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Helicase-Primase Complex of Herpes Simplex Virus Type 1: a Mutation in the UL52 Subunit Abolishes Primase Activity

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The UL52 gene product of herpes simplex virus type 1 (HSV-1) comprises one subunit of a 3-protein helicase-primase complex that is essential for replication of viral DNA. The functions of the individual subunits of the complex are not known with certainty, although it is clear that the UL8 subunit is not required for either helicase or primase activity. Examination of the predicted amino acid sequence of the UL5 gene reveals the existence of conserved helicase motifs; it seems likely, therefore, that UL5 is responsible for the helicase activity of the complex. We have undertaken mutational analysis of UL52 in an attempt to understand the functional contribution of this protein to the helicase-primase complex. Amino acid substitution mutations were introduced into five regions of the UL52 gene that are highly conserved among HSV-1 and the related herpesviruses equine herpesvirus 1, human cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus. Of seven mutants analyzed by an in vivo replication assay, three mutants, in three different conserved regions of the protein, failed to support DNA replication. Within one of the conserved regions is a 6-amino-acid motif (IL)(VIM)(LF)DhD (where h is a hydrophobic residue), which is also conserved in mouse, yeast, and T7 primases. Mutagenesis of the first aspartate residue of the motif, located at position 628 of the UL52 protein, abolished the ability of the complex to support replication of an origin-containing plasmid in vivo and to synthesize oligoribonucleotide primers in vitro. The ATPase and helicase activities were unaffected, as was the ability of the mutant enzyme to support displacement synthesis on a preformed fork substrate. These results provide experimental support for the idea that UL52 is responsible for the primase activity of the HSV helicase-primase complex.

Herpes simplex virus type 1 (HSV-1) encodes seven proteins that are required for viral DNA replication (7–11, 24, 34, 36, 44, 53–56). The functions of several of these polypeptides are now well established. UL9 binds specific sequences within the origins of replication of HSV (19, 20, 39) and also possesses helicase activity (5, 21). UL29 (ICP8) is a single-stranded DNA-binding protein (42, 54). UL30 and UL42 compose a heterodimeric DNA polymerase in which UL30 is the catalytic subunit and UL42 acts to increase the processivity of the enzyme (10–12, 22, 23, 26, 27, 41, 43). Finally, UL5, UL8, and UL52 form a heterotrimeric complex with both 5′ to 3′ helicase and primase activities (13, 15).

The roles of the individual subunits of the UL5-UL8-UL52 complex have not been defined clearly. The complex has been purified from insect cells triply infected with baculoviruses expressing each of these polypeptides and has DNA-dependent ATPase, helicase, and primase activities identical to those obtained from herpesvirus-infected mammalian cells (17, 46). Likewise, a subcomplex containing only UL5 and UL52 has been purified from insect cells and retains DNA-dependent ATPase and helicase activity (6, 18, 46) and the ability to synthesize primers (18, 46). These results indicate that the core primase and helicase activities of the complex are not dependent on UL8.

Examination of the primary structure of the UL5 gene suggests that it is the helicase of the complex. The amino acid sequence of the UL5 subunit contains six conserved motifs that are found in many DNA and RNA helicases (25, 29). Two of these motifs define a nucleotide-binding site (52), consistent with the known ability of UL5-UL52 to hydrolyze ATP. All six conserved motifs have been shown to be critical to the function of the UL5 protein, as mutations within these motifs abolish the ability of an origin-containing plasmid to replicate in a transient replication assay (56). Because the UL5 protein contains the amino acid sequence motifs conserved among other helicases, it seems likely that it contains the helicase active site. Yet UL5 expressed from recombinant baculoviruses in insect cells in the absence of UL52 had less than 1% of the ATPase activity of either the UL5-UL8-UL52 or the UL5-UL52 complex (6, 46).

