Residues within the Conserved Helicase Motifs of UL9, the Origin-binding Protein of Herpes Simplex Virus-1, Are Essential for Helicase Activity but Not for Dimerization or Origin Binding Activity*

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UL9, an essential gene for herpes simplex virus type 1 (HSV-1) DNA replication, exhibits helicase and origin DNA binding activities. It has been hypothesized that UL9 binds and unwinds the HSV-1 origin of replication, creating a replication bubble and promoting the assembly of the viral replication machinery; however, direct confirmation of this hypothesis has not been possible. Based on the presence of conserved helicase motifs, UL9 has been classified as a superfamily II helicase. Mutations in conserved residues of the helicase motifs I–VI of UL9 have been isolated, and most of them fail to complement a UL9 null virus in vivo (Martinez R., Shao L., and Weller S. (1992) J. Virol. 66, 6735–6746). In addition, mutants in motifs I, II, and VI were found to be transdominant (Malik, A. K., and Weller, S. K. (1996) J. Virol. 70, 7859–7866). Here we present the characterization of the biochemical properties of the UL9 helicase motif mutants. We report that mutations in motifs I–IV and VI affect the ATPase activity, and all but the motif III mutation completely abolish the helicase activity. In addition, mutations in these motifs do not interfere with UL9 dimerization or the ability of UL9 to bind the HSV-1 origin of replication. Based on the similarity of the helicase motif sequences between UL9 and UvrB, another superfamily II member with helicase-like activity, we were able to map the UL9 mutations on the structure of the UvrB protein and provide an explanation for the observed phenotypes. Our results indicate that the helicase function of UL9 is indispensable for viral replication, supporting the hypothesis that UL9 is essential for unwinding the HSV-1 origin of replication in vivo. Furthermore, the data presented provide insights into the mechanism of transdominance of the UL9 helicase motif mutants.

Helicases, enzymes that unwind double-stranded nucleic acids (DNA, RNA, or DNA/RNA hybrids), function in essentially every cellular process involving nucleic acids (1, 2). Nucleic acid unwinding is a dynamic process involving ATP binding/hydrolysis, hydrogen bond breakage, and translocation of the helicase along single-stranded nucleic acid, accompanied by conformational changes in the protein molecule itself (1–3). Despite their diversity with respect to sequence, size, oligomeric state, and substrate preference, all helicases possess conserved motifs proposed to play pivotal roles in helicase activity (4). Two superfamilies of helicases, SFI and SFII, have been characterized by the presence of seven conserved motifs. Between these two superfamilies, motifs I and II are well conserved; however, motifs Ia and III–VI exhibit very limited homology (3). Despite this limited sequence similarity, crystallographic information shows remarkable structural similarity between helicases from SFI and SFII (4–7). The structures of helicases from both superfamilies (Rep from Escherichia coli (2), PcrA from Bacillus stearothermophilus (1), NS3 helicase from hepatitis C virus (8–10), UvrB from Bacillus caldodanax (11) and Thermus thermophilus (12), and eIF4a from Saccharomyces cerevisiae (13)) reveal the presence of two RecA-like domains. The conserved helicase motifs are positioned along a cleft that runs between the two Rec A-like domains comprising an ATP-binding site. The adenine moiety of ATP is positioned at the base of the ATP-binding cleft, and phosphates protrude upward inside the cleft. ATP binding in the cleft results in conformational changes leading to cleft closure (8, 14, 15). Many helicases require a divalent metal ion, preferably Mg2+, that is believed to play an important role in the correct positioning of the ATP phosphates in the binding site as well as for the destabilization of the P–O bond, facilitating hydrolysis (16). A conserved lysine from the GS TGKT (motif I) was shown in the crystal structures of several helicases to coordinate ATP β/γ-phosphates, whereas a conserved glutamate from motif II is thought to activate a water molecule involved directly in catalysis of the ATP hydrolysis (14, 17).

The mechanism of helicase action is believed to involve the coupling between several steps including ATP binding, ATP hydrolysis, and binding to nucleic acid. The Rep, PcrA, and NS3 helicase structures have also been solved in complexes with nucleic acids as follows: Rep with ssDNA (2), PcrA with partially dsDNA (18), and the NS3 helicase with ssRNA (8). In all three structures, single-stranded nucleic acid was observed to bind in a cleft almost perpendicular to the ATP-binding site, positioned at the top of the RecA-like domains. Structural and

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1 The abbreviations used are: SFI, superfamily I; SFII, superfamily II; HSV-1, herpes simplex virus, type 1; ssDNA, single-stranded DNA; PMSF, phenylmethylsulfonyl fluoride; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin.

2 In this paper the term “helicase motifs” is used to refer to motifs shared between large number of superfamily II members. It should be noted, however, that many SFII members have not been shown experimentally to exhibit helicase activity despite the presence of these “motifs.”
genetic data suggest that residues from motifs Ia, III, and V are involved in ssDNA/RNA binding (2, 8, 18). Motifs III–VI have been implicated in the coupling between the ATPase and helicase activities and/or in the coupling between ATPase and ssDNA binding activities (8, 19–22).

