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

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The UL5 protein of herpes simplex virus type 1, one component of the viral helicase-primase complex, contains six sequence motifs found in all members of a superfamily of DNA and RNA helicases. Although this superfamily contains more than 20 members ranging from bacteria to mammalian cells and their viruses, the importance of these motifs has not been addressed experimentally for any one of them. In this study, we have examined the functional significance of these six motifs for the UL5 protein through the introduction of site-specific mutations resulting in single amino acid substitutions of the most highly conserved residues within each motif. A transient replication complementation assay was used to test the effect of each mutation on the function of the UL5 protein in viral DNA replication. In this assay, a mutant UL5 protein expressed from an expression clone is used to complement a replication-deficient null mutant with a mutation in the UL5 gene for the amplification of herpes simplex virus origin-containing plasmids. Eight mutations in conserved regions and three similar mutations in nonconserved regions of the UL5 gene were analyzed, and the results indicate that all six conserved motifs are essential to the function of UL5 protein in viral DNA replication; on the other hand, mutations in nonconserved regions are tolerated. These data provide the first direct evidence for the importance of these conserved regions in any member of the superfamily of DNA and RNA helicases. In addition, three motif mutations were introduced into the viral genome, and the phenotypic analyses of these mutants are consistent with results from the transient replication complementation assay. The ability of these three mutant UL5 proteins to form specific interactions with other members of the helicase-primase complex, UL8 and UL52, indicates that the functional domains required for replication activity of UL5 are separable from domains responsible for protein-protein interactions. It is anticipated that this type of structure-function analysis will lead to the identification of protein domains that contribute not only to the enzymatic activities of helicase or primase but also to protein-protein interactions within members of the complex.

Unwinding of duplex DNA and RNA is essential for many biological processes, including replication, recombination, repair, transcription, and translation. This activity is performed in cells by helicases which contain an intrinsic DNA- or RNA-dependent ATPase activity. The hydrolysis of ATP supplies energy for unidirectional translocation along one strand of duplex DNA or RNA, resulting in unwinding of the duplex. The essential role of DNA helicases in DNA replication has been studied in various prokaryotic systems and in the eukaryotic virus simian virus 40 (9, 25, 31, 39). A helicase activity in herpes simplex virus type 1 (HSV-1)-infected cells was first reported in 1988 (7).

Protein sequence analysis has revealed the existence of well-conserved motifs in a superfamily of known and putative helicases, including *Escherichia coli* proteins UvrD, Rep, RecB, and RecD, the yeast helicase PIF, proteins involved in positive-strand RNA virus RNA replication, and four proteins of the herpesvirus family (Fig. 1) (18, 19, 23). 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) (43). In addition, four other conserved motifs (III to VI) have been observed. 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.

The UL5 gene of HSV-1 has been identified as an essential gene for viral DNA replication through the studies of temperature-sensitive (*ts*) (48) and host range mutants (50). Temperature shift experiments with *ts* mutants in the UL5 gene indicate that this gene is required continuously during viral DNA synthesis, suggesting a direct role for the UL5 gene product (48). The existence of a consensus ATP binding site within the gene raised the possibility that this protein may be an ATPase or a helicase (32, 48). It is now clear that the UL5 gene product is part of a complex made up of three viral proteins (the products of the UL5, UL8, and UL52 genes) which exhibits helicase and primase activity (6, 8). These activities can be assembled in vivo in insect cells triply infected with baculoviruses expressing UL5, UL8, and UL52 gene products (3, 10). The coexpression of UL5 and UL52 gene products has been shown to be sufficient for DNA-dependent ATPase and helicase (3) and for helicase and primase (11) activities that are indistinguishable from the three-subunit complex.

Sequence analysis of the UL5 gene indicates that it is a member of the superfamily of helicases described above (17-19, 23). As with other members of this superfamily of helicases, the functional significance of the six conserved motifs in UL5 has not been addressed directly. We report herein our initial attempts to probe the importance of these regions in the UL5 gene by introducing directed amino acid substitutions into each of the conserved regions. Because

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FIG. 1. Conserved motifs in a superfamily of helicases. The top line represents the number of amino acid residues. Six proteins (UL5; *E. coli* proteins Rep, UvrD, RecB, and RecD and the yeast protein PIF) are represented by boxes, with the length of the box corresponding to the size of the protein (18, 19, 23). The six conserved motifs are represented by dark bars. The motifs are numbered as indicated, and the order of the motifs in each protein is identical.

the HSV genome is large (152 kb), it is not convenient or straightforward to introduce a large number of mutations directly into it. Therefore, we established a transient assay system to analyze the function of mutant UL5 gene products expressed from a cloned copy of the gene. Our results indicate that all of the six conserved motifs are essential to the function of the UL5 gene product in HSV DNA replication, thus providing direct evidence for the functional significance of these motifs. Three motif mutations were introduced into the viral genome, and phenotypic analysis of these viral mutants confirms the importance of these motifs in UL5 function. Furthermore, the ability of mutant UL5 proteins to form specific interactions with UL8 and UL52 indicates that the functional domains required for helicase activity are separable from domains responsible for protein-protein interaction. It is anticipated that this type of structure-function analysis will lead to the identification of not only protein domains that contribute to the enzymatic activities of helicase but also domains involved in protein-protein interactions.

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 (45). The KOS strain of HSV-1 was used as the wild-type virus. *ts* mutants of strain KOS, *ts*K13, and *ts*M19 were provided by P. A. Schaffer (Dana-Farber Cancer Institute, Boston, Mass.). The *lacZ* insertion mutant *hr99*, which does not synthesize the UL5 gene product, and the cell line L2-5, which supports the growth of UL5 mutants, are described in the accompanying report (50).

