Biochemical Analyses of Mutations in the HSV-1 Helicase-Primase That Alter ATP Hydrolysis, DNA Unwinding, and Coupling Between Hydrolysis and Unwinding*

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Herpes simplex virus type 1 encodes a heterotrimeric helicase-primase composed of the products of the UL5, UL52, and UL8 genes. UL5 possesses six motifs conserved among superfamily 1 of helicase proteins. Substitutions of conserved residues in each motif abolishes DNA replication in vitro (Zhu, L., and Weller, S. K. (1992) J. Virol. 66, 469–479). Purified UL5-52 harboring a Gly to Ala change in motif V retains primase and helicase activities in vitro but exhibits a higher $K_M$ for single-stranded DNA and lower DNA-dependent ATPase activity (Graves-Woodward, K. L., and Weller, S. K. (1996) J. Biol. Chem. 272, 13629–13635). We have purified and characterized six other subcomplexes with residue changes in the UL5 helicase motifs. Each variant subcomplex displays at least wild type or greater levels of primase and DNA binding activities, but all are defective in helicase activity. Mutations in motifs I and II exhibit profound decreases in DNA-dependent ATPase activity. Mutations in motifs III–VI decrease DNA-dependent ATPase activity 3–6-fold. Since mutations in motifs III, IV, V, and VI do not eliminate ATP hydrolysis or DNA binding, we propose that they may be involved in the coupling of these two activities to the process of DNA unwinding. This analysis represents the first comprehensive structure-function analysis of the conserved motifs in helicase superfamily 1.

A large number of biological processes, including DNA replication, DNA repair, recombination, and transcription, require the action of helicases. DNA helicases catalyze the unwinding of a duplex DNA molecule using the energy provided by DNA-stimulated NTP hydrolysis (reviewed in Refs. 1–4). Many helicases have been shown to self-assemble. For example, the SV40 and polyoma T-antigen proteins (5, 6), the phage T4 gp41 helicase-primase (12, 13), and the bacteriophage T7 gp4 helicase-primase (12, 13), and the bacteriophage T4 gp41 helicase (14) form hexamers. The E. coli helicases Rep (15), helicase II (UvrD) (16), and helicase III (17), HeLa helicase (18) and the herpes simplex virus type 1 UL9 origin binding protein (19) form dimers. Although the exact role of oligomerization in helicase function is not clearly understood, models have been proposed in which formation of higher order structures upon ATP and/or DNA binding is necessary for DNA unwinding (reviewed in Refs. 1, 4).

Herpes simplex virus type 1 (HSV-1) encodes a heterotrimeric complex composed of the products of the UL5, UL52, and UL8 genes. This protein complex has been shown to possess ssDNA-dependent ATPase, 5′ to 3′ DNA helicase, and RNA primase activities (20–23). Although all three subunits are required for DNA replication in vivo (24, 25), the UL8 protein is not absolutely required for helicase and primase activities in some in vitro assays (20, 23). Neither UL5 nor UL52 appears to possess any of these activities when expressed and purified alone (20, 23, 26). The UL52 protein contains a motif conserved in many primases which, when mutated, abolishes primase but not ATPase or helicase activities (27, 28) suggesting that UL52 gene encodes at least a portion of the primase component of the complex.

The UL5 protein possesses six motifs conserved among superfamily 1 of known and/or putative helicase proteins (Fig. 1) (29–32). Consistent with their ability to hydrolyze ATP, all of these proteins contain two highly conserved sequence motifs (motifs I and II) found in many nucleotide binding proteins (30, 31, 33). Motif I is the well known GXGXGKT/S Walker A motif believed to be involved in binding the di- or triphosphate moiety of the nucleotide cofactor (33, 34). Motif II contains a group of hydrophobic residues terminated by an Asp and a Glu residue called the Walker B motif believed to be involved in stabilization of the coordinated Mg$^{2+}$ (33–35). Although the functional significance of motifs III, IV, V, and VI is not well understood for any helicase, the strong conservation of the six motifs suggests that these sequence elements may be important for helicase activity.