The UL9 gene is essential for herpes simplex virus type 1 (HSV-1) replication in vivo (23) and exhibits helicase and origin binding activities in vitro (24, 25). UL9 is composed of at least two domains (Fig. 1) as follows: a C-terminal origin-binding domain (residues 535–851) and an N-terminal helicase domain (residues 1–535) that contains the SP1I-conserved helicase motifs (3). Each domain expressed separately exhibits the expected biochemical activities (26, 27). UL9 has been hypothesized to bind and unwind the HSV-1 origin of replication, creating a replication bubble and promoting the assembly of the viral replication machinery (28–30); however, direct confirmation of this hypothesis has been lacking. The importance of UL9 in HSV-1 replication in vivo has been supported by genetic experiments (23, 31). Studies with temperature-sensitive mutants demonstrated the importance of UL9 during the early stages of viral infection (31). The overexpression of wild type UL9 or the origin-binding domain by itself is inhibitory to HSV-1 replication (32–35). The mechanism of HSV-1 origin unwinding is poorly understood, and it has not been possible to demonstrate the unwinding of duplex origin-containing plasmids in vitro. Previous attempts to unwind oriS-containing helicase substrates in vitro have revealed the requirement for the presence of ssDNA near the origin; moreover, unwinding could be observed only in the presence of HSV-1 ssDNA-binding protein, ICP8 (36, 37). Electron microscopic studies have demonstrated that unwound stem-loop structures form in the presence of UL9 and HSV-1 ssDNA-binding protein (38). Due to the artificial nature of the substrates (36, 37) and the inability to demonstrate unwinding of duplex origin in vitro, the significance of the helicase function of UL9 for HSV-1 replication remains to be determined.

A collection of mutations in conserved residues of UL9 helicase motifs I–VI have been isolated, and most fail to complement the growth of UL9 null virus in vivo (39). In this paper, we report that mutations in motifs I–IV and VI exhibit decreased levels of ATPase activity and most abolish helicase activity, but they do not interfere with UL9 dimerization or the ability of UL9 to bind the HSV-1 origin of replication. Based on the similarity of the helicase motif sequences between UL9 and UvrB, a superfamily II helicase-like enzyme, we were able to map the UL9 helicase motif mutations on the structure of UvrB and provide an explanation for the observed mutant phenotypes (40). Our results indicate that the helicase function of UL9 is indispensable for viral replication in vivo, supporting the hypothesis that UL9 is essential for unwinding at the HSV-1 origin of replication. Furthermore, the data presented provide insights into the mechanism of transdominance of the UL9 helicase motif mutants.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**—Supplemented Grace’s medium and penicillin/streptomycin were purchased from Life Technologies, Inc.; fetal calf serum was from Gemini Biological Products. Restriction enzymes were from New England Biolabs, and protease inhibitors and ampicillin were from Sigma. BaculoGold™ DNA was purchased from PharMingen, and the pFastBac vector and DH10Bac™ competent cells were from Life Technologies, Inc. The SP-Sepharose and Superose 12 HR 10/30 columns were from Amersham Pharmacia Biotech, and the UnoS column was from Bio-Rad.

**Cells**—Spodoptera frugiperda (SF9) insect cells were maintained in Grace’s medium supplemented with 10% (v/v) fetal calf serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin. E. coli DH5α cells were used for plasmid amplification.

**Western Blot Analysis**—Western blot analysis was performed as described previously (41). In brief, samples were resolved by SDS-PAGE and electrotransferred to Hybond™ ECL™ membrane using Tris-glycine/methanol transfer buffer. Membranes were blocked with 5% milk in Tris-buffered saline, incubated with anti-UL9 primary antibody and alkaline phosphatase-conjugated secondary antibody (Promega), and visualized with the alkaline phosphatase color reaction. The following primary antibodies were used as follows: 1) the R250 polyclonal antibody, recognizing the C-terminal residues 841–851, was generously provided by Dr. M. Challberg (National Institutes of Health, Bethesda); 2) RH7, a polyclonal antibody raised against the C-terminal domain of UL9, was generously provided by Dr. Daniel Tenney (Bristol-Myers Squibb Pharmaceutical Research Institute); and 3) 17B, a monoclonal antibody, recognizing the N-terminal 35 amino acids of UL9, was described previously (42).