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. (36). Plasmid pDG2 contains the upstream regulatory element of the ICP6 gene on a 549-bp fragment in the vector Bluescribe (Stratagene, San Diego, Calif.) (see Fig. 1 in the accompanying report [50]). The vector Bluescribe contains an origin of replication from the phage M13 and thus can be packaged as single-stranded DNA in the presence of helper phage, R408 (38). Plasmid pCW8 containing an *XbaI*-to-*KpnI* fragment (sequence coordinates 10636 to 16269) encompassing the UL5 gene (12487C to 15133) was kindly provided by M. D. Challberg. p6UL5, in which the UL5 open reading frame is placed under the control of the upstream regulatory region of the ICP6 gene, is described in the accompanying report (50).

Recombinant plasmids were propagated in *E. coli* DH5 α F' by standard procedures (30).

Site-directed mutagenesis. Single-stranded DNA from p6UL5 was generated following infection with the helper phage R408 (1). Site-directed mutagenesis was performed on single-stranded DNA with the T7-GEN in vitro mutagenesis kit as instructed by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). Mutagenic oligonucleotides were synthesized by using a Cyclone DNA synthesizer (Biosearch Inc., Burlington, Mass.) (Table 1). In addition to incorporating the desired base substitution, many oligonucleotides also contain silent mutations which generate restriction enzyme site polymorphisms; thus, the restriction maps of the mutant genes can be distinguished from wild type. All of the mutations were verified by DNA sequence analysis using Sequenase (United States Biochemical) and the supplier's instructions.

Transient replication complementation (TRC) assay. Vero or L2-5 cells were transfected with the *ori_S*-containing plasmid p100-1 by itself or cotransfected with wild-type or mutant p6UL5 by using a modification of the standard calcium phosphate coprecipitation procedure (21). A mixture of DNAs including 0.2 μ g of p100-1, 1.0 μ g of p6UL5 (where indicated), and 5 μ g of sonicated salmon sperm DNA in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (pH 7.05) was precipitated in the presence of 125 mM CaCl₂ for 20 min at room temperature. Rapidly growing Vero or L2-5 cells (1.5×10^6) were pelleted by low-speed centrifugation and resuspended in 600 μ l of the precipitated DNA mixture, and the cell suspension was incubated in a shaking incubator at 37°C for 30 min. Complete medium was added, and the cells were transferred to a 60-mm tissue culture dish at 37°C. Four hours later, the medium was removed and the dish was washed once with prewarmed phosphate-buffered saline (PBS), incubated in 2 ml of HEPES-buffered saline with 15% glycerol at 37°C for 2 min, and washed twice with PBS before the dish was replenished with complete medium. At 24 h posttransfection, the medium was changed. At 30 h posttransfection, the cells were superinfected with KOS or *hr99* 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 (46). Two micrograms of DNA was analyzed by digestion with *EcoRI* alone or in combination with *DpnI* and then subjected to agarose gel electrophoresis and Southern blot hybridization. The blot was probed with Bluescribe DNA labeled with ³²P as described previously (13).

TABLE 1. Sequences of mutagenic oligonucleotides

Mutation	Oligonucleotides (5'-3') ^a	New restriction site
G-102 to V	GCACGTGCTCTTTACGGATCCAGCGTTGCC	<i>Bam</i> HI
K-103 to A	TGTCTGCACGCACGTGCTCGCTCCGGATCCAGCGTTGCC	<i>Bam</i> HI
Reversion A-103 to K	CGCACGTGCTCTTTCCGGAGCCAGCGTTGC	Loss of <i>Bam</i> HI site
D-249E-250 to AA	CCCAAGGAGGCCGGCAGCAGCGATGACGAT ^b	<i>Nae</i> I
G-290 to S	TCGGCGAGCTCACACACAC	None
R-345 to K	GTTACCGAAATTCGTGCTCGACGCACCTTTTGTGTGT	<i>Eco</i> RI
R-416 to K	CTCCCCCTCTTTTCGTACCTTC	None
E-579 to A	GCCGTTAGTGCTCCCATGC	None
E-757E-758 to AA	AGGGCAGGGGGCGCGCTGCCAGTAAC	<i>Nar</i> I
T-809 to I	CGCGTGATGATCATGGCAA	None
G-815 to A	GTCCAGGCTCAGGCGCTGGGAGC	<i>Alw</i> NI
Y-836 to A	TGGCCACGGCCGCGCTGT	None

^a Desired mutations are represented by double underlining, and silent changes to create new restriction enzyme sites are indicated by single underlining.

^b Three of three isolates containing the desired DE-to-AA mutation as determined by DNA sequencing do not contain the cleavage site for *Nae*I as expected; instead, they contain the wild-type sequence at this position.

Immunoprecipitation and immunoblot analysis. To detect the UL5 protein synthesized from the expression clone p6UL5, immunoprecipitation of [³⁵S]methionine-labeled extracts was performed as described in the accompanying report (50). To detect UL5 protein and protein-protein interactions in cells infected with KOS or viral mutants, a combination of immunoprecipitation followed by immunoblot analysis was performed as described in the accompanying report (50).

Isolation of UL5 motif viral mutants. Infectious *hr99* genomic DNA was prepared as described previously (48). HSV mutants containing motif mutations were recovered by cotransfecting L2-5 cells with infectious *hr99* DNA and mutant versions of p6UL5. Marker transfer experiments were performed as described previously (16) except that white plaques were picked and purified from a background of blue plaques.