Because UL5-UL52 can synthesize primers in the absence of UL8 and UL5 contains all of the motifs found in known helicases, UL52 is the likely candidate for the primase subunit. Experimental evidence for this functional assignment has, however, been difficult to obtain because of the low solubility of this subunit. Unlike with UL5, examination of the primary amino acid sequence of UL52 failed to uncover any significant degree of homology to other proteins in the data bases except for homologs of UL52 found in other herpesviruses. As an alternate approach to the question of the function of UL52, we have undertaken a mutational analysis of this polypeptide to obtain mutants lacking one, but not both, of the activities of the UL5-UL52 complex. We analyzed in detail one mutant, UL52(D628Q). Biochemical analysis of the purified UL5-UL8-UL52(D628Q) complex demonstrated that this mutation completely abolished the ability of the complex to synthesize primers, while it had no effect on the helicase activity of the complex. These results provide the first experimental evidence

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for the assignment of the primase function to the UL52 subunit.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* (Sf9) cells were grown in TMNFH medium (GIBCO) containing 10% fetal bovine serum, 50 μg of gentamicin per ml, and 2.5 μg of amphotericin B (Fungizone) per ml. Recombinant baculoviruses were propagated as previously described (50).

Buffers. Buffer B contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6), 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonic acid, 0.5 mM $MgCl_2$, 10 mM $NaHSO_3$, and 2 μg each of leupeptin and pepstatin A per ml. Buffer C contained 20 mM HEPES (pH 7.6), 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonic acid, 2 μg each of leupeptin and pepstatin A per ml, and 10% glycerol.

Enzyme assays. (i) DNA-dependent ATPase assays. DNA-dependent ATPase assays were performed as described previously (46).

(ii) Assays for lagging-strand synthesis. Assay mixtures for lagging-strand synthesis (25-μl reaction mixtures) contained 25 mM Tris HCl (pH 7.5); 5 mM $MgCl_2$; 25 mM NaCl; 0.1 mM each dGTP, dATP, and dTTP; 0.025 mM dCTP; 33 nM [α-^{32}P]dCTP (6,000 Ci/mmol); 0.05 mM each GTP, CTP, and UTP; 2 mM ATP; 1 mM DTT; 25 fmol of single-stranded pBS(+) DNA (molecules); 1 pmol of HSV DNA polymerase-UL42 (pol-42) complex; various amounts of UL5-UL8-UL52 or UL5-UL8-UL52(D628Q); and 15 pmol ICP8. Reaction mixtures were preincubated with pol-42 for 15 min at 30°C to elongate any self-primed linear molecules in the DNA preparation, then chilled on ice for the sequential addition of various amounts of the wild-type or mutant helicase-primase complex and ICP8, and incubated a further 2 h at 30°C. Reactions were terminated by addition of an equal volume of 1% sodium dodecyl sulfate (SDS), 40 mM EDTA, tRNA (200 μg/ml), and proteinase K (1 mg/ml) and incubation at 37°C for 1 h. The reaction products were precipitated in ethanol and analyzed by electrophoresis in a 0.8% neutral agarose gel and autoradiography. For control reactions, a primer (PB0) homologous to base pairs 826 to 855 of pBS was annealed to the single-stranded DNA, and treatment was the same as for the experimental reactions.

(iii) Direct primase assays. Direct primase assay mixtures (25-μl reaction mixtures) contained 25 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM NaCl, 5 μg of acetylated bovine serum albumin (BSA), 2 mM ATP, 1 mM GTP, 0.1 mM CTP, 2 μM UTP, 130 nM [α-^{32}P]UTP (3,000 Ci/mmol), 1 mM DTT, 600 fmol of single-stranded pBS(+) DNA (molecules), and various amounts of UL5-UL8-UL52 or UL5-UL8-UL52(D628Q). The reaction mixtures were incubated at 30°C for 1.5 h, and the resulting primers were purified on a NENsorb 20 column (Dupont NEN Research Products, Boston, Mass.) according to the manufacturer's recommendations, eluted in 50% MeOH, dried, resuspended in 50% formamide, and analyzed by autoradiography following electrophoresis through an 18% Hydrolink Long Ranger gel (AT Biochem, Malvern, Pa.) containing 7 M urea.

