A Mutation in the C-terminal Putative Zn\(^{2+}\) Finger Motif of UL52 Severely Affects the Biochemical Activities of the HSV-1 Helicase-Primase Subcomplex*

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Herpes simplex virus type 1 encodes a heterotrimeric helicase-primase complex that is composed of the products of the UL5, UL52, and UL8 genes. A subcomplex consisting of the UL5 and UL52 proteins retains all the enzymatic activities exhibited by the holoenzyme in vitro. The UL52 protein contains a putative zinc finger at its C terminus which is highly conserved among both prokaryotic and eukaryotic primases. We constructed a mutation in which two highly conserved cysteine residues in the zinc finger motif were replaced with alanine residues. A UL52 expression plasmid containing the mutation in the zinc finger region is unable to support the growth of a UL52 mutant virus in a transient complementation assay. Wild type and mutant UL5-UL52 subcomplexes were purified from insect cells infected with recombinant baculoviruses. Surprisingly, the mutant protein was severely affected in all biochemical activities tested; no helicase or primase activities could be detected, and the mutant protein retains only about 9% of wild type levels of single-stranded DNA-dependent ATPase activity. Gel mobility shift assays showed that DNA binding is severely affected as well; the mutant subcomplex only retains approximately 8% of wild type levels of binding to a forked substrate. On the other hand, the mutant protein retains its ability to interact with UL5 as indicated by copurification and with UL8 as indicated by a supershifted band in the gel mobility shift assay. In addition, the ability of individual subunits to bind single-stranded DNA was examined by photo-cross-linking. In the wild type UL5-UL52 subcomplex, both subunits are able to bind an 18-mer of oligo(dT). The mutant subcomplex was severely compromised in the ability of both UL5 and UL52 to bind the oligonucleotide; total cross-linking was only 2% of wild type levels. These results are consistent with the proposal that the putative zinc binding motif of UL52 is required not only for binding of the UL52 subunit to DNA and for primase activity but also for optimal binding of UL5 to DNA and for the subsequent ATPase and helicase activities.

Herpes simplex virus type 1 (HSV-1)† encodes a heterotrimeric DNA helicase-primase complex composed of the products of the UL5, UL52, and UL8 genes (1, 2). All three genes are essential for viral DNA replication (3–7). This protein complex has been shown to possess ssDNA-dependent NTPase, 5′ to 3′ DNA helicase, and DNA primase activities (1, 2, 8–10). The HSV-1 helicase-primase complex can be isolated from insect cells that have been simultaneously infected with recombinant baculoviruses that express each of the three subunits (8). A subassembly consisting of the UL5 and UL52 gene products also exhibits all the enzymatic activities of the holoenzyme (11). The UL5 protein contains seven conserved motifs found in all members of helicase Superfamily I which comprises DNA and RNA helicases from bacteria, viruses, and eukaryotes (12). Mutations in conserved residues in the helicase motifs have been shown to abolish the ability of mutant UL5 to support DNA replication in vivo (13). Furthermore, these mutations abolish helicase but not primase activity in vitro (14). The UL52 protein contains several conserved motifs found in other primases including a DDX motif associated with the catalytic activity in other primases (15, 16). Mutations in the DDX motif specifically abolished primase but not ATPase or helicase activities (15, 16). The UL8 gene product has not been associated with any enzymatic activities (9, 11) but can stimulate both the helicase and primase activities of the helicase-primase complex (8, 17–20). UL8 may also be responsible for mediating protein-protein interactions required at the replication fork (20–23). Taken together, these results suggest that UL5 encodes the helicase subunit and that UL52 encodes the primase subunit of the helicase-primase complex; however, neither UL5 nor UL52 appears to possess any of these activities when expressed and purified alone (8, 11). Thus it is not clear whether UL52 contributes a specific function to helicase activity or whether UL5 contributes a function to primase activity. It is also possible that amino acid residues from both polypeptides actually contribute to the catalytic activities of the complex or that each polypeptide needs the other for proper folding and conformation.

Unwinding of duplex DNA by a helicase is an essential step in many biological processes such as DNA replication, DNA repair, recombination, and transcription. Although the precise mechanism of unwinding is unknown for any helicase, it is clear that the unwinding reaction requires the coupling of several simpler events such as ATP binding, ATP hydrolysis, single strand and double strand DNA binding, and translocation along the DNA. One model for helicase activity poses that helicases must utilize at least two distinct DNA-binding sites (24). It is believed that helicases achieve this by forming oligomeric structures, either dimer, hexamer, or multimeric complexes. The Rep protein of Escherichia coli, also a member of Superfamily I, is believed to form a dimer (25, 26) at the replication fork, whereas the helicases of T4 and T7 bacteriophages (27, 28) and SV40 (29, 30) apparently form hexamers or higher order structures. The stoichiometry of the
UL5-UL52-UL8 heterotrimeric complex at the replication fork is not known nor is it known which proteins or domains within each protein are capable of contacting the DNA substrate. It is possible that the UL5 subunit itself contains all the DNA binding regions required for helicase activity and that the UL52 subunit has a DNA binding region associated with primase activity. However, it is also possible that the UL52 protein contains a DNA binding domain required for helicase activity. In this paper we developed a photo cross-linking assay and used it to demonstrate that both UL5 and UL52 subunits of the helicase-primase subcomplex are capable of contacting DNA.