Southern blot analysis of viral DNA. Viral DNA was recovered and analyzed as described in the accompanying report (50).

Analysis of viral DNA synthesis. The ability of mutants to induce viral DNA synthesis was analyzed as described in the accompanying report (50).

RESULTS

Development of a functional assay for UL5 gene mutations. The functional significance of the six conserved putative helicase motifs was investigated by introducing mutations into the most conserved amino acid residues in the motifs. Because of the large size (152 kb) of the HSV genome, it is not convenient or straightforward to introduce a series of site-directed mutations into the viral genome. To facilitate the analysis of the effects of these engineered mutations, we developed an assay to test the function of various UL5 gene mutations expressed from plasmids. The assay was 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 of the necessary *trans*-acting functions are provided. *trans*-acting replication functions can be supplied by superinfection with HSV (40, 41, 46) or by cotransfection with a set of seven cloned HSV genes (4, 22, 47). We showed that infection with *ts* mutants containing mutations in the UL5 gene (*ts*K13 or *ts*M19) fails to support the replication of origin-containing plasmids at the nonpermissive temperature and that the defect can be complemented by the presence of a plasmid expressing the wild-

type version of the UL5 gene product (data not shown). This assay will be referred to as the TRC assay.

The TRC assay was optimized with respect to the virus providing *trans*-acting replication factors and the clone providing functional UL5 protein. To circumvent possible interference or complications due to the presence of a *ts* gene product in the TRC assay, a UL5 insertion mutant, *hr99*, which fails to synthesize UL5 protein (50) was used. To provide a functional UL5 gene product, p6UL5, in which UL5 is expressed from the ICP6 promoter, was used. In the accompanying report (50), we show that the level of expression from p6UL5 is at least 10-fold higher than from pCW8, in which UL5 is expressed from its own promoter. Thus, the use of p6UL5 avoids potential problems associated with inefficient expression of functional UL5 in the TRC assay. Furthermore, the ICP6 promoter is inducible by superinfection with *hr99* (49, 50). Thus, in the TRC assay, all the *trans*-acting factors needed for DNA synthesis except UL5 are provided by *hr99*, and functional UL5 is provided in an inducible manner by p6UL5.

A typical TRC assay is shown in Fig. 2. Vero cells or L2-5 cells, containing the wild-type version of the UL5 gene, were transfected with *ori_S*-containing p100-1 alone or in combination with the UL5 expression clone p6UL5. At 30 h posttransfection, cells were superinfected with KOS or *hr99*. At 40 h posttransfection, total DNA was harvested and digested with *Eco*RI alone (Fig. 2, lanes a) or in combination with *Dpn*I (lanes b). *Eco*RI cleaves p100-1 once, generating a fragment of 2.8 kb (labeled p100-1 in Fig. 2). *Dpn*I recognizes only 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 *Dpn*I (lane 1b), whereas DNA which has replicated in mammalian cells is *Dpn*I resistant (lanes 2b, 4b, and 5b). The presence of *Dpn*I-resistant bands indicates that the plasmid DNA has replicated in Vero cells. When Vero cells were transfected with p100-1 and superinfected with KOS, a *Dpn*I-resistant band of 2.8 kb, representing linearized p100-1, was observed, indicating that KOS can support the replication of this origin-containing plasmid (lane 2b). The smaller *Dpn*I digestion fragments (lanes b) represent input plasmid and serve as internal controls for the efficiency of transfection and recovery. The absence of the 2.8-kb *Dpn*I-resistant band in cells transfected with p100-1 and superinfected with *hr99* indicates that *hr99* cannot support the replication of p100-1 (lane 3b). However, if a functional UL5 product is supplied

FIG. 2. TRC assay. The TRC assay was performed in either Vero or L2-5 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). Lane 1 contains 5 ng of p100-1 containing HSV-1 *oriS* propagated in *E. coli*, and lanes 2 to 5 contain 2 μ g of total cellular DNA harvested from the TRC assay as follows: lane 2, transfection with p100-1 and superinfection with KOS; lane 3, transfection with p100-1 and superinfection with *hr99*; lane 4, transfection with p100-1 and p6UL5 and superinfection with *hr99*; lane 5, transfection of L2-5 cells with p100-1 and superinfection with *hr99*. After restriction enzyme digestion, the samples were subjected to 0.8% agarose gel electrophoresis and blotted onto a GeneScreen Plus membrane. The blot was hybridized with 32 P-labeled Bluescribe plasmid DNA. Positions of the linearized p100-1 and the 6.3-kb fragment from p6UL5 are marked. Small fragments resulting from *DpnI* digestion are also marked.

either by cotransfection with the UL5 expression clone p6UL5 or by transfection of L2-5 cells which contain a wild-type version of the UL5 gene, the defect in *hr99* is complemented (lanes 4b and 5b, respectively). The 6.8-kb band seen with *EcoRI* digestion (lane 4a) represents input p6UL5 plasmid. This plasmid lacks an HSV origin and therefore cannot replicate in Vero cells and is sensitive to *DpnI* digestion (lane 4b). In L2-5 cells, the p6UL5 band is *DpnI* resistant because it is integrated and replicates with cellular DNA (lane 5b). By using this assay, the ability of the UL5 protein expressed from the expression plasmid p6UL5 to complement the replication defect in *hr99* can be tested. This assay provides a convenient method for testing the function of UL5 protein expressed from plasmids bearing wild-type and mutant versions of the UL5 gene.