(iv) Helicase assays. Helicase assay mixtures (20 μl) contained 20 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 2 μg of acetylated BSA, 5 mM DTT, 10% glycerol, 25 fmol of single-stranded M13mp18 DNA (molecules) that was singly primed with a 5'-end-labeled oligodeoxyribonucleotide (45-mer, 22 bases annealed, 23-base 3' tail) (14), and various amounts of UL5-UL8-UL52 or UL5-UL8-UL52(D628Q). The reaction was allowed to proceed for 1 h at 37°C and then stopped by the addition of SDS to 0.1% and EDTA to 20 mM. The reaction products were separated on a 15% nondenaturing polyacrylamide gel and visualized by autoradiography.

(v) Leading-strand replication assays. Leading-strand replication assay mixtures (25 μl) contained 25 mM Tris HCl (pH 7.5); 2 mM $MgCl_2$; 2 mM ATP; 25 mM NaCl; 0.1 mM each dGTP, dATP, and dTTP; 0.025 mM dCTP; 33nM [α-^{32}P]dCTP (6,000 Ci/mmol); 0.05 mM each GTP, CTP, and UTP; 1 mM DTT; 25 fmol of pBS(+) single-stranded DNA (molecules) singly primed with the deoxyribonucleotide primer PB32 (62-mer, homologous to bases 826 through 855 of pBS, with a 32-base nonhomologous 5' tail); 0.25 pmol of HSV pol-42; various amounts of UL5-UL8-UL52 or UL5-UL8-UL52 (D628Q); and 15 pmol of ICP8. The reaction mixtures were preincubated with pol-42 for 15 min at 30°C to produce the preformed fork substrate (open duplex circular plasmid with a free 5' tail) and were placed on ice prior to the sequential addition of the wild-type or mutant helicase-primase complex and ICP8. The reaction mixtures were incubated for a further 1.5 h at 30°C, and reactions were terminated by the addition of an equal volume of 1% SDS, 40 mM EDTA, tRNA (200 μg/ml), and proteinase K (1 mg/ml). Following incubation at 37° for 1 h, the reaction mixtures were ethanol precipitated and the products were separated on a 0.8% alkaline agarose gel. The gel was neutralized, dried and exposed for autoradiography.

In vivo replication assays. The in vivo replication procedure is a modification of the method of Stow (47). Sf9 cells in Grace's medium (GIBCO) containing 10% serum were transfected with $CaPO_4$ (50) with 750 ng of plasmid pMC110 (9), which contains the minimal sequences of ori_s required for replication, and incubated for 4 h at 27°C. The cells were washed and then infected at a multiplicity of infection of 10 each with recombinant baculoviruses expressing the wild-type or mutant versions of UL52 and each of the six other genes required for HSV DNA replication (UL5, UL8, UL9, UL29, UL30, and UL42) for 1 h. The medium was changed, and cells were incubated at 27°C in TMNFH medium for 48 h. Total cellular DNA was prepared. Five micrograms of DNA was digested with *Eco*RI and *Dpn*I, and the presence or absence of unit length (2.7-kb) *Dpn*I-resistant DNA was assayed by Southern blot with radiolabeled pUC18 DNA as a probe.