**Generation of Recombinant Baculoviruses Expressing UL9**—Mutations in the helicase motifs of UL9 were described previously: motif I (UL9-K87A), motif II (UL9-E175A), motif III (UL9-T214S), motif IV (UL9-F303W), motif V (UL9-G354A), and motif VI (UL9-R387K) (39). Hereafter we will refer to the UL9 helicase motif mutants only by the relevant motif number. The motif mutations were initially constructed in p6-119b, a vector containing the ICP6 promoter (39). The wild type UL9 gene was subcloned into a baculovirus transfer vector (pPl0 or pFastBac) as a BamHI fragment. Each of the UL9 mutant genes was cloned into the baculovirus vector by ligation of a 1.1-kilobase pair Nhel-EcoNI fragment from the corresponding p6UL9-119b plasmids (39) to pPl0 or pFastBac-UL9 or pFastBac-UL9 as described previously.

**Recombinant baculoviruses** (Autographa californica nuclear polyhedrosis baculoviruses) expressing wild type or mutant UL9 protein were generated using the baculovirus transfer vectors, described above, and the BaculoGold (PharMingen) or pFastBac (Life Technologies, Inc.) commercial systems according to manufacturers’ instructions. The recombinant baculoviruses were amplified to produce large scale baculoviral stocks, tested for optimal expression in SF9 insect cells, and used further for protein production (see below).

**UL9 Expression and Purification**—SF9 cells in 50% confluent flasks were infected with the recombinant baculovirus of interest, and the cells were harvested at 48 h post-infection by vigorous shaking. The cell pellet was washed once with phosphate-buffered saline, resuspended in Buffer B (20 mM HEPES, pH 7.6, 1 mM EDTA), and homogenized in a Dounce homogenizer. The nuclei were pelleted in a GSA rotor at 3,000 rpm for 10 min at 4 °C. The nuclear pellet was resuspended in 30 ml of Buffer B with 10% sucrose and mixed with an equal volume of 3.4 M NaCl in Buffer B. High speed centrifugation (27,000 rpm, SW 28 rotor, Beckman ultracentrifuge) was used to separate the soluble and insoluble protein fractions. The soluble proteins were precipitated with ammonium sulfate. The majority of UL9 was found to precipitate at 50% saturation. The ammonium sulfate precipitate was resuspended in a minimal volume of Buffer B containing 0.25 mM NaCl and dialyzed overnight against 4 liters of the same buffer. The dialyzed sample was centrifuged to remove insoluble particles and subjected to chromatography on an SP-Sepharose column. The protein bound to the column was eluted with a 0.25–1 M linear gradient of NaCl. UL9 was found to run with a mobility at 0.45 M NaCl. SP-Sepharose was monitored by the Bradford protein assay and Western blot with anti-UL9 antibodies. Fractions containing UL9 were pooled and loaded on a UnoS column. Protein elution and fraction screening were performed as described above for the SP-Sepharose column. UnoS fractions containing UL9 were concentrated using a Microsep™ concentrator (Fall Filtron Corporation) with a 30-kDa cutoff and loaded on a Superose HR 10/30 gel filtration column. The fractions containing UL9 were identified and concentrated as described above. Aliquots of purified protein in buffer B containing 0.25 M NaCl were stored at −70 °C. All solutions used for protein purification contained 1 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 10 mM sodium bisulfite.

**Protein Microweighting**—Protein microweighting was performed in a 50-ml reaction with a monomolecular mass of ~40 kDa seen in Coomassie-stained SDS gels of purified wild type and mutant UL9. Five micrograms of the wild type UL9 preparation was resolved on 10% SDS gel and electrotransferred to Immobilon-PSQ membrane (Millipore; pore size 0.2 μm) using the CAPS/methanol buffer system. The membrane was stained with Coomassie and the band of interest excised. Membrane elution and protein microweighting were performed in the Biological Lab of the University of Massachusetts Medical School, Shrewsbury, MA, by Dr. J. Leszky.

**Limited Proteolysis**—Protease inhibitors used during the protein purification scheme were removed by chromatography on a Sephadex G-75 gel filtration column using 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 1 mM DTT buffer and flow rate of 1 ml/min. Fractions containing UL9 were identified with the Bradford protein assay and...
substrate and 200 nM UL9 protein (unless otherwise stated). The reaction was stopped with the addition of 25 μl binding buffer containing 0.1 mM EDTA, 40% glycerol, 0.1% bromphenol blue, resolved on 8% native polyacrylamide gels, and visualized by autoradiography. Helicase reactions were boiled for 10 min before loading were used as a reference for the mobility of the unwound, and the helicase reaction without UL9 protein was used as a reference for the mobility of the annealed substrate.