UL5 contains six conserved helicase motifs. Sequence analysis of the UL5 gene reveals considerable homology with members of a superfamily of helicases of *E. coli*, *Saccharomyces cerevisiae*, and RNA and DNA viruses (Fig. 1) (17–19, 23). The presence of six well-conserved motifs and the similarity of their positions relative to one another are noteworthy (Fig. 1). In this report, the functional significance of these six conserved motifs in UL5 was examined by the introduction of engineered mutations into the most conserved residue(s) in each motif (Fig. 3).

Motifs I and II. Oligonucleotide mutagenesis was performed on single-stranded DNA from p6UL5 as described in Materials and Methods; oligonucleotides used to generate each mutation are listed in Table 1. The first target for mutagenesis was motif I (also called the A site) containing a sequence (G/AX₄GKS/T) which is highly conserved among most enzymes utilizing ATP and GTP (43). This motif contains a flexible loop believed to be involved in the binding of the pyrophosphate moiety of nucleoside triphosphates (NTPs). Two invariant residues were targeted for mutagenesis; the first mutation resulted in an alteration of G-102 to a

FIG. 3. Site-directed mutagenesis of the UL5 gene. The UL5 protein is represented by a shadowed box, and the six conserved motifs are represented by dark bars. Arrows under the UL5 protein indicate the positions of site-directed mutations. The substituted amino acids in three mutations in nonconserved regions are marked with arrows. The amino acid sequences of the six conserved motifs from six proteins (UL5, Rep, UvrD, RecB, RecD, and PIF) are shown (18, 19, 23); the numbers between the amino acid sequences refer to the number of the residues separating each motif. The most conserved residues within each motif are listed below the amino acid sequences. Conserved residues were subjected to mutagenesis, and the amino acid substitutions are shown.

FIG. 4. TRC assay of UL5 proteins with site-directed mutations in motifs I and II. The TRC assay was performed as described in Materials and Methods and the legend to Fig. 2. In each sample, p100-1 was used in transfection either alone (lane control) or in combination with p6UL5 containing wild-type UL5 (lane p6UL5) or various mutant constructs of p6UL5 as indicated. Transfected cells were superinfected with *hr99*.

valine residue (G102V), and the second resulted in an alteration of K-103 to alanine (K103A) (Fig. 3). Both mutagenic oligonucleotides also generate a novel *Bam*HI site, and the introductions of both the targeted motif mutation and the novel restriction site into p6UL5 were confirmed by DNA sequencing (data not shown). The motif I mutations were tested for their ability to complement *hr99* in the TRC assay as described above. As shown in Fig. 4, wild-type UL5 (p6UL5) can complement *hr99* in its ability to support the replication of p100-1 in Vero cells (lane p6UL5); however, mutants G102V and K103A fail to do so (lanes G102V and K103A, respectively). The transfection efficiencies of both p100-1 and p6UL5 in each assay are roughly similar, as indicated by the presence of the small *Dpn*I-sensitive fragments (lanes b); as described above, these bands represent input DNA. The loss of UL5 function in G102V and K103A demonstrates the importance of conserved motif I. Furthermore, a reversion of the K103A mutation back to wild type (A103K) results in the recovery of UL5 function (lane A103K), confirming that the observed loss of function of K103A is due to the targeted mutation at position 103.

The second region targeted for mutagenesis was motif II, a region containing one or two negatively charged residues (D or DE) at the COOH terminus of a β strand; the negatively charged residue(s) is believed to interact with the Mg^{2+} associated with the β or γ phosphate of a purine nucleotide substrate (motif II) (14, 26). To assess the significance of this motif, we replaced both the aspartic acid at residue 249 and the glutamic acid at residue 250 with two alanine residues (Fig. 3). The plasmid carrying mutant DE250AA was unable to complement *hr99* in the replication assay (Fig. 4), indicating that the presence of negatively charged amino acids within this motif is indeed essential.

Motifs III to VI. In addition to the well-known motifs I and II, four other motifs are shared among over 20 proteins which have known or putative helicase activity (19, 23). However, unlike motifs I and II, the functional significance of motifs III to VI has not been previously addressed either genetically or biochemically. To determine whether these

FIG. 5. TRC assay of UL5 proteins with site-directed mutations in motif II, IV, V, and VI. For details, see the legend to Fig. 4. Pairs of samples are marked with the names of the p6UL5 constructs used in transient transfections.

four motifs are essential for UL5 function, we introduced alterations into each conserved region (Fig. 3). Motif III contains an invariant glycine at position 290 which was replaced with a serine (G290S). A conservative alteration replacing the invariant arginine residue at position 345 with lysine (R345K) was introduced into motif IV. An *Eco*RI site was introduced into p6UL5 together with the R345K mutation, and as expected, the band representing p6UL5 is smaller in size (Fig. 5). Two mutations were introduced into motif V, one replacing threonine 809 with isoleucine (T809I) and the other replacing glycine 815 with alanine (G815A). Each of the mutations in motifs III, IV, and V resulted in failure to complement *hr99* in the replication assay (Fig. 5).

Motif VI can be divided into two components, one well conserved among RNA and DNA helicases of this superfamily and the other a single tyrosine found in known DNA helicases but absent in RNA helicases (23). A tyrosine in this position is found in *E. coli* enzymes UvrD, Rep, RecB, and RecD, in the yeast enzyme PIF, and in the UL5 counterparts of the herpesviruses Epstein-Barr virus, human cytomegalovirus, varicella-zoster virus, and HSV. To assess its importance, we changed the tyrosine at position 836 of UL5 to an alanine (T836A). Mutation T836A completely abolishes the ability of the plasmid to complement *hr99* for UL5 function (Fig. 5, lane Y836A).