Single amino acid substitutions in the most conserved residues in the helicase motifs (Fig. 1) have been shown to abolish the ability of UL5 to support DNA replication in vivo (36) suggesting that these conserved residues are essential. We have previously shown that a Gly to Ala substitution in motif V lowers the ssDNA-dependent ATPase activity of UL5-52 and does not dramatically alter primase activity in vitro; surprisingly, however, this mutant was found to exhibit near wild type levels of helicase activity in vitro (37). The observation that a defect in a conserved helicase motif resulted in a mutant protein which retained helicase activity led us to consider the importance of these conserved motifs in helicase function. It is likely that the mechanism of helicase involves the coupling of various partial reactions such as ATP binding, ATP hydrolysis, ssDNA, single-stranded DNA; Sf9, Spodoptera frugiperda; kbp, kilobase pairs; kb, kilobases; SSB, single-stranded DNA binding protein.

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DNA binding, and protein translocation. We predict that the conserved helicase motifs may be critical for some of these partial reactions; therefore, we coexpressed six more helicase variants of UL5 with the UL52 protein, purified each subcomplex, and compared the biochemical activities of each subcomplex with respect to the wild type enzyme. Unlike the G815A motif V mutant, all six of these additional mutants completely lacked helicase activity. We have shown that motif I is directly involved in ATP binding and/or hydrolysis, and motif II appears to be required for coupling of DNA binding to ATP hydrolysis. Mutations in motifs III, IV, V, and VI do not eliminate ATP hydrolysis nor affect DNA binding and therefore may be involved in the coupling of these two activities to the process of DNA unwinding.

EXPERIMENTAL PROCEDURES

Reagents

Supplemented Graces’ media, fetal calf serum, 10% Pluronic® F-68, and the Bac-to-Bac® recombinant baculovirus kit were purchased from Life Technologies, Inc. BacuLoGold™ DNA was obtained from Pharmingen. Penicillin/streptomycin solution and ampicillin were from Sigma. The MonoQ® HR 5/5 strong anion exchange column and the SP-Sepharose™ weak anion exchange column were from Pharmacia Biotech Inc. Radiolabeled nucleotides were purchased from Amersham. L-1-tosylamido-2-phenylethylchloromethylketone. Buffer A was 20 mM NaH2PO4, 10 mM sodium bisulfite, 5 mM MgCl2, 5 mM Na2EDTA, and 0.1% (v/v) deoxycholate. All buffers were filtered through a 0.22-μm membrane and equilibrated with Buffer B containing 0.1 M NaCl. All other biochemical reagents were purchased from Sigma.

Buffers

Buffer A was 20 mM NaH2PO4, 10 mM sodium bisulfite, 5 mM MgCl2, 5 mM Na2EDTA, and 0.1% (v/v) deoxycholate. Buffer B was 20 mM NaH2PO4, 10 mM sodium bisulfite, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotonin, 0.5 mg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone, 0.7 mg/ml 1-tosylamido-2-phenylmethyl chloromethyl ketone. Buffer C was 200 mM NaH2PO4, 20% (v/v) glycerol, and 0.5 mM EDTA. All buffers were filtered through a 0.22-mm membrane and degassed before use.

Cells, Viruses, and DNA

Spodoptera frugiperda (Sf9) cells were grown in Graces’ insect medium containing 10% (v/v) fetal calf serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin, and 0.1% (v/v) Pluronic® F-68 in a six-liter flask (100 rpm). When the cell density reached 2 × 10^6 cells/ml (doubling time no greater than 24 h), recombinant baculoviruses were added to each culture and allowed to attach to the cells by shaking at 50 rpm at 27°C. After 60 min the cultures were diluted to 1 × 10^6 cells/ml and shaken at 100 rpm for 48 h at 27°C. Cells were harvested by centrifugation at 1000 × g in a GSA rotor, washed with cold serum-free media, resuspended in 40 ml of ice-cold Buffer A, and allowed to swell on ice for 15 min. Cells were dounced by 15 strokes with a type B pestle. Nuclei were pelleted by centrifugation at 1000 × g. Cytosolic extracts were clarified by centrifugation at 48,000 × g in an SS34 rotor. The UL5-2 subcomplexes (both wild type and variant) were fractionated from the cytosolic extract by precipitation with 1 M ammonium sulfate on ice for 2 h. The precipitated protein pellets were resuspended in a minimal volume of Buffer B containing 0.1 M NaCl, dialyzed to removed ammonium sulfate, and reclarified by centrifugation at 48,000 × g.