The UL52 protein contains a putative zinc finger motif at its C terminus that is highly conserved among herpesviruses and also other primases such as the bacteriophage primases, mouse, and yeast primases (31, 32). Zinc finger motifs have been shown to be responsible for template recognition (32). In a study of the bacteriophage T7 primase, the motif is essential in vivo. The mutant protein subcomplex exhibits severe defects in DNA binding, ATPase, helicase, and primase activities. These results suggest the essential role of the putative zinc finger motif in biochemical activities of the helicase-primase subcomplex.

**Experimental Procedures**

**Reagents**

Supplemented Grace's medium, 10% Pluronic®F-68 and the Bac-to-Bac™ recombinant baculovirus kit were purchased from Life Technologies, Inc. Fetal calf serum was obtained from Atlanta Biologicals. Penicillin/streptomycin solution, ampicillin, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin were purchased from Sigma. The 20-ml HiLoad 16/10 SP Sepharose Fast Flow column was from Amersham Pharmacia Biotech. The 12-ml Uno Q (Q-12) column was from Bio-Rad. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. The oligonucleotides were synthesized by Life Technologies, Inc. The oligonucleotide substituted with 5-iodo deoxyuridine was synthesized by Cruachem (Dulles, VA). Long Ranger™ 50% acrylamide was purchased from Biotechnology. Oligonucleotides were synthesized by Life Technologies, Inc. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by Life Technologies, Inc. Fetal calf serum was obtained from Atlanta Biologicals.

**Buffers**

Buffer A consists of 20 mM HEPES, pH 7.6, 1.0 mM diithiothreitol (DTT), 10 mM sodium bisulfite, 5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μM leupeptin, 1 μM pepstatin, and 2 μM aprotinin. Buffer B contains 20 mM HEPES, pH 7.6, 1.0 mM DTT, 10% (V/V) glycerol, and 0.5 mM EDTA. All buffers were passed through a 0.22-μm filter and degassed before use.

**Cells and Viruses**

Suitable Grac’s medium, 10% Pluronic®F-68 and the Bac-to-Bac™ recombinant baculovirus kit were purchased from Life Technologies, Inc. Fetal calf serum was obtained from Atlanta Biologicals. Penicillin/streptomycin solution, ampicillin, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin were purchased from Sigma. The 20-ml HiLoad 16/10 SP Sepharose Fast Flow column was from Amersham Pharmacia Biotech. The 12-ml Uno Q (Q-12) column was from Bio-Rad. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. The oligonucleotides were synthesized by Life Technologies, Inc. The oligonucleotide substituted with 5-iodo deoxyuridine was synthesized by Cruachem (Dulles, VA). Long Ranger™ 50% acrylamide was purchased from Biotechnology. Oligonucleotides were synthesized by Life Technologies, Inc. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by Life Technologies, Inc. Fetal calf serum was obtained from Atlanta Biologicals.

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**Cells and Viruses**

**Spodoptera frugiperda** (SF9) cells were maintained at 27 °C in Grace’s insect medium containing 10% fetal calf serum, 0.33% lactalbumin hydrolysate, 0.33% yeastolate, 0.1 mg/ml streptomycin, and 100 units/ml penicillin. Recombinant Autographa californica nuclear polyhedrosis baculovirus expressing HSV-1 UL5, AcmNPV/UL5, was a kind gift from Dr. Robert Lehman (Stanford University School of Medicine, Stanford), and recombinant baculovirus expressing UL52, AcUL52, was a kind gift from Dr. Nigel D. Stow (Medical Research Council Virology Unit, Glasgow, UK). Viral stocks were amplified in SF9 cells grown in suspension as described previously (14). Stocks were titrated by determining the volume of viral stock that gave the maximum level of recombinant protein expression on 1 × 10⁸ SF9 cells at 48 h postinfection. African green monkey kidney cells (Vero, American Type Culture Collection, Rockville, MD) were propagated as described previously (39). The B-L-1 cell line which is permissive for UL52 mutants and the UL52 mutant virus, hrl14, containing a loc2 insertion were described in Ref. 6.

**Plasmids**

The plasmid pcDNA1-UL52 containing the UL52 gene under the control of the CMV promoter was described previously (40). The amplification vector plasmid pF1-CMV was generously provided by Ann D. Kwong (see Ref. 41). In order to generate an ampiclon vector capable of expressing UL52 (pF1-UL52), pcDNA1-UL52 was digested with BamHI and HpaI, and the 3.5-kb fragment was subcloned into pF1-CMV digested with EcoRV and BglII.

**Construction of Point Mutation**

Mutations were constructed by a two-step polymerase chain reaction method (42). The outside primers were OZnFM3 (5’ GCCCGGACGACGCGAG- CACTTTCCGAACA 3’) and OZnM4 (3’ GCGCCGCAGACGAGCG- TTAGACA 3’). The mutagenic primers were as follows: OZnM1 (5’ CAGACCCATCCTGGCAGAACACGCAACC 3’ and OZnM2 (5’ TGCTCTGCTACCTTTCCGAGGATCCTGCTGACACAAGGA 3’). The mutations resulting in coding changes are underlined; a silent change resulting in the introduction of a BamI site is indicated by a double underline. The mutated polymerase chain reaction products were digested with BpiI and HindIII, and the 903-base pair fragment of subcloned into pF1-UL52. The clone was confirmed by the presence of the new BamI restriction site. Sequencing of the 903-base pair region confirmed that no spontaneous mutations were introduced by polymerase chain reaction. The mutant plasmid was designated as pF1-UL52(CC3,4AA).