**Filter Binding Assay**—A double filter nitrocellulose filter binding assay was performed to evaluate the ability of wild type and mutant UL9 proteins to bind the HSV-1 origin of replication. Each reaction contained 1× filter binding buffer (50 mM HEPES, pH 7.6, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol), 0.2 mM NaCl, 0.4 mM DNA substrate (unless otherwise stated), 100 μg/ml BSA, 2 mM ATP, 1 mM MgCl₂, and 1 mM DTT. The reaction was heated to 70 °C for 10 min and slowly cooled to room temperature. The annealed substrate was incubated for 30 min at 37 °C in the presence of 1 mM EDTA and purified twice on Sepharose 4B column to separate the annealed substrate from the free. The helicase assay was performed in HEPES-based buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 0.5 mg/ml BSA, 0.2 mM NaCl) for 30 min at 37 °C. Each helicase reaction (50 μl) contained 1 mM helicase substrate and 200 μl UL9 protein (unless otherwise stated). The reaction was stopped with the addition of 25 μl buffer containing 0.1 mM EDTA, 40% glycerol, 0.1% bromphenol blue, resolved on 8% native polyacrylamide gels, and visualized by autoradiography. Helicase reactions boiled for 10 min before loading were used as a reference for the mobility of the unwound, and the helicase reaction without UL9 protein was used as a reference for the mobility of the annealed substrate.

**ATPase Assay**—ATPase activity was monitored by the Malachite Green/ammonium molybdate colorimetric assay as described previously (24). A 5'-32P-end-labeled (46), resulting in a partially double-stranded substrate with a 23-nucleotide 5'-ends was used as a DNA effector at 20 μM final concentration in nucleotides, unless otherwise stated.

**RESULTS**

**Generation of Recombinant Baculoviruses and Purification of Wild Type and Mutant UL9 Proteins**—The baculovirus expression system was shown to produce functional wild type UL9 protein exhibiting ATPase, helicase, and origin binding activities (24, 25). To evaluate the importance of conserved residues in the UL9 helicase motifs, we constructed recombinant baculoviruses expressing wild type and mutant versions of the UL9 protein (Fig. 1). All recombinant baculoviruses except the motif V mutant expressed intact full-length protein as detected by Western blot analysis using antibodies against the N terminus (17B) and the C terminus (R250) of UL9 (data not shown). Wild type and mutant UL9 proteins were purified to greater than 80% homogeneity (Fig. 2) as seen by Coomassie-stained SDS-PAGE. A contaminating band with a molecular mass of ~40 kDa was observed in all preparations. Microsequencing of the first five N-terminal amino acids revealed that the contaminating protein is most likely an early 39-kDa protein (Swiss-Prot accession number P11042) from *A. californica* nuclear polyhedrosis virus, the baculovirus used for protein expression. It is known that this gene is required for very late gene expression and that the protein is associated with the nuclear matrix (48). Our results (shown below) indicate that the contaminant did not interfere with the biochemical assays performed in this study. For example, a comparison of the kinetic parameters for wild type ATPase activity with already published values and the broad spectrum of effects on ATPase activity seen in various mutant UL9 preparations show that the early 39-kDa protein does not influence the ATPase assay. In addition, the absence of helicase activity in most mutant preparations indicates that the contaminant protein does not exhibit helicase activity.

All purified proteins except the motif IV mutant behaved similarly during the course of purification, eluting from the ion-exchange columns as a single peak (data not shown). In contrast, the motif IV mutant did not elute as a discrete peak but was found in the majority of the fractions eluted from the ion-exchange columns, independent of the salt concentration of the eluting gradient. Such behavior suggests that the mutation may alter the overall distribution of surface charges on the molecule and consequently its ability to bind to the ion-exchange column. Indeed, in gel filtration experiments, the motif IV mutant protein was found to aggregate (see below). Our attempts to express the motif V mutant protein, previously shown to be unstable in mammalian cells (40), did not yield any stable full-length protein. The wild type UL9 and mutant UL9 to bind the origin of replication was monitored by a dot blot filter binding assay (47), also using nitrocellulose and DEAE-cellulose membranes. A gel-purified Mspl fragment of p-100-1 plasmid containing OriS was labeled as described above and used as DNA substrate. After filtration, the nitrocellulose and DEAE-cellulose membranes were air-dried, and DNA retention was quantitated with a PhosphorImager.

**FIG. 1. Domain structure of UL9.** The UL9 protein is thought to be composed of two domains. The helicase domain (residues 1–534) is presented as an open box and the origin-binding domain (residues 535–851) is represented by a gray box. The black boxes represent the helicase motifs (numbered M–MVII). The sites of mutations in each helicase motif used in this study are shown, as is the OB mutation, a four-amino acid insertion (RIIRA) after residue 591 (66).
prestained markers were used as molecular weight standards, and their positions are depicted on the left.

ized in the N-terminal two-thirds of the protein. Gel filtration chromatography was performed as a step in the purification of UL9 as well as to determine the oligomeric state of UL9 helicase motif mutants. Wild type UL9 was found to behave as reported previously (24), eluting from the Superose HR 10/30 gel filtration column as a wide peak centered around a position corresponding to a molecular mass of 180 kDa (Fig. 3). All motif mutants except the motif IV mutant showed a similar elution pattern (Fig. 3). The motif IV mutant was shown to aggregate, eluting as a discrete peak corresponding to a molecular mass above 443 kDa. Since the gel filtration column used is linear only up to 300 kDa (Amersham Pharmacia Biotech), it was not possible to determine precisely the molecular weight of the aggregates. Aggregates were also observed in the preparation of the motif VI mutant, but in this case most of the mutant protein eluted as a dimer. These results argue that the helicase motifs are most likely not involved in UL9 dimerization. This result provides support for the idea that residues outside the helicase motifs are involved in protein-protein interactions (2).