The wild type UL5-2 and the UL5G815A-52 subcomplexes, the UL3 protein, the UL29 protein (single-stranded DNA binding protein, SSB), and the Ul30-42 DNA polymerase complex were purified as described previously (37). All other variant UL5-2 subcomplexes were purified as follows. The dialyzed sample was loaded onto a 20-ml SP-Sepharose® column equilibrated with Buffer B containing 0.1 M NaCl, and the column was washed with 20 column volumes of the equilibration buffer. One-tenth of each 2.5-ml fraction was analyzed by SDS-polyacrylamide gel electrophoresis. The protein solution was then loaded onto a 1-ml MonoQ® column equilibrated with Buffer B containing 0.1 M NaCl. The column was washed with 5 ml of Buffer B containing 0.2 M NaCl, and the UL5-2 complex was eluted using a 20-ml linear gradient of Buffer B containing 0.2–1 M NaCl. Fractions containing the UL5-2 subcomplex were identified by both ATPase assay and by SDS-polyacrylamide gel electrophoresis. The

**Fig. 1.** The HSV-1 UL5 protein has six motifs conserved within helicase superfamily I. The six conserved helicase motifs in the UL5 protein are numbered I–VI and are represented by black boxes. The conserved amino acids in each motif, the residue which was replaced, and the residue change are shown. The replaced residues are underlined.

**Table 1.** The HSV-1 UL5 protein has six motifs conserved within helicase superfamily I. The six conserved helicase motifs in the UL5 protein are numbered I–VI and are represented by black boxes. The conserved amino acids in each motif, the residue which was replaced, and the residue change are shown. The replaced residues are underlined.
clearest fractions containing the UL5-52 complex were pooled and frozen in small aliquots at -70 °C. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent.

Enzyme Assays

Direct Primerases—RNA primer synthesis reactions (25 μl) were performed as described previously using 1 pmol (molecules) of a 50-base DNA oligonucleotide template containing a preferred primerase initiation site and 2 pmol of the UL5-52 subcomplex (wild type or variant) (37). Reactions were allowed to proceed for 60 min at 30 °C, stopped by the addition of 1 μl of 0.25 M EDTA, dried by Speed Vac, and resuspended in 10 μl of 50% (v/v) formamide, 0.01% (v/v) bromphenol blue. Samples were boiled for 2 min, and the reaction products were separated on an 18% Long Ranger 6, 7 μm urea gel. The gels were dried and exposed to film at room temperature.

ATPase assays—ATPase reactions (50 μl) were performed as described previously using 7 mM ATP, 5 mM MgCl₂, 0.1 mM ss M13mp18 DNA (nucleotides), and 0.45 pmol of UL5-52 subcomplex (4.5 pmol of UL5-52 for reactions done in the absence of ssDNA) (37). Formation of inorganic phosphate was determined by the addition of 0.8 ml of an acidic ammonium molybdate solution containing malachite green (39) and the subsequent addition of 0.1 ml of 34% sodium citrate. The absorbance at 660 nm was determined. Nanomoles of inorganic phosphate released per reaction were determined from a standard curve.

Helicase assays—Helicase reactions (50 μl) contained 20 mM Na⁺ HEPES (pH 7.6), 1 mM dithiothreitol, 5 mM MgCl₂, 7 mM ATP, 0.1 mg/ml bovine serum albumin, 10% glycerol, and 0.64 pmol (molecules) of the forked DNA substrate (diagrammed in Fig. 5A). The forked DNA substrate was constructed by heat denaturing and annealing 80 pmol of the helicase 30-mer oligo (5’ CAGTCACAGCTTGGTATTAAGAGGCGGC- CAGTTATAGCTGATAAGACTGGC 3’ radiolabeled at the 5’ end with [32P]P and 80 pmol of unlabeled helicase 30/3Pr oligo (5’ GTGC- GCCACCTCTCGTATTTAGCTTCGCCGTTTTACACCGTGTG- ACTG 3’). The underlined residues are those which are homologous and make up the duplex region of the molecule. Reactions containing 2 pmol of the UL5-52 subcomplex (wild type or variant) and 6 pmol of the UL8 protein were allowed to proceed for 30 min at 37 °C and terminated by the addition of one-fifth volume of stop solution (0.25 mM EDTA (pH 8), 40% glycerol, 0.1% bromphenol blue). Reaction products were separated by electrophoresis on an 8% nondenaturing acrylamide, 0.11% Bis-gel in 1 x Tris-borate-EDTA at 180 V at 4 °C until the bromphenol blue had migrated approximately halfway down the gel. Dried gels were exposed to film at room temperature.