**Construction of the Baculovirus Recombinant Harboring the UL52 Mutant Gene**

pF1-UL52(CC3,4AA) was digested with HindIII and NotI, and the resulting 3.7-kb fragment containing the UL52(CC3,4AA) gene was cloned into pFastBac1, a baculovirus transfer vector (4.8 kb, Life Technologies, Inc.), to generate UL52(CC3,4AA)/FastBac. This transfer vector was then used to generate a baculoviral stock, AcUL52(CC3,4AA), capable of expressing the mutant UL52 protein as described previously (14).

**Protein Expression and Purification**

Two liters of SF9 cells were grown in suspension at 27 °C in Grace’s insect medium as described previously (14). The wild type UL5UL52 and the UL5-UL52(CC3,4AA) subcomplexes were purified essentially as described earlier except that a UnoQ (Bio-Rad) column was used in place of the Mono Q column (14). Cells were Dounced using 15 strokes of a tight-fitting pestle in buffer A, and the cytosolic extracts were clarified by centrifugation at 35,000 × g for 30 min. The UL5-UL52 subcomplexes were fractionated from the cytosolic extract by adding equal volume of buffer B containing 0.2 mM NaCl and 2 mM ammonium sulfate on ice for 4 h. The resultant protein pellets were resuspended in buffer B containing 0.1 mM NaCl and dialyzed against the same buffer. The dialed sample was loaded onto a 20-ml SP-Sepharose column equilibrated with buffer B containing 0.1 mM NaCl, and the column was washed with 5 column volumes of the equilibration buffer. Fractions containing the UL5-UL52 subcomplex were identified by both ATPase assay and SDS-polyacrylamide gel electrophoresis. The UL5-UL52 subcomplex elutes from the column in the void volume. Pooled fractions from SP-Sepharose were loaded onto a 12-ml Uno Q column equilibrated with buffer B containing 0.1 mM NaCl. The column was washed with 60 ml of buffer B containing 0.1 mM NaCl, and the protein was eluted using a 185-mI linear gradient of buffer B containing 0.1–1 mM NaCl.

**Enzyme Assays**

**ATPase Assay**—ATPase assays (50 μl) were performed using 5 mM ATP, 5 mM MgCl₂, 0.1 mM mSMnp18 DNA (nucleotides), and 0.45 pmol of UL5-UL52 complex (4.55 pmol in the absence of ssDNA) as described previously (14). Protein concentrations of the purified recombinant helicase subcomplex per reaction were determined from a standard curve. Kinetic parameters for ATPase activity were calculated as described previously (43). The enzyme activity is expressed as a micromole/min per mg of protein.

**Helicase Assay**—Reaction mixtures (50 μl) contained 20 mM Na⁺ HEPES, pH 7.6, 1 mM DTT, 5 mM MgCl₂, 7 mM ATP, 0.1 mg/ml bovine serum albumin, 10% glycerol, and 0.64 pmol of the forked DNA substrate as described previously (14). The forked DNA substrate was added to the reaction mixture, and the mixture was incubated at 37 °C. The reaction was terminated after 30 min by the addition of 10 mM EDTA to a final concentration of 0.5 mM. The reaction was then precipitated by the addition of 1 vol of 100% ethanol for 15 min at −20 °C. The DNA was dried, redissolved in water, and the radioactivity was measured using a scintillation counter.
constructed by heat denaturation and annealing 80 pmol of the helicase 48/5S oligo (5'-CAAGGTAAGTATGGCGCCCGCCGTGCTGATTGCTGAAGCCG 3') radiolabeled at its 5'-end with [γ-32P]ATP and 80 pmol of the unlabeled 48C/5S oligo (5'-CGAAGGTAAGTATGGCGCCCGCCGTGCTGATTGCTGAAGCCG 3'). The underlined residues are complementary and create a duplex region of the molecule. After annealing, the products were subjected to electrophoresis on a 8% nondenaturing polyacrylamide gel, and the forked substrate was purified by electroelution and ethanol precipitation. Reactions containing varying amounts (1-8 pmol) of the UL52 subcomplex (wild type or mutant) were allowed to proceed for 30 min at 37 °C and were analyzed as described previously (14).

**Direct Primase Assay**—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 primer initiation site and various amounts (1 and 2 pmol) of wild type or mutant protein (14).

**Gel Mobility Shift Assay**

Gel mobility shift assays were essentially performed as described previously (14). The reaction mixture (25 μl) contained 20 mM Na+ HEPES, pH 7.6, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10% glycerol, 1 mM EDTA, 1 pmol (molecules) of the forked DNA substrate labeled at its 5'-end with [γ-32P]ATP, and 4 pmol of the UL52 subcomplex (wild type or mutant) with or without 12 pmol of the UL8 protein. The reaction was allowed to proceed for 10 min on ice and was terminated by the addition of 0.1 volume of stop solution (80% glycerol, 0.1% bromphenol blue). Reaction products were analyzed on a 4% nondenaturing acrylamide, 0.11% Bis-gel at 150 V at 4 °C. The gel was dried and exposed to film at ~70 °C.

