639.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1990. Current protocols in molecular biology. John Wiley & Sons, New York.
 3. Azzaria, M., E. Schurr, and P. Gros. 1989. Discrete mutations introduced in the predicted nucleotide-binding sites of the *mdr1* gene abolish its ability to confer multidrug resistance. *Mol. Cell. Biol.* **9**:5289–5297.
 4. Bramhill, D., and A. Kornberg. 1988. A model for initiation at origins of DNA replication. *Cell* **54**:915–918.
 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. Carmichael, E. P., M. J. Kosovsky, and S. K. Weller. 1988. Isolation and characterization of herpes simplex virus type 1 host range mutants defective in viral DNA synthesis. *J. Virol.* **62**:91–99.
 7. Carmichael, E. P., and S. K. Weller. 1989. Herpes simplex virus type 1 DNA synthesis requires the product of the UL8 gene: isolation and characterization of an ICP6::lacZ insertion mutation. *J. Virol.* **63**:591–599.
 8. Challberg, M. D. 1986. A method for identifying the viral genes required for herpesvirus DNA replication. *Proc. Natl. Acad. Sci. USA* **83**:9094–9098.
 9. Chartrand, P., C. S. Crumpacker, P. A. Schaffer, and N. M. Wilkie. 1980. Physical and genetic analysis of the herpes simplex virus DNA polymerase locus. *Virology* **103**:311–326.
 10. Chen, E. Y., and P. H. Seeburg. 1985. Supercoil sequencing: a fast and simple method for sequencing DNA. *DNA* **4**:165–170.
 11. Coen, D. M., D. P. Aschman, P. T. Gelep, M. J. Retondo, S. K. Weller, and P. A. Schaffer. 1984. Fine mapping and molecular cloning of mutations in the herpes simplex virus DNA polymerase locus. *J. Virol.* **49**:236–247.
 12. Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman. 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. *J. Virol.* **37**:191–206.
 13. Crute, J. J., T. Tsurumi, L. Zhu, S. K. Weller, P. D. Olivo, M. D. Challberg, E. S. Mocarski, and I. R. Lehman. 1989. Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. *Proc. Natl. Acad. Sci. USA* **86**:2186–2189.
 14. Dambaugh, T. R. 1992. Personal communication.
 15. Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* **67**:1759–1816.
 16. Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz. 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. Natl. Acad. Sci. USA* **84**:16–20.
 17. Dean, F. B., M. Dodson, H. Echols, and J. Hurwitz. 1987. ATP-dependent formation of a specialized nucleoprotein structure by simian virus 40 (SV40) large tumor antigen at the SV40 replication origin. *Proc. Natl. Acad. Sci. USA* **84**:8981–8985.
 18. Deb, S., and S. P. Deb. 1991. A 269-amino-acid segment with a pseudo-leucine zipper and a helix-turn motif codes for the sequence-specific DNA-binding domain of herpes simplex virus type 1 origin-binding protein. *J. Virol.* **65**:2829–2838.
 19. Dodson, M., F. B. Dean, P. Bullock, H. Echols, and J. Hurwitz. 1987. Unwinding of duplex DNA from the SV40 origin of replication by T antigen. *Science* **238**:964–967.
 20. Domier, L. L., K. M. Franklin, M. Shahabuddin, G. M. Hellmann, J. H. Overmeyer, S. T. Hiremath, M. F. E. Siaw, G. P. Lomonosoff, J. G. Shaw, and R. E. Rhoads. 1986. The nucleotide sequence of tobacco vein mottling virus RNA. *Nucleic Acids Res.* **14**:5417–5430.
 21. 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**:17167–17173.
 22. Elias, P., and I. R. Lehman. 1988. Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. *Proc. Natl. Acad. Sci. USA* **85**:2959–2963.
 23. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* **132**:6–13.
 24. Fierer, D. S., and M. D. Challberg. 1992. Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. *J. Virol.* **66**:3986–3995.
 25. Fry, D. C., S. A. Kuby, and A. S. Mildvan. 1986. ATP-binding site of adenylate kinase: mechanistic implications of its homology with *ras*-encoded p21, F₁-ATPase, and other nucleotide-binding proteins. *Proc. Natl. Acad. Sci. USA* **83**:907–911.
 26. Gallo, M. L., D. I. Dorsky, C. S. Crumpacker, and D. S. Parris. 1989. The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. *J. Virol.* **63**:5023–5029.
 27. Goldstein, D. J., and S. K. Weller. 1988. An ICP6::lacZ insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. *J. Virol.* **62**:2970–2977.
 28. Goldstein, D. J., and S. K. Weller. 1988. Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. *Virology* **166**:41–51.
 29. Gorbalenya, A. E., and E. V. Koonin. 1988. One more conserved sequence motif in helicases. *Nucleic Acids Res.* **16**:7734.
 30. Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov. 1988. A conserved NTP-motif in putative helicases. *Nature (London)* **333**:22–23.
 31. 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.
 32. 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.
 33. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467.
 34. Higgins, D. F., M. P. Gallagher, M. L. Mimmack, and S. R. Pearce. 1988. A family of closely related ATP-binding subunits from prokaryotic and eukaryotic cells. *Bioessays* **8**:111–116.
 35. Hloch, P., G. Scheidner, and H. Stahl. 1990. Complete cDNA sequence of the human p68 protein. *Nucleic Acids Res.* **18**:3045.