Mutations in the Helicase Motifs Do Not Drastically Affect the Overall Conformation of the UL9 Molecule—Limited proteolysis using proteinase K was performed to evaluate the effect of the introduced mutations on the overall conformation of the UL9 protein. Since the yield of the UL9 protein is a limiting factor for extensive proteolytic studies, we took advantage of the availability of anti-UL9 antibodies recognizing the N terminus (17B) and the extreme C terminus (R250) of UL9. Western blot analysis was used to follow the fate of the proteolytic fragments containing each of the termini. Proteinase K digestion produced two major C-terminal fragments (Fig. 4A, asterisks) and one major N-terminal fragment (Fig. 4B). All UL9 mutants, except the motif VI mutant, showed the same general proteolytic pattern as wild type suggesting that the mutations introduced did not significantly alter the overall conformation of the protein. Motif VI digestion did not generate a detectable N-terminal fragment (Fig. 4B, lanes 24–26) suggesting that the mutation induces local conformational changes in the UL9 molecule. These changes most probably affect the accessibility of the extreme N terminus, where the antibody epitope maps, since the Western blot analysis with the anti-C-terminal antibody showed that a C-terminal fragment slightly shorter than the full-length UL9 was present throughout the entire time course (Fig. 4A, lanes 24–26). Some differences in the kinetics of the proteolysis were evident (Fig. 4A, compare lanes 1–4 (wild type UL9) with lanes 19–22 (motif IV) and Fig. 4B, compare lanes 1–4 (wild type UL9) with lanes 19–22 (motif IV) and lanes 23–26 (motif VI). Motif IV produced less stable C-terminal fragments that were almost completely degraded by
Mutations in All Helicase Motifs Affect the Ability of UL9 to Bind/Hydrolyze ATP—The ATPase activity of wild type and mutant UL9 proteins was examined using the Malachite Green/ammonium molybdate colorimetric assay (44). Time courses of intrinsic (Fig. 5A) and ssDNA-stimulated (Fig. 5B) ATPase activity were performed to determine the relevant rate constants (Fig. 5C). Wild type UL9 was found to hydrolyze ATP with a rate constant of 2.96 min$^{-1}$ in the absence of ssDNA and with a rate constant of 27.1 min$^{-1}$ in the presence of ssDNA. M13 ssDNA stimulated wild type ATPase activity—9-fold (Table I). These findings are consistent with previously published reports (25). The intrinsic ATPase activity of the motif III mutant ($k_{cat}$ $2.64$ min$^{-1}$) was comparable to the wild type ($k_{cat}$ $2.96$ min$^{-1}$), whereas it is almost completely abolished for the motif II mutant ($k_{cat}$ $0.27$ min$^{-1}$). The motif VI mutant retained 70% of the wild type intrinsic ATPase activity, motif I retained 50%, and motif IV retained 30% (Fig. 5C compare the black bars). A more significant effect was seen on the ssDNA-dependent ATPase activity. All mutants retained only 25% or less of the wild type ssDNA-stimulated ATPase activity (Fig. 5C, compare the gray bars). All mutants except motif II exhibited lower than wild type stimulation by ssDNA (Table I and below). The $K_m$ values for ATP and M13 ssDNA were determined in the context of ssDNA-stimulated ATPase activity. The values calculated for wild type (Table I) were comparable to values reported previously (50). Surprisingly, the $K_{M_{ATP}}$ value for motif I was found to be 8-fold lower than wild type suggesting higher affinity for ATP binding (see “Discussion”).

Our efforts to assess directly the effect of the helicase motif mutations on the ATP binding affinity of UL9 by UV-cross-linking failed due to extensive UL9 degradation in the course of UV irradiation (data not shown). The $K_m$ ssDNA for motif II is 5-fold lower than that for wild type (Table I). The $V_{max}$ of the ssDNA-stimulated ATPase reaction was reduced as little as 3-fold for the motif III mutant and as much as 10-fold for the motif I mutant (Table I). Although aggregated, the motif IV mutant retained some ATPase activity (Fig. 5 and Table I), confirming that aggregation is most likely due to local conformational changes perhaps caused by difficulties in accommodating a bulkier tryptophan residue instead of phenylalanine. In summary, mutations in all helicase motifs except motif III significantly affected the ATPase function of UL9. The motif III mutant protein exhibited wild type levels of the intrinsic ATPase activity and only a moderate reduction of the ssDNA stimulated ATPase activity.