**Transient Complementation Assay**

The transient complementation assay was performed as described previously (44). Freshly trypsinized exponentially growing Vero cells (1 x 10⁶) were transfected in solution with 8 μg of either pF1-UL52, or pF1-UL52(CC3,4AA). At 24 h post-transfection, the cells were superinfected with hr114 at a multiplicity of infection of 10 pfu per cell. At 16 h post-infection at 34 °C, progeny viruses were harvested and titrated on the complementing BL-1 cell line.

**Transfection and Immunoblot**

Vero cells (1 x 10⁶) were transfectected as described above and superinfected with hr114 at a multiplicity of infection of 10 pfu per cell at 24 h post-transfection. At 16 h post-infection at 34 °C, the cells were collected by centrifugation at 2000 rpm for 10 min in a Beckman TJ-6 centrifuge. For infection, 1.5 x 10⁸ Vero cells in 60-mm plates were infected with KOS or with hr114 at a multiplicity of infection of 10 pfu per cell. Cell pellets were rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3) and resuspended in 50 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (50 mM Tris·HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). The samples were analyzed by 8% SDS-PAGE, and proteins were blotted onto ECL nitrocellulose membrane (Amersham Corp., Buckinghamshire, UK). ECL (enhanced chemiluminescent) Western blotting analysis was performed according to the manufacturer’s (Amersham Pharmacia Biotech) instructions. An antibody detected against the C-terminal 10 amino acids of UL52, 1248 (generously provided by Dr. Mark Chalberg) was used as primary antibody at a dilution of 1:250.

**Photo Cross-linking**

A photo cross-linking experiment was performed with oligo(dT)₁₄ in which the 5th thymidine residue from the 5'-end was substituted with 5-iododeoxyuridine; the substituted oligo was labeled at its 5'-end with [γ-32P]ATP. A 4-pmol aliquot of UL52-UL52 subcomplex (wild type or mutant) was incubated with 1.0 pmol of the labeled substituted oligo in 20 mM Na+ HEPES, pH 7.6, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10% glycerol, and 1 mM EDTA for 10 min on ice before irradiation. An IK series He-Cd laser (IK 3302R-E, KIMMON, Kimmon Electric Co., Ltd.) was used to achieve monochromatic 325 nm light. The laser beam output was 34 milliwatts measured with a power meter, Mentor MA10, Scientech® (Scientech, Inc., Boulder, CO). Sample volumes of 1200 μl were irradiated in a methacrylate cuvette (Fisher brand; catalog number 14-385-938) at room temperature. At different time points 40-μl aliquots were withdrawn, boiled for 5 min in SDS-PAGE loading buffer, and subjected to SDS-PAGE on an 8% gel. The gels were dried and exposed to film at ~70 °C.

**RESULTS**

**Site-directed Mutagenesis of the Zinc Finger Motif of UL52**—Site-directed mutagenesis was used to explore the functional significance of the putative zinc finger region of the UL52 protein. The sequence of the HSV-1 UL52 gene product was compared with its homologs from 10 other herpesviruses, and a putative zinc finger was identified beginning at Cys993 (Cys-X₁₅-Heis-X₉₋₁₅-Cys-X₂₋₄-Cys). Three cysteine residues, Cys993, Cys1023, and Cys1028 within this region are totally conserved among all UL52 homologs and are designated as C1, C3, and C4, respectively (Fig. 1). Histidine 993 (designated as H2) was also conserved in 9 out of 10 UL52 homologs. A similar potential metal-binding site was also highly conserved in DNA primases of bacteriophages, other eukaryotic viruses, prokaryotes, and eukaryotes (31, 32). Sequence-specific recognition of DNA by proteins containing this type of zinc binding motif has been studied in many systems (33–37). In this study, the third and fourth cysteine residues of the zinc finger motif (C3 and C4) were substituted with alanine residues. The mutant gene was cloned into an amplicon expression vector under the control of the CMV promoter [pF1-UL52(CC3,4AA)]. To analyze the ability of the UL52 mutant to support DNA synthesis, Vero cells were transfected with pF1-UL52(CC3,4AA) and subsequently superinfected with the UL52 mutant virus, hr114. After 16 h of infection the supernatant was titered for virus production on the permissive BL-1 cell line. The wild type virus was not detected.
TABLE I

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Titer (pfu/ml)</th>
<th>Complementation index</th>
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<tbody>
<tr>
<td>pF1/CMV</td>
<td>1.2 x 10^4</td>
<td>256</td>
</tr>
<tr>
<td>pF1-UL52</td>
<td>3.2 x 10^6</td>
<td></td>
</tr>
<tr>
<td>pF1-UL52(CC3,4AA)</td>
<td>7.3 x 10^3</td>
<td>0.59</td>
</tr>
</tbody>
</table>

plasmid, pF1-UL52, complemented hr114 very efficiently (complementation index of 256, Table I). The mutant construct, however, was unable to support the growth of the UL52 insertion mutant (complementation index less than 1, Table I). Vero cells were transfected with wild type and mutant constructs, and cell lysates were examined by Western blot analysis to determine steady state levels of the UL52 protein. Although the polyclonal antibody used to detect the UL52 peptide cross-reacts with many proteins in mock-infected cell extracts, it is clear that a band corresponding to UL52 is present in cells transfected with plasmids expressing wild type and mutant proteins but absent in cells transfected with vector alone (Fig. 2, compare lanes 2 and 3 with lane 1). Furthermore, the UL52 band is present in Vero cells infected with KOS but not in cells mock-infected or infected with the null virus hr114 (Fig. 2, compare lane 5 with lanes 4 and 6). We conclude that cells transfected with plasmid expressing the mutant version of UL52 (pF1-UL52(CC3,4AA)) are able to express full-length UL52 protein at wild type levels. This result indicates that the mutant protein did not exhibit any gross conformational changes that affected the stability of the protein in Vero cells.