Construction of UL52 mutants. The *Sph*I site in the polylinker of pGEM5Zf(−) (Promega, Madison, Wis.) was changed to a *Bgl*II site by oligonucleotide-directed mutagenesis (32) to yield pGEM5Bgl. The 5' end of the wild-type UL52 gene was modified by oligonucleotide-directed mutagenesis so that an *Nco*I site was created at the site of the initiator methionine codon. The UL52 gene was then inserted between the *Nco*I and *Eco*RV sites of pGEM5Bgl. The UL52 coding sequence of the resultant plasmid, pGEM/UL52, was sequenced in its entirety and compared to the coding sequence of the UL52 gene of pNN5 (55), which contained the original gene derived from the KOS strain of HSV-1. The two sequences were identical, although there were 16 single-nucleotide substitutions compared with the published sequence of HSV-1 strain 17 (37). Single-amino-acid substitutions were introduced into the UL52 coding sequence by oligonucleotide-directed mutagenesis (32). The oligonucleotides used (complementary to the sense strand of the UL52 gene), along with the resultant amino acid substitutions in UL52, are listed in Table 1.

Each pGEM/UL52 mutant was sequenced to verify the presence of the correct base changes. Each mutant allele was ligated into the polylinker of the baculovirus vector, pVL1392,

TABLE 1. Oligonucleotides used

Sequence[a]	Amino acid mutation[b]
CCAGGCTTTTTTGGTCCGGCCC	E239Q
GAATATCTCGTTGAGATTCACGTAC	R612L
GCCGTTGAATATCTGGTTGCGATTCAC	E614Q
GGCGATGTCGAGCTGCAGGATGATG	D628Q
CATGCGCTTTCGAAAAAATAACAGG	K677E
CCCGCAGTCCAATATTGTCGGTGCAGG	K759N
GGGCATGCACACCTGCAGTCCGATC	R763Q
CAAAATACGGCAAGAGCAGGCTGTG	R823L

[a] The underlined base indicates the position at which the sequence was altered to yield the indicated amino acid change.

[b] The first letter designates the amino acid subject to mutation, the number indicates its position, and the second letter designates the amino acid to which it is changed.

and was recombined into the *Autographa californica* nuclear polyhedrosis virus genome with BaculoGold linearized baculovirus DNA (PharMingen, San Diego, Calif.) as the target DNA. At least two rounds of plaque purification were performed on each recombinant virus.

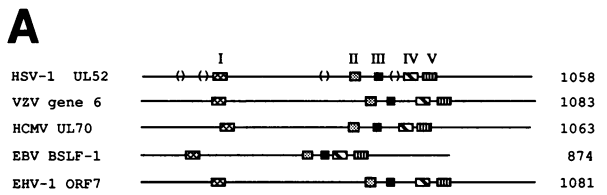
Protein purification. The purification of UL5-UL8-UL52 was essentially as previously described (46), except that the size exclusion chromatography step was omitted. The protein was estimated to be about 95% homogeneous by SDS-polyacrylamide gel electrophoretic analysis. The mutant UL5-UL8-UL52(D628Q) protein was purified by two different methods. The complex was purified as previously described for the wild-type complex and was also purified by a modification of the method of J. Crute (11a). Briefly, 40 225-cm^2 flasks of Sf9 cells were infected at a multiplicity of infection of 10 each with recombinant baculoviruses containing the genes for UL5, UL8 and UL52(D628Q). Cytoplasmic extract (350 mg of total protein) was prepared by the lysis of the cells in buffer B. The cytoplasmic extract was spun at 70,000 × *g* for 15 min and combined with an equal volume of buffer C with 200 mM NaCl and 2 M $(NH_4)_2SO_4$ on ice for 4 h. The resultant protein pellet (18 mg) was resuspended in buffer C containing 200 mM NaCl, dialyzed to equilibrium against buffer C containing 100 mM NaCl, and applied to a MonoQ HR 5/5 column (Pharmacia). Proteins were eluted with a 20-ml linear gradient of 0.1 to 1 M NaCl in buffer C. The fractions containing UL5-UL8-UL52(D628Q) protein (eluting at approximately 400 mM NaCl) were identified by Coomassie blue staining of an SDS-polyacrylamide gel. These fractions (containing 4.5 mg of protein) were pooled, concentrated to 1/4 volume (0.5 ml), and applied to a size exclusion column (Biosil SEC 250; Bio-Rad Laboratories) in buffer C (pH 7.0) containing 250 mM NaCl and 0.01% Nonidet P-40. The fractions containing the peak of UL5-UL8-UL52(D628Q) protein (3 mg) were pooled and frozen at −80°C.