DNA gel shift assay—DNA gel shift assays (25 μl) contained 20 mM Na⁺ HEPES (pH 7.6), 1 mM dithiothreitol, 5 mM MgCl₂, 1.28 pmol (molecules) of the forked DNA substrate (diagrammed in Fig. 5A). Reactions containing 4 pmol of the UL5-52 subcomplex (wild type or variant) plus or minus 12 pmol of the UL8 protein were allowed to proceed for 5 min at room temperature and terminated by the addition of one-tenth volume of stop solution (80% glycerol, 0.1% bromphenol blue). Reaction products were separated by electrophoresis on a 4% nondenaturing acrylamide, 0.11% Bis-gel at 180 V at 4 °C until the bromphenol blue had migrated approximately halfway down the gel. Dried gels were exposed to film at room temperature.

Leading strand DNA replication assay—Leading strand DNA replication assays (25 μl) were performed as described previously using 25 pmol of pBS + single-stranded DNA molecules singly primed with a 62-base oligodeoxyribonucleotide primer (28, 37). The replication fork substrate was generated by incubation of the singly primed DNA with 0.25 pmol of HSV-1 polymerase (UL30-42) for 15 min. 2 pmol of the UL5-52 subcomplex (wild type and variant), 6 pmol of the UL8 protein, and 15 pmol of UL29 (SSB) were then added to the reactions. After 60 min at 30 °C, an equal volume of 1% SDS, 10 mM EDTA, and 1 mg/ml proteinase K was added. After incubation at 37 °C for 60 min, the reaction products were precipitated and resuspended in 25 μl of alkali loading buffer (100 mM NaOH, 1 mM EDTA, 10% glycerol, 0.01% bromoresol green), and reaction products were separated on a 0.8% alkaline agarose gel.

Generation of Figures

 Autoradiograms were scanned using the Microtek ScanMaker II on an Apple Macintosh computer. Photos were generated using Adobe Photoshop® version 3.0.

RESULTS

Generation of Recombinant Baculovirus and Purification of Both Wild Type and Variant UL5-52 Subcomplexes—To examine the contribution of conserved residues in the six helicase motifs of UL5 toward the activities exhibited by the helicase-primase complex in vitro, recombinant baculoviruses containing each mutant were constructed. All recombinant baculoviruses were able to express full-length UL5 protein which reacted with polyclonal α-UL5 antisera in a Western blot (data not shown). Each UL5-52 subcomplex was expressed in SF9 cells infected with recombinant baculoviruses and purified to 90–95% homogeneity (Fig. 2). The biochemical properties of each variant subcomplex was then compared with the wild type subcomplex in vitro.

Conserved Helicase Motif Residues Are Not Essential for the Primase Activity of the UL5-52 Subcomplex—To determine if any of the conserved helicase motifs are required for primase activity, both the wild type and the variant helicase-primase subcomplexes were assayed for primer synthesis using a template containing a preferred primerase initiation site (40). As shown in Fig. 3, all of the variant UL5-52 subcomplexes can catalyze the synthesis of short (8 and 9 nucleotide) primers in the absence of the UL8 protein. Quantitation of the amount of primer synthesized by each subcomplex in three independent sets of assays revealed that mutations in all of the helicase motifs cause an increase in the primase activity. The most dramatic increases in activity were seen with the motif III (lane d) and motif IV (lane c) variant proteins (Table I). These data are consistent with the notion that the UL5 helicase motifs are not required for the in vitro primase activity of the HSV-1 helicase-primase complex. Possible reasons for stimulation of primase activity by helicase motif mutations will be discussed below.

Mutations in the UL5 Helicase Motifs Alter ATPase Activity—The HSV-1 helicase-primase possesses both intrinsic and ssDNA-dependent ATPase activity (20, 23, 41, 42). To test the role of the UL5 helicase motifs in ATP hydrolysis, ATPase activities of each motif variant subcomplex were compared with the wild type subcomplex both in the presence and absence of ssM13 DNA (Fig. 4 and Table I). All of the variant subcomplexes retained linear kinetics of ATP hydrolysis for at least 1 h except for the motif VI variant containing a Y836A mutation (data not shown); both the intrinsic and ssDNA-dependent ATPase activity of the motif VI variant was stable for only the first 15 min. Replacement of Gly-102 in motif I with a Val resulted in a 10-fold decrease in the intrinsic ATPase activity. All of the