**Generation of Recombinant Baculovirus and Purification of Both Wild Type and Mutant UL5-UL52 Subcomplexes**—To examine the contribution of Cys^1023 and Cys^1028 in the putative zinc finger motif of UL52 toward the biochemical activities of the UL5-UL52 subcomplex in vitro, a recombinant baculovirus containing the UL52(CC3,4AA) gene was constructed (AcUL52(CC3,4AA)). Sf9 cells infected with AcUL52(CC3,4AA) expressed full-length UL52 that reacted with the mono-specific polyclonal antibody (1248) in a Western blot (data not shown). A subcomplex consisting of wild type UL5 and mutant UL52 was obtained by infecting Sf9 cells with the appropriate recombinant baculoviruses, and the subcomplex was purified to approximately 80% homogeneity (Fig. 3, lane 2). The same purification scheme was used to purify the wild type UL5-UL52 subcomplex and resulted in approximately 95% homogeneity (Fig. 3, lane 1). The ability of the mutant UL52 to copurify with UL5 indicates that the mutant protein is still able to interact with UL5 and suggests that no gross alterations in conformation have occurred. The purification scheme, however, resulted in a lower level of purity for mutant as compared with wild type subcomplex, indicating that the mutant protein may tend to aggregate more than the wild type version.

**The Zinc Finger Mutation Abolishes Helicase Activity of the UL5-UL52 Subcomplex**—Mutations in the conserved DXXD primase motif of UL52 were shown to abolish primase but not ATPase or helicase activities of the heterotrimeric complex in vitro (15, 16). Although this result indicated that primase activity was likely specified at least in part by the UL52 subunit itself, it remained a possibility that UL52 also contributes to the helicase activity of the helicase-primase complex. In order to address this question, the zinc finger mutant was assayed for its ability to displace a short strand of DNA from a forked substrate. Various concentrations of wild type and mutant proteins were incubated with the ^32P-labeled forked substrate in the presence of ATP and MgCl2, and the reaction products were analyzed by native gel electrophoresis. Fig. 4 indicates...
that helicase activity was present in all four protein concentrations of wild type UL5-UL52 subcomplex, but no strand displacement was observed in reactions containing the mutant complex. Thus, surprisingly, the CC3,4AA mutation in the zinc finger motif abolished helicase activity. This result suggests that UL52 may contribute an activity that is essential for helicase function.

The Intrinsic and ssDNA-dependent ATPase Activity of the Mutant Subcomplex Is Severely Compromised—The HSV-1 helicase-primase possesses both intrinsic and ssDNA-dependent ATPase activity (2, 8, 9). In order to test the role of the zinc finger motif of UL52 in ATP hydrolysis, ATPase activity of UL5-UL52(CC3,4AA) was compared with the wild type subcomplex both in the presence and absence of ssM13mp18 DNA (Fig. 5 and Table II). ATP hydrolysis followed a linear time course up to 40 min under these assay conditions for both wild type and mutant UL5-UL52 subcomplexes. The mutant protein was severely compromised in both intrinsic and ssDNA-dependent ATPase activities, exhibiting a 7.8-fold decrease in turnover rate (Kcat) for DNA-independent ATPase activity and an 11.6-fold decrease in Kcat for ssDNA-dependent ATPase activity as compared with wild type. The severity of this defect was unexpected and suggests that UL52 contributes to the ATPase activity of the complex. Whether the zinc finger mutation has affected the conformation of the subcomplex in a way that severely affects the hydrolysis of ATP or whether the zinc finger itself is required for some aspect of the hydrolysis reaction remains to be determined. In order to determine whether the mutation has affected binding of ATP and DNA to the subcomplex, the Km values for ssDNA and ATP were also determined for wild type and mutant helicase-primase subcomplexes (Table II). The values for Km (DNA and ATP) are not significantly different for the wild type and mutant proteins and are consistent with the previously reported values (43, 45, 46); the UL5-UL52(CC3,4AA) subcomplex showed a 1.3-fold increase in the Km for ssDNA and 1.1-fold increase in the Km for ATP. This result indicates that the ability to bind ATP and ssDNA as measured in the ATPase assay is not compromised in the mutant subcomplex containing a zinc finger mutation.

The Zinc Finger Mutation Abolishes Primase Activity—To determine if the putative zinc finger motif is required for primase activity, both the wild type and the mutant UL5-UL52 subcomplexes were assayed for primer synthesis using a template containing a preferred primase initiation site mapped from pBS plasmid DNA (14, 43). As shown in Fig. 6, wild type DNA can synthesize short (8–9 nucleotide) primers in the absence of UL8, but no primer synthesis was detected for the mutant protein complex.