Nonconserved regions of UL5. Mutations in nonconserved regions of UL5 were introduced to determine whether the UL5 gene product can tolerate similar changes outside the putative functional motifs (Fig. 3). Thus, an arginine-to-lysine mutation at position 416 (R416K), a glutamic acid-to-alanine substitution at position 579 (E579A), and a double replacement of two glutamic acid residues at 757 and 758 to two alanine residues (EE758AA) (Fig. 3 and Table 2) were tested as described above. All three mutant plasmids were able to complement *hr99* as well as did the wild type (Fig. 6). This result indicates that the UL5 protein can tolerate changes outside the conserved motifs.

Detection of mutant UL5 gene products. The loss of function of mutations in the six conserved motifs of UL5 described above could in principle be due either to changes in global protein conformation or to changes in localized

TABLE 2. Plaquing efficiencies of KOS, *hr99*, *hr99G201V*, *hr99K103A*, and *hr99R345K* on Vero and L2-5 cells^a

Virus	Cell line	Titer (PFU/ml)
KOS	Vero	5×10^9
	L2-5	5×10^9
<i>hr99</i>	Vero	$<1 \times 10^3$
	L2-5	5×10^8
<i>hr99G102V</i>	Vero	$<1 \times 10^3$
	L2-5	5×10^8
<i>hr99K103A</i>	Vero	$<1 \times 10^3$
	L2-5	3×10^8
<i>hr99R345K</i>	Vero	$<1 \times 10^3$
	L2-5	6×10^8

^a Plaquing efficiencies were measured by determining the titers of virus stocks on monolayers of Vero or L2-5 cells as indicated.

residues essential for function. Changes in global protein conformation often affect protein stability, resulting in degradation (37). To demonstrate that the loss-of-function mutations still encode a stable UL5 protein, UL5 expression from mutant constructs was examined in transient transfection experiments. Cells were transfected with wild-type pUL5 or mutant versions of the plasmid and superinfected with *hr99*. Cells were labeled with [³⁵S]methionine, and cell extracts were immunoprecipitated with anti-UL5 antiserum (α-UL5) as described in Materials and Methods. Cells transfected with salmon sperm DNA alone and superinfected with *hr99* fail to synthesize a protein of the expected molecular size, 99 kDa (Fig. 7, leftmost lane). When cells were transfected with pUL5 and superinfected with *hr99*, a protein of 99 kDa was specifically precipitated by α-UL5 (Fig. 7, lane Wild type). In the absence of superinfection, cells transfected with pUL5 fail to synthesize detectable amounts of the UL5 gene product (data not shown). Thus, consistent with results presented in the accompanying report (50), *hr99* is able to provide *trans*-inducing factors which stimulate expression of pUL5. Cells transfected with UL5 constructs containing mutations in motifs I through VI produce UL5 protein recognized by α-UL5 in amounts similar to those produced by the wild type after superinfection with *hr99*

FIG. 6. TRC assay of UL5 proteins with site-directed mutations in the nonconserved regions. For details, see the legend to Fig. 4. Pairs of samples are marked with the names of the constructs used in transient transfections.

FIG. 7. Detection of UL5 proteins in transient transfection by immunoprecipitation with α-UL5. Equal amounts (12 μg) of pUL5 with mutations that abolished the UL5 function (as marked) were used to transfect Vero cells, and the cells were then superinfected with *hr99*. In the leftmost lane, 12 μg of salmon sperm DNA was used. Transfected cells were labeled and processed for immunoprecipitation as described in the accompanying report (50). Positions of ¹⁴C-labeled rainbow protein molecular weight markers (Amersham, Arlington Heights, Ill.) electrophoresed in parallel are shown on the left.

(Fig. 7). All mutant proteins showed electrophoretic mobilities identical to that of the wild-type protein. Thus, although these mutations abolish the ability of UL5 to complement *hr99* in the replication assay, the stabilities of the mutant UL5 gene products are comparable to that of the wild type. This result suggests that the loss of function seen in the mutant constructs is most likely due to changes in residues involved in UL5 function rather than disruptions which affect the three-dimensional structure and destabilize the protein.

Isolation of viral mutants with UL5 motif mutations. The transient replication complementation test has provided a convenient way of assessing the effects of motif mutations on the function of UL5 protein in viral DNA replication. To confirm that this transient assay indeed serves as a reasonable facsimile for viral infection, we introduced several motif mutations into the viral genome and tested their effects in the context of viral infection. The isolation of mutants with UL5 motif mutations was facilitated by the availability of the UL5 null mutant *hr99* (50). *hr99* contains an ICP6::lacZ insertion near the N terminus of the UL5 gene and forms blue plaques on the UL5 permissive cell line L2-5. Viruses containing motif mutations were recovered following cotransfection of L2-5 cells with infectious *hr99* DNA and plasmids carrying motif mutations (see Materials and Methods). Homologous

FIG. 8. Genome structures of mutants *hr99*, *hr99G102V*, *hr99K103A*, and *hr99R345K*. Diagrams showing the mutations in each viral mutant are shown in relation to an *XbaI*-to-*KpnI* fragment (sequence positions 10636 to 16261) which spans the UL5 open reading frame. The UL5 open reading frame is shown as a hatched box with an arrow indicating the direction of translation within the HSV genome. The positions of site-directed mutations and the new restriction sites generated concurrently in *hr99G102V*, *hr99K103A*, and *hr99R345K* are marked by arrows. The positions of DNA probes for Southern blot hybridization are shown; the N-N probe and the M-M probe are DNA fragments generated by *NaeI* and *MluI* digestion, respectively. Restriction enzyme recognition sites: B, *BamHI*; G, *BglII*; K, *KpnI*; M, *MluI*; P, *PstI*; X, *XbaI*.