2 J. Gottlieb and M. D. Challberg, manuscript in preparation.

3 J. Gottlieb and M. D. Challberg, unpublished results.
other variant subcomplexes exhibited wild type levels of intrinsic ATPase activity. While the wild type subcomplex shows a 15-fold increase in ATP hydrolysis in the presence of 0.1 mM ssDNA, the ATPase activities of neither the motif I nor the motif II variant subcomplexes could be stimulated by ssDNA. Subcomplexes containing mutations in helicase motifs III, IV, V, and VI exhibited 3–6-fold decreases in ssDNA-dependent ATPase activity. Thus, although mutations in any of the motifs appear to decrease ssDNA-dependent ATPase activity, only the DE to AA mutation in motif II completely abolishes the stimulation by ssDNA and only the G to V mutation in motif I decreases both the intrinsic and ssDNA-dependent activities.

All Motif Variants Except Gly815 in Motif V Abolish DNA Helicase Activity—The helicase activities of the wild type and variant subcomplexes were measured using a forked substrate containing 30 base pairs of duplex DNA (diagrammed in Fig. 5A).2 Unwinding of duplex regions of 30 base pairs or greater requires not only the UL5-UL52 subcomplex but also UL8; it is therefore likely that helicase assays employing this substrate represent a more stringent test of full helicase function than assays that employ substrates containing shorter regions of duplex. Unwinding of substrates of the latter type is not dependent on the presence of UL8.2

In the experiment shown in Fig. 5B, the helicase-primase subcomplex and UL8 were incubated with the radiolabeled DNA substrate in the presence of ATP and MgCl2, and the reaction products were separated by native gel electrophoresis. The results show that all variants except the Gly to Ala mutation in motif V (lane m) abolish DNA helicase activity as measured by this assay. Thus, although mutation in motifs III, IV, VI, and T to I mutation in motif V retain significant (18–38%) ssDNA-dependent ATPase activity, these proteins are not able to catalyze this DNA unwinding reaction in vitro.

Motif Variants That Lack Helicase Activity Still Bind to the Forked Helicase Substrate—One explanation for the lack of DNA helicase activity exhibited by most of the motif variants is that mutations in these motifs alter the binding of the UL5-52 subcomplex to the DNA helicase substrate. The ability of the helicase-primase complex to bind to the forked helicase substrate was tested using an electrophoretic mobility shift assay.3 Fig. 6 shows an analysis of the DNA binding properties of each subcomplex. Quantitation of three individual gel shift experiments shows that all of the mutations bind the forked DNA as well as wild type, and in fact, all of the mutations except the G to A and T to I mutations in motif V (lanes I, m, n, and o) increase binding of UL5-52 to the forked DNA by at least 2-fold. Therefore, the inability of these proteins to unwind the forked DNA helicase substrate is not due to the inability to interact with the substrate but due to the inability to couple ATP hydrolysis and DNA binding in a manner that leads to DNA unwinding.

Motif Variants That Lack Helicase Activity Are Also Unable to Carry Out Leading Strand DNA Synthesis In Vitro—All of the assays described previously measure properties of the helicase-primase subcomplex which are independent of the other HSV-1 DNA replication proteins. The ability to replicate a DNA substrate containing a preformed replication fork in vitro requires six of the seven essential HSV-1 replication proteins, DNA polymerase (UL30-42), ssDNA binding protein (UL29), and the heterotrimeric helicase-primase (UL5-52-8) (28). This assay requires the helicase to unwind long stretches of duplex DNA (up to greater than 20 kb) and may also depend on protein-protein interactions with the other members of the HSV-1 replication machinery.

Each subcomplex was incubated with the preformed fork

![Table I](https://example.com/tables/table1.png)