The Zinc Finger Mutant Binds to a Forked Helicase Substrate and Can Interact with UL8—One possible explanation for the lack of DNA helicase and primase activities of the mutant subcomplex is that this mutation alters binding to DNA. The ability of the mutant and wild type UL5-UL52 subcomplexes to bind to the forked helicase substrate was tested using a gel mobility shift assay (Fig. 7). The wild type UL5-UL52 subcomplex can efficiently shift the substrate as previously reported (Fig. 7, lane 3) (14). Furthermore, as seen previously, addition of UL8 to the binding reaction resulted in a supershift to a slower migrating species (Fig. 7, lane 4). The mutant UL5-UL52 subcomplex was also able to shift the forked substrate but with much lower efficiency (8.3% of wild type levels, Fig. 7, lane 5). Addition of UL8 resulted in the disappearance of the UL5-UL52 shifted species and the appearance of a faint diffused supershifted band (Fig. 7, lane 6). A darker exposure of lanes 5 and 6 is shown in lanes 7 and 8, respectively. The disappearance of the UL5-UL52 shifted species in the presence of UL8 suggests that the interaction of the UL5-UL52 subcomplex with UL8 has not been compromised.

Both UL5 and UL52 Subunits of Wild Type Helicase-Primase Complex Can Be Efficiently Cross-linked to a Short DNA Oligonucleotide—The DNA-binding sites within helicase-primase complex have not been mapped. The previous methods for
Table II
Kinetic parameters for ssDNA-dependent and DNA-independent ATP hydrolysis for wild type and mutant UL5-UL52 subcomplex

<table>
<thead>
<tr>
<th>UL5-UL52 subcomplex</th>
<th>ssDNA-dependent $K_m$</th>
<th>DNA-independent $K_m$</th>
<th>$K_m$ for ATP</th>
<th>$K_m$ for ssDNA</th>
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<tbody>
<tr>
<td>Wild type</td>
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<tr>
<td>Mutant</td>
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| Wild type | 555 ± 14 | 39 ± 3 | 0.56 ± 0.04 | 0.11 ± 0.02 |
| Mutant    | 48 ± 5   | 5 ± 2  | 0.62 ± 0.05 | 0.15 ± 0.03 |

$^a$ 5 mM ATP, 5 mM MgCl$_2$, and 0.1 mM ssDNA in nucleotide.
$^b$ 5 mM ATP, 5 mM MgCl$_2$, and no DNA.
$^c$ Varying concentration of ATP·MgCl$_2$ (1:1) from 0.025 to 10 mM and 0.1 mM ssDNA.
$^d$ 5 mM ATP, 5 mM MgCl$_2$, and varying concentrations of ssDNA from 0.01 to 0.1 mM.

Analyzing protein-DNA interactions such as filter binding and gel shift experiments have not been able to determine whether UL5 or UL52 or both subunits contact DNA. In order to test the contribution of individual subunits in DNA binding, a photo cross-linking assay was performed using a 5-iododeoxyuridine-substituted oligonucleotide and a He-Cd laser as a light source. In previous studies, substituted oligonucleotides have been cross-linked to associated proteins using UV-mediated cross-linking (47–49); however, UV light sources (which emit 254 nm light) result in inefficient cross-linking and considerable degradation of both protein and nucleic acid. More recent studies show that higher yields of protein cross-linked to DNA or RNA can be obtained using higher wave lengths of light (308–325 nm) which result in much less degradation of both protein and nucleic acid (50, 51). In this study a He-Cd light source that emits at 325 nm was used to photo cross-link the UL5-UL52 subcomplex to a 32P-end-labeled 18-mer oligo(dT) molecule in which 5-iododeoxyuridine was substituted for one of the thymidine residues. Wild type and mutant subcomplexes were irradiated at room temperature for various periods with the He-Cd laser. SDS-PAGE and subsequent autoradiography of the wild type subcomplex revealed two major bands that migrate at positions that correspond to UL5 and UL52 (Fig. 8, lanes 1–5). The identity of these two major bands as UL5 and UL52 was confirmed by Western blot analysis (data not shown). At the 2-, 4-, and 10-min time points, the intensity of the UL52 band was stronger (2–2.5 fold) than that of the UL5 band; however, by 60 min, the intensities of the two labeled bands were almost similar. The mutant UL5-UL52 subcomplex exhibited very little cross-linking to either full-length UL5 or UL52 (Fig. 8, lanes 6–10). Both wild type and mutant subcomplexes were subjected to SDS-PAGE and Coomassie staining after cross-linking to confirm that equivalent amounts of proteins in the subcomplexes were present in both preparations (data not shown). Quantification of counts cross-linked to full-length UL5 and UL52 for both mutant and wild type is shown in Fig. 9, A and B, respectively. In the wild type subcomplex, cross-linking to UL52 appeared to be slightly more efficient than to UL5. The total number of counts cross-linked to both UL5 and UL52 in the mutant subcomplex was only 2% of wild

![Image](http://www.jbc.org/)

**Fig. 6.** Comparison of the abilities of UL5-UL52 and UL5-UL52(CC3,4AA) to carry out RNA primer synthesis. RNA primase activity on a preferred primase initiation site template catalyzed by wild type and mutant UL5-UL52 subcomplex was measured as described under “Experimental Procedures” and analyzed by electrophoresis on a 18% urea gel. Lane 1 represents the reaction in absence of enzyme. Lanes 2 and 3 represent reactions containing 1 and 2 pmol of wild type enzyme, respectively. Lanes 4 and 5 represent reactions containing 1 and 2 pmol of mutant protein, respectively.