Western immunoblot analysis. Western immunoblot analysis was performed with antipeptide sera specific for UL5, UL8, and UL52 proteins as previously described (40).

Computerized generation of figures. Autoradiograms and photographs were scanned with the Agfa Arcus Plus scanner and an Apple Macintosh computer. Images were generated with Adobe Photoshop, version 2.5.

RESULTS

Mutagenesis of UL52. To identify potentially important regions within UL52 to target for mutagenesis, the sequence of the HSV-1 UL52 gene was compared with its homologs in four herpesviruses: varicella-zoster virus, Epstein-Barr virus, human cytomegalovirus, and equine herpesvirus 1. HSV-1, varicella-zoster virus, and equine herpesvirus 1 are alphaherpesviruses and appear to be closely related evolutionarily, while human cytomegalovirus is a betaherpesvirus and Epstein-Barr virus is a gammaherpesvirus. We identified five regions of at least 10 amino acids possessing 30% identity and ≥50% homology (i.e., identical or strongly conserved residues) between HSV UL52 and each of the other four viral homologs. Figure 1A shows diagrammatically the location of these conserved regions in the five proteins. In an attempt to define more precisely potentially



B

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HSV UL52        227  SPFWFLSKFGPDEKSLVLTTRYY  249          E239Q
VZV gene 6      222  SPFWYIVRFGPSEKTLVLATRYY  244
EHV-1 ORF7      227  TPFWFASKFGPGEREIVLATRYY  249
EBV BSLF-1      154  SARWFISTFGSHEAQFVLVTAAY  176
HCMV UL70       258  SPSWFISVFGHTEGQVLLTMAYY  280
                     || *||  **  *  ||*   *

HSV UL52        672  PCYFFKSACRP  682          K677E
VZV gene 6      713  PCYFYKTACPE  723
EHV-1 ORF7      696  PCYFYKTSCPP  706
EBV BSLF-1      544  PVYFFKSACPP  554
HCMV UL70       672  PVYFFKSACPP  682
                     * **|*||*

HSV UL52        752  PVCSCTDKIGLRVCMPVPA  770      K759N
VZV gene 6      791  LPCNCKEKIGFRVCVPIPN  809      R763Q
EHV-1 ORF7      789  DACECTEKMGFRVTVPVPP  807
EBV BSLF-1      565  PFCICTGKLGFRVITPLPG  583
HCMV UL70       745  PYCRCKGKIGLRIITPFPA  763
                       * * |*|*|*|  * *

HSV UL52        810  LIDTGVYAHGHSLRLPYF  827      R823L
VZV gene 6      849  FIDTGVYSHGHSLRLPFF  866
EHV-1 ORF7      847  FIDTGVYSHGRSLRLPFF  864
EBV BSLF-1      624  LFDSGVYHAGRCIRLPHT  641
HCMV UL70       803  SLDTGIYHHGRSVRLPYM  820
                      |* *|*  *|||***

HSV UL52        607  QMYVNRNEIFNGALAITNIILDLD  630      R612L
VZV gene 6      648  QLYINRNELFNINLIITNLILDVD  671      E614Q
EHV-1 ORF7      631  QMYINRNEIFNSSLAVSNIILDVD  654      D628Q
EBV BSLF-1      482  QLFVSRHEYFNPRLPVCNLVLDLD  505
HCMV UL70       612  QFYYTRHEVFNERLPVFNFVADFD  635
                     *||| *|* **  * | *|| *|*