The Helicase Activity of UL9 Was Abolished by Mutations in All Helicase Motifs Except Motif III—The effect of the helicase motif mutations on the helicase activity of UL9 was evaluated using an in vitro unwinding assay. In the absence of ATP, no unwinding was observed (data not shown). When ATP was...
supplied to the reaction, only wild type and the motif III mutant exhibited helicase activity (Fig. 6A). To compare the helicase activities of these two proteins, we performed unwinding reactions varying the amount of UL9 protein added. We found that the amount of unwound DNA correlated with the amount of UL9 present in the reaction and that the helicase activity of the motif III mutant was comparable to that of wild type (Fig. 6B). PhosphorImager quantitation indicated that when UL9 (wild type or the motif III mutant) was present at a concentration of 200 nM, 60% of the helicase substrate is unwound. When UL9 was present at 100 or 50 nM, the percentages of unwound substrate were 35 and 20%, respectively. Thus, the mutations in all conserved helicase motifs of UL9, except motif III, abolished the helicase function of the UL9 protein; the motif III mutant protein exhibited helicase activity comparable to that of wild type UL9, which may not be surprising taking into account the conservative nature of the substitution of serine with threonine.

The phenotype of the motif III mutant is intriguing. It exhibited wild type levels of intrinsic ATPase and helicase activity, and a moderate defect in ssDNA-stimulated ATPase activity. Despite possessing wild type levels of in vitro helicase activity, it complemented the growth of hr94, the UL9 null virus, in vivo only partially (39). One possible explanation for this apparent discrepancy may reflect the different nature of the assays, in vitro versus in vivo. Alternatively, the ssDNA stimulated ATPase activity may play a role in addition to its proposed role in the helicase function of UL9. Another possibility is that the mutation may alter crucial protein-protein or protein-DNA interactions essential for replication but not measured by the assays used. Any of these effects may explain the partial complementation phenotype.

**Mutations in the Helicase Motifs of UL9 Do Not Alter Its Origin-specific DNA Binding Activity**—To determine the effect of the helicase motif mutants on the origin-binding function of UL9, a double membrane filter binding assay was performed. Our analysis indicated that none of the characterized mutations in the helicase motifs of UL9 interferes with the specificity of the origin binding (Fig. 7). When MspI-digested p-100-1 plasmid (Fig. 7, lane 15) was used as a substrate in the filter binding reaction, only the OriS-containing fragment was selectively retained on the nitrocellulose membrane by the wild type and all motif mutant proteins (Fig. 7, lanes 8–14) even though multiple labeled plasmid fragments with similar length were present.

To quantify the origin binding abilities of wild type and mutant proteins, we chose to compare OriS binding of wild type and mutant UL9 at protein concentrations (1, 4, and 10 nM), shown to be in the linear range of the binding curve of UL9 to an OriS-containing DNA fragment (24). Our experiments indicated that all characterized helicase motif mutants, except motif IV mutant, bind OriS with wild type efficiency at all concentrations examined (Fig. 8). The binding of the wild type UL9 protein was plotted as 100%. At 1 nM, 5% of the DNA substrate in the reaction was bound by wild type UL9. At 4 and 10 nM, the percentages of bound substrate were 12 and 30, respectively. The differences observed between wild type and mutant UL9 proteins, with the exception of the motif IV mutant protein, were less than 2-fold. The motif IV mutant exhibited a 4-fold lower than wild type binding efficiency to OriS at the 10 nM protein concentration. This result may reflect the tendency of the mutant protein to aggregate, which is not predominant when the protein is at lower concentrations. In conclusion, all UL9 motif mutant proteins, except motif IV, exhibited near wild type ability to bind OriS.

**DISCUSSION**

Previous genetic studies of UL9 helicase motif mutants showed that the conserved residues are important for UL9 function in vivo since none of the mutants were able to complement fully the growth of the UL9 null mutant (hr94). In fact,
partial complementation was observed only for the motif III (UL9-T214S) mutation, which we now show retains helicase activity (Fig. 6). However, when the same position was mutated to alanine, the UL9 function was abolished completely (39). The biochemical analysis reported in this study correlates very well with the genetically observed phenotypes of the helicase motif mutants (39). Thus, the most likely reason for the inability of mutants in motifs I–IV and VI to complement the growth of the UL9 null virus is that helicase activity is completely abolished. Although direct evidence for UL9 unwinding of the origin of replication in vivo is still lacking, the most straightforward explanation for our results is that UL9 plays an indispensable role in HSV-1 origin unwinding.

*Functions of the Conserved Helicase Motifs*—The UL9 helicase motif mutants were designed based on the conservation of the targeted residues within helicase motifs of UL9 homologs (39) before any structural information about helicases was available. Sequence alignment (Fig. 9A) of the helicase motifs of UL9 with the helicase motifs of UvrB allowed us to map the mutated residues in UL9 on the structure of UvrB (Fig. 9B) and gain additional insights into the molecular basis behind the observed mutant phenotypes. UvrB is a member of nucleotide excision repair pathway, which exhibits limited helicase activity only when complexed with UvrA (51, 52). Intriguingly, like UL9, UvrB is not able to separate long DNA duplexes (52). Thus neither UvrB nor UL9 when expressed alone exhibit very robust helicase activity on natural substrates. It is tempting to speculate that similarities in the biochemical properties of UL9 and UvrB may be due to their structural similarities.