recombination between *hr99* DNA and the UL5 sequences results in viral genomes which have lost the *lacZ* gene and therefore form white plaques in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (15). Since new restriction sites were introduced by silent nucleotide changes at positions next to many of the motif mutations (Table 1), the presence of the mutations could be determined by restriction analysis of viral DNA from the white plaque isolates. We have introduced two mutations in motif I (G102V and K103A) and one mutation in motif IV (R345K) into viral genomes, resulting in the isolation of mutants *hr99G102V*, *hr99K103A*, and *hr99R345K*, respectively (Fig. 8). In each case, over 50% of the white plaque isolates contained the desired mutation, as determined by restriction enzyme analysis. KOS, *hr99*, and the three motif mutant DNAs were analyzed by restriction enzyme digestion and Southern blot hybridization (Fig. 9). DNAs from *hr99G102V* and *hr99K103A* would be expected to contain a novel *BamHI* site, and *hr99R345K* would be expected to contain a novel *EcoRI* site, as diagrammed in Fig. 8. In Fig. 9A, KOS, *hr99*, *hr99G102V*, and *hr99K103A* DNAs were digested with *BamHI* and probed with the N-N probe (an *NaeI* fragment; Fig. 8). Wild-type KOS contains the expected 9.8-kb band (Fig. 9, lane 1). In *hr99*, the size of the corresponding fragment increases to approximately 14 kb as a result of the ICP6::*lacZ* insertion (lane 2). In both *hr99G102V* and *hr99K103A*, the new *BamHI* sites are clearly present, as indicated by the disappearance of the 14-kb band and the appearance of 3.0- and 6.8-kb bands (lanes 3 and 4, respec-

FIG. 9. Southern blot analysis of viral DNA from KOS and three UL5 mutants. (A) Viral DNAs were digested with *BamHI*, and the blot was hybridized with the N-N probe (see Fig. 8). Lanes: 1, KOS; 2, *hr99*; 3, *hr99G102V*; 4, *hr99K103A*. (B) Viral DNAs were digested with *EcoRI* and probed with the M-M probe (see Fig. 8). Lanes: 5, KOS; 6, *hr99*; 7, *hr99R345K*. Sizes of the fragments are as marked.

FIG. 10. Analysis of viral DNA synthesis by KOS and four viral mutants. Vero or L2-5 cells were infected with the indicated virus at a multiplicity of infection of 5 PFU per cell for 18 h at 37°C. A series of fivefold dilutions of each cell suspension was spotted onto a GeneScreen Plus membrane. Cells were lysed on the membrane, and the membrane was hybridized with 32 P-labeled *EcoRI* F fragment as described in the accompanying report (50).

tively). The intensities of the 3.0- and 6.8-kb bands differ depending on the position of each fragment with respect to the probe. In Fig. 9B, KOS, *hr99*, and *hr99R345K* DNAs were digested with *EcoRI* and probed with the M-M probe (an *MluI* fragment; Fig. 8). As predicted, KOS contains a 16.7-kb band (lane 5). The 2.2- and 15.9-kb bands seen in *hr99* DNA (lane 6) are expected and are due to internal *EcoRI* sites within the ICP6::lacZ insert itself. The presence of a new *EcoRI* site in *hr99R345K* (lane 7) is confirmed by the appearance of 1.5- and 15.2-kb bands. The large fragments in lanes 6 and 7 are very faint because of the small region of homology with the M-M probe.

Phenotypic analysis of motif mutants *hr99G102V*, *hr99K103A*, and *hr99R345K*. Mutants *hr99G102V*, *hr99K103A*,

and *hr99R345K* were propagated on L2-5 cells, and the resulting stocks were titrated on L2-5 and Vero cells. As shown in Table 2, these mutants could form plaques efficiently in L2-5 cells; however, all three mutants are unable to form plaques on Vero cells. Thus, these motif mutations severely impair the normal growth on Vero cells, suggesting that the mutations affect UL5 functions essential for virus growth. The ability of mutants *hr99G102V*, *hr99K103A*, and *hr99R345K* to synthesize viral DNA was analyzed by dot blot hybridization to a representative HSV DNA probe (32 P-labeled HSV-1 strain KOS *EcoRI* F fragment) as described in Materials and Methods. Figure 10 shows that all three mutants were unable to synthesize viral DNA in Vero cells, whereas the inability to synthesize viral DNA was fully complemented to wild-type levels by growing in the permissive cell line L2-5.

Motif mutants synthesize stable UL5 protein which retains its ability to interact with UL8 and UL52 specifically. As mentioned above, loss-of-function mutations could be due either to changes in global protein conformation or to changes in localized residues essential for function. Therefore, stability of the mutant UL5 proteins in the context of viral infection was examined. Extracts from mutant and wild-type virus-infected cells were immunoprecipitated with α -UL5, and the immunocomplexes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with α -UL5 as described in Materials and Methods. Figure 11A shows that while *hr99* did not synthesize detectable UL5 protein, all three motif mutants were capable of synthesizing full-length UL5 proteins at levels similar to that of the wild-type virus KOS. Thus, consistent with results of transient transfection experiments described above, the mutations do not appear to affect the stability of the UL5 peptide.