Yeast Primase 1          107  LVFDID  113
Mouse Primase (p49)      105  LVFDID  111
Phage T7 Primase         140  LMFDMD  146
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FIG. 1. Regions of homology between HSV-1 UL52 and its homologs in other herpesviruses. (A) The positions of regions spanning at least 10 amino acids and possessing 50% or greater homology between HSV-1 and the related herpesviruses varicella-zoster virus (VZV), equine herpesvirus 1 (EHV-1), Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV) are indicated by the variously hatched boxes. These regions of UL52 include amino acids 227 to 249 (I), 607 to 630 (II), 672 to 682 (III), 752 to 770 (IV), and 810 to 827 (V). Four regions of UL52 of greater than 20 amino acids with no counterpart in EBV are indicated in parentheses. (B) Alignment of the five conserved regions shown in panel A. At the bottom of each alignment, invariant residues are indicated by an asterisk and conservative changes are indicated by a vertical line. Targeted amino acids in each region are indicated in boldface, and the actual amino acid changes introduced in each region are shown to the right with the original amino acid (single-letter designation) preceding the position number and the replacement following the position number. The motif that is conserved between UL52 and other primases is also shown. The numbers preceding and following each sequence show the positions of the regions in their respective proteins.

important amino acid residues within these regions, the sequences of known viral, prokaryotic, and eukaryotic primases were searched for homology to the conserved regions of UL52. One potential motif that is imperfectly conserved between phage T7 primase, yeast primase 1, the p49 subunit of mouse primase, and the five herpesvirus homologs was identified. This 6-amino-acid motif, (IL)(VIM)(LF)DhD (where h is a hydrophobic residue), is located at amino acids 625 to 630 of UL52, within conserved region II (Fig. 1B). Single-amino-acid substitutions were introduced into each of the five conserved regions of UL52, as illustrated in Fig. 1B. Recombinant baculoviruses carrying each mutated UL52 gene were identified by their expression of full-length, immunoreactive UL52 protein. The protein expressed by each mutant UL52 recombinant was stable, showing steady-state levels of UL52 protein comparable to that expressed by the wild-type recombinant when analyzed by Western blot (data not shown).

The biological activity of UL52 mutants. The baculovirus recombinants expressing the mutated versions of UL52 were used to assess the ability of the mutant polypeptides to support DNA synthesis in vivo. A plasmid containing a functional origin of replication from HSV-1 (ori_s) can be amplified following transfection of Sf9 cells when the cells are subsequently infected with a mixture of baculovirus recombinants each encoding one of the seven essential HSV-1 replication proteins (47). DNA replication in this system is dependent on each of the seven genes that are necessary for viral replication in mammalian cells (9, 55), and the system is useful for assessing the biological activities of mutants of these genes (47). To analyze the abilities of the UL52 variants to support DNA synthesis, Sf9 cells were transfected with pMC110 (9), an ori_s-containing plasmid, and subsequently infected with a mixture of recombinant baculoviruses containing the mutated UL52 gene and each of the other six herpesvirus genes required for replication. After 48 h of infection, the plasmid DNA was isolated and digested with the restriction enzyme *Dpn*I. Replication of the transfected plasmid DNA results in the loss of adenine methylation and consequent resistance to *Dpn*I digestion. The infected cells were also examined by Western blot analysis for the steady-state levels of UL52 polypeptides; as expected, the mutant UL52 polypeptides were expressed in the coinfected cells at levels equal to that of the wild-type polypeptide (data not shown).

The mutant proteins fell into three classes with respect to their ability to support plasmid replication in this system (Fig. 2). Mutants E239Q and R612L supported replication as well as the wild-type UL52. Conversely, mutants D628Q, R763Q, and R823L completely failed to support replication. Mutants E614Q, K677E, and K759N had an intermediate phenotype, supporting replication about 25 to 40% as well as the wild type. Because mutant D628Q was totally defective in supporting replication in the in vivo assay and because it contained the mutation within the motif we identified in known primases, this protein variant was chosen for further biochemical characterization.