**ATP Binding and Hydrolysis**—Motifs I and II (Walker box A and B, respectively) were shown previously to play an important role in ATP binding/hydrolysis by mutational analysis and crystallographic studies (reviewed in Refs. 4, 6, and 53). The motif I Lys-87 residue of UL9 corresponds to Lys-45 in UvrB, seen in the crystal structure to contact the γ-phosphate of ATP (11). In UL9, the substitution of Lys-87 with alanine removes a positively charged long side chain. The alanine side chain would not be able to coordinate the phosphate of the ATP, which would explain the observed defects in UL9 ATPase and helicase activities. One might imagine that the defects are due to impaired ATP binding since the coordination of the phosphates is abolished. Surprisingly, the $K_m$ value for ATP as seen in the ATPase assay (Table I) was 8-fold lower than that for wild type suggesting higher affinity for ATP binding. The same phenomenon was observed for the NS3 (21) and the PcRA helicases (14). This apparent discrepancy is not well understood. Soultnas et al. (14) suggested that the lower $K_m$ may reflect “changes in rate constants associated with conformational changes or product release.”

The glutamate at position 175 from motif II (Walker box B) of UL9 corresponds to Glu-339 from UvrB, seen to be oriented toward the ATP molecule bound but at distance too great to be involved in direct interaction (11). A glutamate in Walker box B of two other ATP-binding proteins (RecA (17) and PcrA (14)) was hypothesized to be responsible for the activation of a water molecule involved in attacking the P–O bond. Indeed, in PcrA, when this glutamate was replaced with an alanine, a 70-fold decrease of the $k_{cat}$ for ssDNA-stimulated ATPase activity and no significant effect on $K_m$ for ATP were observed. In UL9 the same type of mutation resulted in almost undetectable intrinsic ATPase and a 6-fold reduction of the ssDNA-stimulated ATPase activity. Surprisingly, the ssDNA stimulation of the ATPase activity was 4-fold higher than that for wild type (Table 1), which correlates with a 5-fold lower $K_m$ for ssDNA. A phenotype similar to that of the motif II mutant has been observed previously for the E221Q mutation in UvrD (54). There is no straightforward explanation for the increased ssDNA stimulation, and the lower $K_m$ value for ssDNA since motif II has not been observed in any helicase/nucleic acid structure in close proximity to the ssDNA-binding cleft. It is possible that the mutant protein undergoes a conformational change upon ssDNA binding and/or it is involved in hydrogen bonds or electrostatic interactions with other residues involved directly or indirectly in ssDNA binding. Alternatively, the inability of the mutant to hydrolyze ATP could lead to arrest of the protein molecule in an ATP/ssDNA-bound state. Thus, each mutant protein molecule may spend a longer time on ssDNA due to the slower rate of release from the complex, which is seen as an indirect effect on the $K_m$ for ssDNA. In summary, our results confirm the importance of motifs I and II in ATP binding/hydrolysis and indicate that motif II may also mediate allosteric effects of ssDNA on ATPase activity.

**Coupling of the ssDNA-binding ATPase and Helicase Activities**—In addition to ATP binding and hydrolysis, multiple events have to take place for helicase unwinding to proceed. ssDNA binding activity, unwinding, and translocation along the unwound DNA are tightly interrelated and coordinated with ATP binding/hydrolysis. Limited information is available about the specific mechanism of action of any helicase, but mutational studies have suggested that motifs III–VI may play a role in the coupling of ATP binding/hydrolysis to ssDNA binding and helicase activities (7, 15, 19–21, 53, 55).

Motif III is positioned along the ATP-binding cleft (Fig. 9B) and can be pictured also as a bridge between the ATP-binding and ssDNA-binding clefts suggesting a role in coupling the ATPase activity with DNA binding and/or helicase activity. The phenotype of the D248N mutant of motif III from *E. coli* helicase II (UvrD) supports the notion that motif III is at the crossroads between the ATPase and ssDNA binding activities. This mutant was found to be defective in the formation of binary complexes with ATP and DNA separately but formed a wild type tertiary complex (UvrD-ATP-ssDNA) as seen from the corresponding ATP/ssDNA binding studies (20).

We report herein that UL9 motif III mutant behaves as wild type in all activities except ssDNA-stimulated ATPase. The $K_m$ values for ATP and ssDNA are comparable with the wild type
null mutant, cell lines with high copy number exhibited lower levels of complementation. High concentrations of UL9 appear to be inhibitory in an plaque reduction (transdominance) assay, wild type UL9 is inhibitory. Malik et al. (65) reported that although cell lines containing a low copy number of the UL9 wild type gene were observed, but all ATP binding/hydrolysis kinetic parameters were comparable with wild type (22). In contrast, when Gln-54 from the same motif was mutated, no effect on the ssDNA binding was observed, but the ATPase activity was decreased (22).