As demonstrated in the accompanying report (50), the interaction between UL5 and other members of the helicase-primase complex, UL8 and UL52, can be demonstrated in KOS-infected cells by coimmunoprecipitation. We examined whether UL5 peptides with mutations in motifs I and IV are capable of forming specific immunocomplexes with UL8 and UL52. Extracts from Vero cells infected with KOS or UL5 motif mutants *hr99G102V*, *hr99K103A*, and *hr99R345K* were immunoprecipitated with α -UL5 as described above. Immunocomplexes were subjected to SDS-PAGE and im-

FIG. 11. Interactions of UL5 proteins from KOS and four viral mutants with UL8 and UL52 as detected by coimmunoprecipitation. Vero cells were mock infected or infected with the indicated virus at a multiplicity of infection of 10 PFU/ml for 18 h at 34°C. Cells were harvested, and extracts were subjected to a combination of immunoprecipitation and immunoblotting as described in the accompanying report (50). Extracts were immunoprecipitated with α -UL5, and immunocomplexes were divided into three aliquots and subjected to SDS-PAGE in parallel. Each blot was probed with either α -UL5 (A), α -UL8 (B), or α -UL52 (C). Positions of the UL5 and immunoglobulin G (IgG) proteins are marked in panel A; positions of the UL8 and UL52 proteins are marked in panels B and C, respectively. Positions of molecular weight markers are indicated.

munoblotted with α -UL8 or α -UL52 as described in the accompanying report (50). Figures 11B and C demonstrate that mutant peptides specified by *hr99G102V*, *hr99K103A*, and *hr99R345K* retain the ability to interact with both UL8 and UL52. In Fig. 11B, cells infected with the three motif mutants *hr99G102V*, *hr99K103A*, and *hr99R345K* contain a smaller band in addition to the expected UL52 gene product. This band is not present in KOS-infected cells and is not reproducibly seen in motif mutant-infected cells. Its origin is not clear.

Thus, the behavior of the motif mutants with respect to viral DNA synthesis and stability of mutant peptides is consistent with the results obtained in transient assays. These results thus confirm the validity of the TRC assay for testing the function of UL5 in viral DNA synthesis.

DISCUSSION

The functional dissection of genes by site-directed mutagenesis has provided many insights into the evolutionary relationship and domain organization of many protein molecules. In this study, we initiated a structure-function analysis of the UL5 protein. The presence of six conserved motifs in UL5 shared by a large superfamily of helicases with six conserved motifs provided logical targets for site-specific mutagenesis. The results presented demonstrate that all six motifs are in fact essential for the function of UL5 in DNA replication.

TRC assay. Structure-function analysis involves the introduction of a series of specific mutations into a gene. For the HSV system, it is not always convenient and straightforward to introduce a large number of specific mutations directly into the viral genome. To facilitate our analysis of UL5 protein mutants, we developed a convenient assay for UL5 function in transiently transfected cells. The TRC assay is based on the demonstration that a plasmid bearing an HSV origin of replication can be amplified if all necessary *trans*-acting functions are supplied (4, 40, 41, 46, 47). In our study, all of the *trans*-acting factors except UL5 are provided by the null mutant *hr99* (50), and wild-type or mutant versions of UL5 are provided by the expression plasmid p6UL5. Thus, the ability of mutant versions of UL5 to complement *hr99* in the amplification of origin-containing plasmids provides a functional assay for UL5.

All six conserved motifs of the UL5 gene are essential. Protein sequence analysis has revealed that the HSV UL5 (as well as the UL5 gene counterparts in other herpesviruses) is a member of a superfamily of DNA and RNA helicases which share a set of six well-conserved motifs (18, 19, 24). This remarkable conservation of motifs within helicases from bacteria, yeast, and mammalian cells and viruses suggests that these motifs are important for helicase function; however, their significance has not been directly demonstrated. In this report, we show that mutations in each of the conserved motifs of UL5 completely abolish UL5 function in the TRC assay, whereas similar mutations into nonconserved regions of the UL5 gene have no effect on UL5 function. The effect of amino acid substitutions on UL5 function could in theory be due to either a change in a residue that is involved in function or a change in protein conformation potentially leading to instability of the protein. We consider the latter possibility unlikely since wild-type levels of full-length UL5 protein have been detected in cells transiently transfected with plasmids expressing loss-of-function mutations in the UL5 gene (Fig. 7).

To confirm that transient transfection experiments reflect

events which occur in infected cells, three motif mutations (G102V, K103A, and R345K) were introduced into the viral genome. This allowed us to confirm the validity of the TRC assay and to analyze single amino acid substitutions in the UL5 gene in the context of the viral infection. Introduction of motif mutations into the viral genome was facilitated by the existence of the *lacZ* insertion mutant *hr99*, which provides a color screen for recombinant viruses. The phenotypes of mutants *hr99G102V*, *hr99K103A*, and *hr99R345K* fully support the results obtained from the TRC assay in that these mutants fail to synthesize viral DNA when grown in Vero cells, although wild-type levels of mutant proteins are present in infected cells. Consistent with their inability to synthesize viral DNA, these three mutants also fail to form plaques on Vero cells.