**Table I**

| Purified UL5-UL52 subcomplex | Primate activitya | ATPase activityb | Helicase activityc | DNA binding activityd | Helicasedependent DNA replication | In vivoe | In vitrof |
|-----------------------------|------------------|------------------|-------------------|----------------------|----------------------------------|---------|---------|
|                            | %                | +ssDNA | −ssDNA | %            | +UL8 | −UL8 |                  |                  |
| Wild type                   | 100              | 100    | 100    | 100         | 100  | 100  | +               | +                |
| Motif I                     | 194 ± 22         | 0.8 ± 0.2 | 10.0 ± 2.1 | 3.2 ± 1.5 | 516 ± 102 | 238 ± 32 | −      | −                |
| Motif II                    | 596 ± 17         | 4.7 ± 0.3 | 61.1 ± 5.5 | 3.2 ± 0.1 | 329 ± 49 | 190 ± 36 | −      | −                |
| Motif III                   | 3632 ± 745       | 17.6 ± 2.9 | 78.1 ± 9.7 | 3.8 ± 1.9 | 540 ± 5 | 337 ± 40 | −      | −                |
| Motif IV                    | 900 ± 180        | 38.4 ± 3.2 | 86.7 ± 6.7 | 2.9 ± 1.8 | 601 ± 66 | 340 ± 65 | −      | −                |
| Motif V (G)                 | 296 ± 67         | 36.1 ± 3.6 | 86.7 ± 27 | 94 ± 6 | 153 ± 23 | 103 ± 14 | −      | −                |
| Motif V (T)                 | 317 ± 35         | 30.1 ± 3.2 | 86.7 ± 6.7 | 3.7 ± 0.8 | 132 ± 31 | 110 ± 7 | +     | −                |
| Motif VI                    | 438 ± 99         | 38.4 ± 2.6 | 119.8 ± 6.7 | 5.7 ± 1.2 | 281 ± 18 | 199 ± 57 | +     | −                |

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a Primase reactions were performed in triplicate as described under “Experimental Procedures.” The results from a typical experiment are shown in Fig. 3.

b ATPase reactions were performed in duplicate as described under “Experimental Procedures.” The actual rate constants and standard errors are shown in Fig. 4.

c Helicase reactions were performed in duplicate in the presence of the UL8 protein as described under “Experimental Procedures.” One typical experiment is shown in Fig. 5B.

d DNA gel-shift reactions were performed in triplicate in the presence and absence of the UL8 protein as described under “Experimental Procedures.” One typical experiment is shown in Fig. 6.

e In vivo transient replication complementation experiments have been previously published (37).

f Leading-strand DNA replication reactions were performed in duplicate in the presence of the UL8 protein as described under “Experimental Procedures.” One typical experiment is shown in Fig. 7.
absence of ATP (wild type and UL5 variant) was measured as described under “Experimental Procedures.” Reactions were performed in the presence (black bars) and absence (white bars) of 100 mM ssM13 DNA, and rate constants (min⁻¹) were determined by a least squares fit using the computer program Kaleidagraph²⁸.

In this study we report that although all but one of the mutations in the helicase motifs of UL5 drastically affect helicase activity, they did not eliminate primase activity. Somehow, in fact, all of the motif mutants displayed increased primer synthesis, and in some cases this effect was quite dramatic. We suggest two possible general explanations for the increased primase activity displayed by the helicase motif mutants. First, the increased primase activity may reflect alterations in subunit interactions resulting from small changes in the structure of the mutant UL5 polypeptides. Although the functional relationships of the three subunits of the HSV helicase-primase complex are not completely known, previous results have indicated that the full function of the complex is dependent on mutual interactions between the three subunits. Purified UL5 protein has minimal ATPase and helicase activities in the absence of UL52, and UL52 expressed alone is insoluble (20, 23, 43). UL5 has been shown to stimulate both activities (44, 45). We view this explanation as less likely because all of the helicase motif mutants resulted in increased primase activity; if simple structural changes were responsible, we would have expected that at least some of the mutants would have exhibited decreased primase activity. Alternatively, it is possible that the increases in primase activity are a direct result of decreases in helicase activity and reflect the fact that a single heterotrimeric helicase-primase complex bound to DNA cannot carry out helicase and primase activities simultaneously (helicase activity moves in the 5' to 3' direction along the lagging strand template, while primase activity necessarily occurs in the 3' to 5' direction along the template). According to this view, there are several possible ways in which helicase mutations could lead to increased primase activity. First, there may be competition between the helicase site and the primase site for binding to DNA. Thus, mutation of the helicase motifs of the UL5 polypeptide may disrupt binding of the UL5 helicase subunit to DNA, increasing the likelihood of DNA binding at the primase active site. We found no difference in DNA binding of any of the helicase variants compared with wild type, but the gel shift assay that we employed to analyze DNA binding may
not distinguish between binding at the helicase site and binding at the primase site. Second, translocation of the complex by helicase action may move the primase away from preferred primase recognition sites before productive binding by primase to these sites can occur. In the primase assays employed in this work, we used a 50-base oligonucleotide containing an optimal primase recognition site as template. It will be of interest to test the mutant proteins on longer, natural DNA templates. Finally, it is possible that the primase activity of the complex is regulated by the UL5 helicase subunit as a means of coordinating leading and lagging strand DNA synthesis at the replication fork. In this model, the helicase would exist in two structural states, active and inactive. The active structure would inhibit primase activity, and the inactive structure would stimulate primase activity. The helicase motif mutations may shift the UL5 structure toward the “inactive” state, leading to increased primase activity. It is interesting to note, however, that the G815A mutant that exhibits wild type helicase activity also exhibits a 3-fold stimulation in primase activity. Clearly, more work will be required to test these ideas.