**Fig. 7.** The CC3,4AA mutation reduces the ability of the UL5-UL52 subcomplex to gel shift the forked DNA substrate. DNA gel shift reactions were performed using 1 pmol of the radiolabeled forked DNA substrate, 4 pmol of the UL5-UL52 subcomplex (wild type or mutant) in the presence or absence of 12 pmol of the UL8 protein. The samples were incubated for 10 min on ice and analyzed by 4% nondenaturing polyacrylamide gel electrophoresis as described under “Experimental Procedures.” Lane 1 represents the reaction in absence of any protein. Lane 2 shows the reaction that contained only the UL8 protein. Lanes 3 and 4 represent reactions that contained the wild type UL5-UL52 subcomplex alone and in the presence of UL8, respectively. Lanes 5 and 6 represent similar reactions that contained mutant UL5-UL52 subcomplexes alone and in the presence of UL8, respectively. Lanes 7 and 8 represent a darker exposure of lanes 5 and 6, respectively.
type levels. In this case, however, the number of counts cross-linked to UL5 was higher than to UL52. This suggests that binding to UL52 was more severely compromised than binding to UL5, although both were severely affected.

In addition to the two major bands corresponding to UL5 and UL52, both wild type and mutant preparations contained labeled bands that migrated faster than full-length UL5 and UL52. In the case of wild type, a band of 47 kDa was seen at the 60-min time point (Fig. 8, lane 5), and in the mutant preparation, predominant bands corresponding to 47 and 60 kDa were observed at all time points (Fig. 8, lanes 7–10). These smaller bands may represent degradation product of UL5 or UL52; however, these forms do not react with several different polyclonal and monoclonal UL5 and UL52 antibodies.2 Alternatively, it is possible that the mutant preparation contains one or more contaminating proteins that can cross-link to DNA very efficiently; as mentioned above, the purity of the mutant preparation is lower than that of wild type.

In summary, we have developed a cross-linking assay that indicates that both UL5 and UL52 can contact DNA. The mutant subcomplex is severely compromised in the ability of both UL52 and UL5 to bind to DNA. These results will be discussed further below.

DISCUSSION

In this report the putative zinc binding domain (Cys988-X9-X1023-His993-X40-Cys1028) of the UL52 subunit was analyzed by site-directed mutagenesis. Several observations have been made. 1) The replacement of Cys1023 and Cys1028 with alanine residues completely abolished the growth of UL52 mutant virus in a transient complementation assay, suggesting that this motif is important for the growth of the virus. 2) Biochemical analysis of a purified UL5-UL52 subcomplex expressed in insect cells infected with recombinant baculoviruses revealed several defects: the mutant subcomplex fails to exhibit helicase and primase activities and only retains about 9% wild type levels of ssDNA-dependent ATPase activity. 3) Overall DNA binding activity as measured by gel mobility shift analysis also indicates that the UL5-UL52 subcomplex is severely compromised (8.3% of wild type levels). 4) A newly developed photo cross-linking assay demonstrates that both UL5 and UL52 can be cross-linked to an 18-mer of oligo(dT) indicating that both subunits in the wild type subcomplex can contact DNA. In the wild type preparation, UL52 can be cross-linked slightly more efficiently than UL5. 5) Both subunits of the mutant UL5-UL52 subcomplex exhibit significant defects in their ability to bind DNA (total cross-linking at 2% of wild type levels), and UL52 binding is decreased even more than UL5 binding.

Previous reports indicated that the DXD motif in UL52 (located between residues 610 and 636), which is also found in other primases, is probably required for the catalytic activity of the primase (15, 16). Until this report, no other important regions of the UL52 protein had been identified experimentally. We have replaced two invariant cysteine residues in a putative zinc binding region of UL52, and our results indicate that amino acids 1023 and 1028 are indeed important for UL52 function. It is possible that by mutating these two amino acids, changes in the overall conformation of UL52 have occurred that result in the observed defects in the UL5-UL52 subcomplex. However, several observations suggest that gross conformational changes have not occurred. 1) The mutant UL52 can be detected in transfected Vero cells at wild type levels suggesting that no gross alterations in stability have occurred. 2) The mutant subcomplex still copurifies from insect cells infected with recombinant baculoviruses expressing wild type UL5 and mutant UL52, indicating that mutant UL52 can still interact with UL5. 3) The purified mutant UL5-UL52 complex containing the mutant version of the UL52 gene can still interact with UL8 as detected in the gel mobility shift assay; this may indicate that the C terminus of UL52 is not necessary for its interaction with either UL5 or UL8. 4) The purified mutant UL5-UL52 complex exhibits similar K_m values for ATP and ssDNA for ATP hydrolysis, indicating that the binding of these cofactors is not altered with respect to the ATPase assay. This result may seem contradictory to the observation that DNA binding of the subcomplex as determined by gel shift and photo cross-linking assays is severely compromised. This apparent discrepancy may be due to different substrates used in each assay. The gel shift assays were performed using a 48-mer synthetic forked substrate, and cross-linking assays were carried out using an 18-mer oligo(dT) substrate, whereas the kinetic parameters were determined using m13mp18 single-stranded DNA. It has been reported that the kinetic parameters of the ATPase activity of the UL5-UL52 subcomplex are affected by the length of the oligonucleotide cofactor (52). It is also possible that other differences in the assay conditions such as the presence or absence of ATP may affect the DNA-binding properties of the mutant protein subcomplex. In any case, our results taken together suggest that it is unlikely that the overall conformation of the protein is significantly altered by these amino acid substitutions. However, it is possible that subtle conformational changes have occurred especially within the putative zinc finger domain itself. In fact, since other similar zinc fingers whose structures are known have been shown to fold into discrete domains capable of binding zinc and DNA (33, 53–56), it is possible that the mutation in UL52 results in a local alteration of conformation in the C terminus of UL52 which does not affect its global conformation and that this alteration in the C terminus is responsible for the observed changes in activity. Further experiments will be needed to clarify these points. At this point, we can only say that the alteration of two C-terminal cysteine residues of UL52