 36. Hodgman, T. C. 1988. A new superfamily of replicative proteins. *Nature (London)* **333**:22–23.
 37. Hodgman, T. C. 1988. A new superfamily of replicative proteins. *Nature (London)* **333**:578. (Erratum.)
 38. Koff, A., J. F. Schwedes, and P. Tegtmeyer. 1991. Herpes simplex virus origin-binding protein (UL9) loops and distorts the viral replication origin. *J. Virol.* **65**:3284–3292.
 39. Koff, A., and P. Tegtmeyer. 1988. Characterization of major recognition sequences for a herpes simplex virus type 1 origin-binding protein. *J. Virol.* **62**:4096–4103.
 40. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
 41. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
 42. la Cour, T. F. M., J. Nyborg, S. Thirup, and B. F. C. Clark. 1985. Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by X-ray crystallography. *EMBO J.* **4**:2385–2388.
 43. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 44. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* **240**:1759–1764.

45. Lane, D. 1988. Enlarged family of putative helicases. *Nature (London)* **334**:478. (Letter.)
46. Liu, Q., and W. C. Summers. 1988. Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. *Virology* **163**:638-642.
47. Malik, A. K., R. Martinez, L. Muncy, E. P. Carmichael, and S. K. Weller. Genetic analysis of mutations in the HSV-1 UL9 origin specific DNA binding protein: isolation of an ICP6::lacZ insertion mutant. *Virology*, in press.
48. Malik, A. K., and S. K. Weller. 1992. Unpublished results.
49. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. Marchetti, M. E., C. A. Smith, and P. A. Schaffer. 1988. A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. *J. Virol.* **62**:715-721.
51. Matz, B., J. H. Subak-Sharpe, and V. G. Preston. 1983. Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 using cloned restriction endonuclease fragments. *J. Gen. Virol.* **64**:2261-2270.
52. Naumovski, L., and E. C. Friedberg. 1986. Analysis of the essential and excision repair functions of the *RAD3* gene of *Saccharomyces cerevisiae* by mutagenesis. *Mol. Cell. Biol.* **6**:1218-1227.
53. 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.
54. 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.
55. Pakula, A. A., and R. T. Sauer. 1989. Genetic analysis of protein stability and function. *Annu. Rev. Genet.* **23**:289-310.
56. Parris, D. S., A. Cross, L. Haarr, A. Orr, M. C. Frame, M. Murphy, D. J. McGeoch, and H. S. Marsden. 1988. Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. *J. Virol.* **62**:818-825.
57. Purifoy, D. J. M., R. B. Lewis, and K. L. Powell. 1977. Identification of the herpes simplex virus DNA polymerase gene. *Nature (London)* **269**:621-623.
58. Purifoy, D. J. M., and K. L. Powell. 1981. Temperature-sensitive mutants in two distinct complementation groups of herpes simplex virus type 1 specify thermolabile DNA polymerase. *J. Gen. Virol.* **54**:219-222.
59. Rabkin, S. D., and B. Hanlon. 1991. Nucleoprotein complex formed between herpes simplex virus UL9 protein and the origin of DNA replication: inter- and intramolecular interactions. *Proc. Natl. Acad. Sci. USA* **88**:10946-10950.
60. Russell, M., S. Kidd, and M. R. Kelley. 1986. An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene* **45**:333-338.
61. Shao, L., and S. K. Weller. 1992. Unpublished results.
62. Song, J. M., B. A. Montelone, W. Siede, and E. C. Friedberg. 1990. Effects of multiple yeast *rad3* mutant alleles on UV sensitivity, mutability, and mitotic recombination. *J. Bacteriol.* **172**:6620-6630.
63. Spaete, R. R., and N. Frenkel. 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**:295-304.
64. Stahl, H., P. Droege, and R. Knippers. 1986. DNA helicase activity of SV40 large tumor antigen. *EMBO J.* **5**:1939-1944.
65. 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.
66. Stow, N. D., and E. C. Monagle. 1983. Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130**:427-438.
67. Sung, P., D. Higgins, L. Prakash, and S. Prakash. 1988. Mutation of lysine-48 to arginine in the yeast *RAD3* protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. *EMBO J.* **7**:3263-3269.
68. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
69. Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight. 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* **2**:718-729.
70. Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153**:3-11.
71. 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.
72. Weeda, G., R. C. A. van Ham, W. Vermeulen, D. Bootsma, A. J. van der Eb, and J. H. J. Hoeijmakers. 1990. A presumed DNA helicase encoded by *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. *Cell* **62**:777-791.
73. Weir, H. M., J. M. Calder, and N. D. Stow. 1989. Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. *Nucleic Acids Res.* **17**:1409-1425.
74. Weller, S. K. 1990. Genetic analysis of HSV genes required for genome replication, p. 105-135. *In* E. Wagner (ed.), Herpesvirus transcription and its regulation. CRC Press, Boca Raton, Fla.
75. Weller, S. K., E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer. 1987. Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of HSV-1 U_L DNA. *Virology* **161**:198-210.
76. 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.
77. 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 *ori_L*, a herpes simplex virus type 1 origin of DNA synthesis. *Mol. Cell. Biol.* **5**:930-942.
78. Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg. 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *J. Virol.* **62**:435-443.
79. Yohda, M., S. Ohta, T. Hisabori, and Y. Kagawa. 1988. Site-directed mutagenesis of stable adenosine triphosphate synthase. *Biochim. Biophys. Acta* **933**:156-164.
80. 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.
81. Zhu, L., and S. K. Weller. 1992. The six conserved helicase motifs of the UL5 gene product, a component of the herpes simplex virus type 1 helicase-primase, are essential for its function. *J. Virol.* **66**:469-479.