Although the precise mechanism by which any helicase unwinds DNA or RNA is not known in detail, it is clear that the entire process will require the coupling of simpler events such as ATP binding, hydrolysis, and single- and double-stranded DNA binding. Thus, for instance, ATP binding and hydrolysis are an intrinsic property of all DNA and RNA helicases (reviewed in Refs. 1, 4). The UL5 protein contains the conserved Walker A (motif I) and Walker B (motif II) motifs found in ATP-binding proteins (33). Structural studies of ATP-binding proteins including the G protein Ha-ras p21 (46) elongation factor Tu (47–49), the ATP binding domain of the RecA protein (35), and adenylate kinase (34) indicate that the e-amino group of the conserved Lys in UL5 motif I results in the loss of ATPase and helicase activity and an increase in primase activity. It has not been determined if nucleotide can still bind to this variant protein. Although mutations in all of the conserved helicase motifs decrease ssDNA-dependent ATP hydrolysis, only replacement of the conserved Gly-102 residue in motif I abolishes intrinsic ATPase activity (Fig. 4). This variant also has no DNA helicase activity (Fig. 5B) but can still bind the helicase DNA substrate (Fig. 6). Taken together, these observations are consistent with a role of both the lysine and glycine in motif I in ATP hydrolysis. However, a role in ATP binding cannot be ruled out.

Another aspect of ATP binding involves the binding of the Mg$^{2+}$ ion which is believed to be stabilized by at least two residues in the A-motif, i.e. the Ser or the Thr, and one residue in the B-motif, the Asp or the Gln residue (33–35). An inability to properly bind and/or coordinate Mg$^{2+}$ may lead to a defect in nucleotide binding and/or hydrolysis. Mutagenesis of both the Asp and the Gln residues in motif II inactivates the ssDNA-dependent but not the intrinsic ATPase activity of the helicase-primase but does not significantly alter the K_m for the ATP-Mg complex (data not shown) suggesting that binding is not altered. Similar results have been obtained for DNA helicase II (UvrD) (62), RNA helicase eukaryotic initiation factor-4A (58), and DNA helicase NS-1 (63) and the vaccinia virus early transcription factor (50) in which mutation of one or both of these acidic residues in motif II decreases ATP hydrolysis but does not substantially affect ATP binding. Results from the DNA gel shift experiments suggest that the motif II variant subcomplex can still interact with DNA in the absence of ATP (Fig. 6). Addition of ATP or non-hydrolyzable ATP did not substantially alter the gel shifts for any of the subcomplexes (data not shown). Replacement of the conserved acidic residue in the

4 J. Crute, personal communication.
DnaK B-motif decreases both the peptide stimulation of ATP hydrolysis and the dissociation of the DnaK-peptide complex even in the presence of high concentrations of ATP (64, 65). The authors proposed that motif II is involved in coupling ATP hydrolysis to substrate binding by a protein conformational change which requires both ATP and Mg$^{2+}$ binding (64, 65). Replacement of the acidic residues by an uncharged Ala may affect either the electrostatic environment or the position of the Mg$^{2+}$ in the ATP binding site. We propose that these acidic residues in motif II are not essential for either ATP or DNA binding but play a key role in the proper coordination of ATP, Mg$^{2+}$, and DNA essential for productive ATP hydrolysis. The mutations in motif II may prevent a DNA-induced conformational change of UL5 required for the stimulation of ATPase activity. To test this model, assays that detect possible conformational changes induced in the presence of ATP, Mg$^{2+}$, and DNA will have to be developed.