has profound effects on the function of the UL5-UL52 subcomplex. Interestingly, in the case of the T7 helicase-primase encoded by the T7 gene 4, a mutation in the zinc motif of the primase has been shown to affect both primase and helicase activities (32). In that example, however, the mutant helicase-primase exhibits wild type levels of nucleotide hydrolysis activity.

The precise mechanism of helicase action is not known in detail, but it can be imagined that the entire process requires the coupling of subreactions such as ATP binding, ATP hydrolysis, single- and double-stranded DNA binding, translocation along DNA, and coupling between ATP hydrolysis and DNA unwinding. ATP binding and hydrolysis are an intrinsic property of all DNA and RNA helicases, and the UL5 protein contains the conserved Walker A and Walker B motifs that are found in ATP-binding proteins (57). Our observation that a mutation in the putative zinc binding region of UL52 exhibited severe defects in both intrinsic and ssDNA-dependent ATPase activities was therefore unexpected. Although UL5 and UL52 have been considered as the helicase and primase subunits of the complex, respectively, our results indicate that the UL52 subunit may be required for optimal ATPase activity. It is possible that the subcomplex that forms in the presence of the mutant UL52 protein exhibits subtle conformational changes that directly affect ATPase activity. Alternatively, the putative zinc binding region of UL52 may contribute amino acid residues that play a direct role in ATPase catalysis or, as discussed below, the putative zinc binding region may be involved in DNA binding that is required for optimal ATPase activity.

The DNA binding domains on the UL5-UL52-UL8 helicase-primase have not been mapped. The UL5 subunit itself may contain all the DNA binding regions required for helicase activity, and the UL52 subunit would be expected to specify its own DNA binding region for primase activity. However, it remains a possibility that UL52 contributes a DNA-binding site to the unwinding process. Previous attempts to map the DNA binding domains in UL5 were frustrating because of our inability to distinguish between the individual contributions of the two subunits toward DNA binding. By analogy with other superfamily helicases, especially those for which structural information is available (58), it was anticipated that some of the conserved motifs of UL5 (especially motif IA, motif III, and motif V) may be involved in contacting DNA. However, mutations in these motifs were still able to gel-shift a forked sub-

Fig. 9. Quantification of cross-linking data. Data from the Fig. 8 quantified using a PhosphorImager, and the total counts were plotted against the time of irradiation. Panel A shows the wild type subcomplex (UL5, △ and UL52, □); panel B shows the mutant subcomplex (UL5, △ and UL52, □).
strate at wild type or higher levels (14). The gel mobility shift assay, however, measures total subcomplex binding, and it seemed likely that binding by UL52 may mask any defect in binding by UL5. Therefore, a photo cross-linking assay was developed that can distinguish the individual contributions toward DNA binding. In this study we have shown that both UL5 and UL52 subunits of the helicase-primase complex can bind DNA. Furthermore, we have shown that the mutant protein subcomplex is defective in both UL52 and UL5 binding. The defect in UL5 binding is consistent with the observation that a similar zinc binding motif in the T7 DNA primase is involved in template recognition (32). The defect in UL5 binding was unexpected however. One explanation for these results is that the mutation in the putative zinc finger domain affects the DNA-binding properties of UL52 which in turn affects the DNA-binding properties of the UL5 component of the UL5-UL52 complex. Alternatively, it is possible that both subunits together form a DNA-binding site and that binding to both subunits are required for optimal DNA binding of the complex.

In summary, we have demonstrated that the conserved cysteine residues (Cys1023 and Cys1028) in the putative zinc finger motif of UL52 are essential for its biological activity. We show that the overall DNA-binding properties of the mutant protein as measured by gel mobility shift and photo cross-linking are severely compromised. The simplest explanation for our results is that the lack of helicase and primase activities in the mutant protein is the consequence of reduced affinity of the UL52 subunit for DNA. Thus the putative zinc finger motif of UL52 may be important not only for the DNA-binding property of the primase subunit but also for the complex as a whole. In any case, the results reported in this paper suggest that it would be unwise to assign helicase function to the UL5 subunit and primase function to the UL52 subunit; it is clear that we must consider these two polypeptides together and that they have a more complex relationship than was originally anticipated.

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A Mutation in the C-terminal Putative Zn^{2+} Finger Motif of UL52 Severely Affects the Biochemical Activities of the HSV-1 Helicase-Primase Subcomplex
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