In all SFI and SFII helicases studied to date, mutations in helicase motifs I–VI exhibit defects in ATPase activity that can be explained at least in part by the nature of the ATP-binding cleft, whose architecture is shaped by residues of the helicase motifs. It appears that the helicase molecule (in general) is a very dynamic structure. Kinetic studies of Rep (59, 60), UvrD (20), and NS3 helicase (61, 62) predict that the ATP and the ssDNA binding are multistep processes accompanied by conformational changes. In UvrD and Rep these conformational changes have been confirmed by limited proteolysis (20, 63).

**The Mechanism of Transdominance of UL9 Helicase Motif Mutants**—The study of transdominant mutants has provided valuable information about protein functional domains and has facilitated the understanding of the molecular mechanisms underlying many biological processes. The finding that the N-terminal domain of UL9 expressed by itself is a functional helicase (26, 64) and the C-terminal domain is a monomer capable of binding the origin of replication (49) creates an opportunity for distinguishing the roles of these two domains in HSV-1 infection. Previously, in a plaque reduction (transdominance) assay, wild type UL9, when overexpressed, was found to be inhibitory; mutants in motifs I, II, and VI were transdominant; mutants in motifs III and IV were neutral, and the mutant in motif V was potentiating (Table II and Ref. 40). Several other lines of evidence also indicate that overexpression of wild type UL9 is inhibitory. Malik et al. (65) reported that although cell lines containing a low copy number of the wild type UL9 gene could efficiently complement hr94, a UL9 null mutant, cell lines with high copy number exhibited lower levels of complementation. High concentrations of UL9 appear to be inhibitory in an *in vitro* helicase assay utilizing DNA substrates involving elements of the HSV-1 origin of replica-

![Fig. 8](http://www.jbc.org/) The abilities of wild type and mutant UL9 proteins to bind OriS are comparable. Wild type (WT) and mutant UL9 proteins were purified from insect cells infected with recombinant baculoviruses, and a double membrane filter binding assay was performed as described under “Experimental Procedures.” All binding reactions contain 0.4 nM labeled OriS fragment. % bound DNA was plotted with 100% described under “Experimental Procedures.” All binding reactions contain and a double membrane filter binding assay was performed as de-
It is believed that the inhibitory effect of the overexpressed UL9 is mediated mainly by the origin-specific DNA binding function of UL9, since transfection with the C-terminal domain of UL9 and wild type infectious DNA can severely reduce the efficiency of plaque formation (34, 40, 66). Moreover, when a mutant, UL9-OB, carrying an insertion mutation (RIRA) in the C-terminal origin-binding domain (Fig. 1) known to disrupt origin binding activity was cotransfected with wild type infectious DNA, no inhibition was observed (40, 66). The inhibitory properties of the overexpressed wild type UL9 are consistent with a model in which HSV-1 DNA replication occurs in two stages (28). According to this model, early in infection viral replication is origin-dependent and occurs by a mecha

The motif III mutant protein is a dimer with wild type levels of helicase activity. In plaque reduction assays it appears as a functional equivalent of wild type UL9, and it behaves as wild type in transdominance assay (neutral). Another neutral mu-
Biochemical Characterization of UL9 Helicase Motif Mutants

**Table II**

Summary of the properties of wild type and mutant UL9

| UL9 Phenotype in transdominance assay$^a$ | % plaque reduction$^b$ | Ability to dimerize | Helicase activity | Ori binding activity | Protein stability$^c$ |
|----------------------------------------|-----------------------|---------------------|-------------------|---------------------|---------------------|
| Wild type                              | Inhibitory            | 40                  | +                 | +                   | +                   |
| C-terminal domain                      | Inhibitory            | 85$^d$              | +                 | +                   | +                   |
| MI                                     | Transdominant         | 85                  | +                 | +                   | +                   |
| MIH                                    | Transdominant         | 98                  | +                 | +                   | +                   |
| MIII                                   | Neutral               | 38                  | +                 | +                   | +                   |
| MIV                                    | Neutral               | 40                  | Aggregated        | +                   | +                   |
| MV                                     | Potentiating          | 180                 | ND$^e$            | ND                  | ND                  |
| MVI                                    | Transdominant         | 92                  | +                 | +                   | +                   |

$^a$ The transdominance assay phenotypes were originally reported by Malik et al. (40).

$^b$ 100% represent the number of plaques observed in cells transfected with wild type infectious DNA alone.

$^c$ Protein stability was determined by transient transfection of Vero cells followed by Western blot analysis (39).

$^d$ The gel filtration data were originally reported by Elias et al. (48).

$^e$ MIV mutant shows a defect in Ori binding at high protein concentrations (see Fig. 8 and text).

$^f$ ND, not determined due to instability of the protein.

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