Although none of the mutations in motifs III through VI affect the intrinsic ATPase activity, each partially reduce the ssDNA-dependent ATPase activity catalyzed by the helicase-primase (Fig. 4). With the exception of one mutation in motif V (G815A), these variant motifs are all unable to unwind DNA (Fig. 5B) or support helicase-dependent DNA replication (Fig. 7). However, all of the variant subcomplexes bind the DNA helicase substrate (Fig. 6). These variants are either intrinsically defective in the unwinding of DNA or are defective in the coupling ATP hydrolysis and DNA binding in such a way as to lead to DNA unwinding. Although it is presumed that helicases are able to couple the energy of ATP hydrolysis to the unwinding of DNA, it is not clear precisely how this occurs. For instance it is possible that the mutants which can hydrolyze ATP and bind but not unwind DNA may be defective in coupling ATP hydrolysis to processive translocation or in coupling processive translocation to actual unwinding. Either of these defects could be due to improper protein conformational cycling of the protein. The data presented here do not distinguish between these possibilities.

To our knowledge this study represents the first comprehensive mutational analysis of any superfamily 1 helicase. Structure-function analyses have been reported for several helicases in other superfamilies. For example, in the UvrB protein (superfamily 2), motifs IV and V are important in the induction of ATPase activity by UV-damaged DNA (66). Mutation of motif V in the RAD3 helicase (superfamily 2) results in an increase in recombination between short homologous sequences (67). The eIF-4A RNA helicase (superfamily 2) mutation of motif III impairs RNA unwinding but not RNA binding or ATP hydrolysis (58), whereas mutation of motif VI impairs RNA binding and ATP hydrolysis (68). Motif VI has also been implicated in DNA binding in the vaccinia virus early transcription factor (superfamily 2) (69). Mutations that uncouple ATPase and the helicase activities have been shown for the T7 helicase-primase (Dnab superfamily although none are present in known helicase superfamilies (70)). Although all of the helicase superfamilies have conserved Walker A and Walker B motifs, motifs III through VI are not well conserved between each superfamily. Structure-function analyses of helicases from the different helicase superfamilies have not led to the definitive assignment of function to the conserved motifs even within each superfamily. It has been proposed that helicase function is dependent on multimerization of the active polypeptide and involves the cycling of ATP hydrolysis and DNA binding leading to translocation and DNA unwinding (reviewed in Refs. 1, 3, 4). These models are based on studies with the hexameric helicases E. coli Dnab, SV40 T-antigen, the T7 gp4 helicase-primase, and the T4 gp41 helicase and the dimeric E. coli Rep helicase. The HSV-1 heterotrimeric helicase-primase appears to represent a unique helicase in its subunit structure. It will be of considerable interest to determine the exact stoichiometry of the HSV-1 helicase-primase with DNA as well as the ability of the UL5 variant proteins to form multimeric structures on DNA. Experiments to determine for instance whether the helicase-primase functions as heterotrimer or as a dimer of trimers are in progress. It is of interest to note that two other members of helicase superfamily I, E. coli Rec B and RecD, form a heterotrimetric complex with RecC which is involved in homologous recombination (reviewed in Refs. 3, 71, 72). Although it appears that HSV-1 DNA replication and DNA recombination may be closely linked, HSV-1 does not appear to encode a specific recombination dependent helicase. Thus, it will also be of interest to understand the role of the UL5 helicase in recombination.

In summary, we have presented data that confirm that at least six of the seven helicase motif mutations which abolish the in vivo activity of UL5 are due to the inability of the protein to unwind DNA. The one mutant (G815A mutation in motif V) which retains DNA helicase activities in vitro may affect coordination of leading and lagging strand DNA synthesis or modulation of essential protein-protein interactions required for an essential step in DNA replication other than helicase activity (37). In the present study we show that this glycine is not crucial for the interaction of the subcomplex with UL8. Further experiments will be required to fully understand the nature of the defect in this variant. In this study we found that not only did mutations which abolish helicase activity not eliminate primase activity, all of the motif mutants displayed increased primer synthesis, and in some cases this effect was quite dramatic. Furthermore, our results suggest that motif I is directly involved in ATP binding and/or hydrolysis; motif II appears to be required for coupling of DNA binding to ATP hydrolysis; and mutations in motifs III, IV, V, and VI do not eliminate ATP hydrolysis nor affect DNA binding and therefore may be involved in the coupling of these two activities to the process of DNA unwinding.

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