As we have shown in the accompanying report (50), UL5 protein expressed during viral infection can be assayed for its ability to associate with UL8 and UL52 proteins by a combination immunoprecipitation and immunoblot procedure. To date we have not been able to observe this association in cells transiently transfected with expression plasmids, presumably because of the sensitivity of our detection methods (51). In this report, we show that UL5 proteins expressed from the three UL5 motif mutants retain the ability to interact with UL8 and UL52 as assayed by coimmunoprecipitation. The ability of these mutant UL5 proteins to associate specifically with UL8 and UL52 indicates that the conserved motifs I and IV are unlikely to be involved directly in the interaction between components of the helicase-primase complex. In addition, these results indicate that the tertiary structure of the mutant proteins has not been grossly altered by the mutations. Taken together, our results suggest that the loss of UL5 function exhibited by the eight mutations in motifs I through VI was due to changes in residues required for UL5 function. These results provide the first demonstration of the functional importance of motifs conserved within members of the large superfamily of helicases.

Possible roles of the six conserved motifs. The importance of motifs I and II in NTP-binding proteins has been recognized for some time on the basis of (i) nuclear magnetic resonance and X-ray diffraction data (14, 26) and (ii) genetic analyses in which replacement of residues in motifs I and II abolishes the ATPase activity of NTP-binding proteins. Interestingly, mutation of the invariant lysine in motif I of the yeast RAD3 protein to an arginine residue resulted in a protein which could still bind ATP but was defective in hydrolysis (42). Similarly, mutations introduced into the invariant glycine or lysine of motif I in the multidrug resistance gene of the mouse (*mdr1*) resulted in the abolition of drug resistance; again these mutants still retained the ability to bind ATP but failed to hydrolyze it (2). Some motif I mutations, on the other hand, 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 (29). Thus, motif I has been strongly implicated in binding and hydrolysis of NTPs in several known NTP-binding proteins. The invariant negatively charged residues in motif II have not received much attention to date. Our results for UL5 indicated the absolute requirement for the negatively charged residues in motif II, while two similar mutations (EE to AA and E to A) introduced into two nonconserved regions of the protein did not affect UL5 function.

Little is known about the biological significance of the other four motifs shared by this superfamily of known and

putative helicase proteins. Our results demonstrate that these four conserved motifs are indeed essential for the function of UL5. In addition to an NTP-binding fold, one might expect that a helicase would contain several other functional regions, including one which contacts the nucleic acid substrate. It is interesting to note that motif VI contains certain residues found only in putative DNA helicases but not in RNA helicases; for example, the tyrosine in motif VI of UL5 is shared by *E. coli* proteins UvrD, Rep, RecB, and RecD, the yeast helicase PIF, and three other herpesviruses, varicella-zoster virus, Epstein-Barr virus, and human cytomegalovirus (18, 19, 24). Our results demonstrate that this tyrosine is indeed important for UL5 function, since the substitution of tyrosine 836 with an alanine residue abolished the activity of UL5 in the replication assay. Further biochemical analysis of this mutant will be necessary to determine whether DNA binding is affected (see below). An additional region of sequence conservation located between motifs I and II and designated motif Ia has also been associated with presumed DNA but not RNA helicases (17, 24, 27).

It is of interest to note that the superfamily described above does not contain all known helicases. A second helicase superfamily (SF2) which contains proteins from *E. coli*, yeast, insect, and mammalian cells, poxviruses, herpesviruses, and positive-strand RNA viruses has been identified (20, 28). A degree of similarity exists between SF1 and SF2, although there are substantial differences. One member of the SF2 family, the RAD3 protein, has been studied through spontaneous and artificial mutations (42). All mutations impairing its activity in DNA repair or essential functions fell exactly within the conserved motifs I to V (33–35). This finding strongly demonstrates the functional importance of these conserved motifs of the SF2. Recently, the presence of these conserved motifs in a newly identified and sequenced protein encoded by the human ERCC-3 gene was taken as evidence for a potential DNA-unwinding function (44). Although it will be necessary to demonstrate experimentally that the product of the human ERCC-3 gene is indeed a helicase, the results provided in this report provide experimental support for the notion that such conserved motifs are good predictors of regions likely to be important for function.

The results presented in this report clearly demonstrate that it will be possible to carry out a detailed structure-function analysis of the HSV UL5 gene product. UL5 viral mutants with single amino acid substitutions have been isolated which are incapable of carrying out viral DNA synthesis but are still able to form specific complexes with other viral proteins. The next step will be to assay mutant viral proteins for other activities in order to define domains required for various functions required of a helicase, including ATP-binding, ATPase, nonspecific DNA-binding, and helicase activities. Direct biochemical assays have been hampered by the lack of a convenient overexpression system which is easily manipulated genetically. The only expression system for UL5, UL8, and UL52 which has consistently generated active helicase or primase enzyme is the baculovirus system (3, 11), and this system has not been convenient for genetic analysis. Experiments to express functional helicase or primase subunits in a genetically manipulable system are under way. This type of structure-function analysis will be applied not only to the UL5 gene but also to the UL8 and UL52 genes and should facilitate the assignment of function to the individual members of the complex; for instance, it should be possible to determine which protein,

UL5 or UL52, has intrinsic primase activity and to map the protein domains responsible for this activity. Furthermore, the results presented in this and in the accompanying report (50) indicate that in addition to determining the protein domains responsible for helicase and primase activity, it will be possible to map the sites required for interaction with other members of the helicase or primase. The fact that interaction between members of the helicase-primase complex appear to be essential for enzymatic activity and the fact that all three subunits are required *in vivo* for viral DNA replication suggest that domains of interaction may provide useful targets for novel antiviral drugs. A precedent for this strategy comes from the finding that a peptide derived from the large subunit of HSV ribonucleotide reductase inhibits reductase activity, evidently by interference with subunit interactions (5, 12).

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