The D628Q mutation has no effect on helicase function. Sf9 cells were triply infected with recombinant baculoviruses expressing wild-type UL5 and UL8 and the D628Q UL52 variant. A heterotrimeric complex containing these three polypeptides was purified from the infected cells according to two different purification protocols as discussed in Materials and Methods. The results of experiments using proteins purified with each protocol were identical (data not shown). Figure 3A shows a silver-stained gel of fractions from the final step in the purification of UL5-UL8-UL52(D628Q). We estimate that the protein is 80% pure. The protein concentration of the mutant

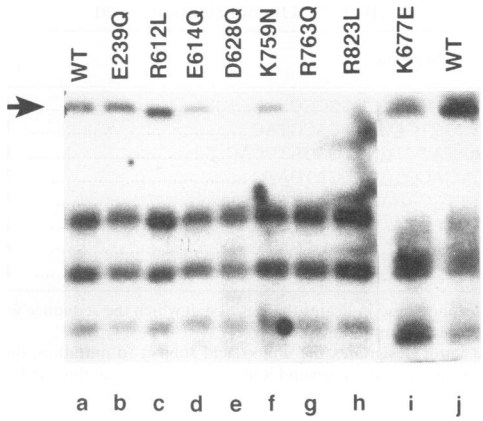

FIG. 2. Ability of UL52 mutants to support DNA replication in vivo. Sf9 cells were transfected with pMC110, which contains one copy of ori_s of HSV-1. The cells were subsequently infected with a mixture of baculoviruses expressing UL5, UL8, UL9, UL29, UL30, UL42, and either the wild-type (WT) or the indicated mutant variant of UL52. After 48 h, total DNA was isolated and 5 μg of DNA from each sample was digested with *Eco*RI and *Dpn*I. Following electrophoresis and transfer to a GeneScreen Plus membrane, the immobilized DNA was probed with ^{32}P-labeled pUC18 DNA. The presence of a full-length 2.7-kb *Dpn*I-resistant band (indicated by the arrowhead) indicates that the plasmid was amplified in the insect cells. The data shown in lanes a to h and in lanes i and j are derived from two different experiments.

complex was determined by dye binding and confirmed in comparison with the wild-type protein by quantitative Western blot analysis (data not shown).

As all of the helicase motifs lie in the UL5 subunit, we anticipated that the D628Q mutant complex might retain helicase and ATPase activities. Therefore, individual column fractions from the final purification step were also assayed for these enzymatic activities. As illustrated in Fig. 3, both ATPase activity and helicase activity were readily detected and both precisely coeluted with the peak of UL5-UL8-UL52(D628Q) protein, strongly suggesting that the observed helicase activity is due to an intrinsic activity of the mutant UL5-UL8-UL52 complex. To determine whether the mutation has a quantitative or qualitative effect on the helicase activity of the complex, we performed a detailed comparison of the activities of the wild-type and D628Q complexes in three assays with increasing degrees of complexity. Thus, we measured specific ATPase activity, the ability of the complex to displace short oligonucleotide primers from single-stranded DNA (helicase assay), and the ability of the complex to support strand displacement synthesis of DNA at a preformed replication fork in conjunction with pol-42 and ICP8 (leading-strand assay).

We compared the ATPase specific activities of the wild-type and mutant enzyme complexes by a colorimetric assay that measures the production of free phosphate. Five picomoles of wild-type or mutant D628Q complex was incubated in the presence of ATP and denatured calf thymus DNA for 1 h at 37°C. Table 2 shows the average ATPase activity of each preparation as determined from duplicate experiments. The specific ATPase activity of the mutant complex is 98% of the wild-type activity.

To compare the helicase activities of the mutant and wild-type complexes, 0.25 and 1 pmol of each complex were assayed in a standard oligonucleotide displacement assay (14). Quantitation of the radioactivity present in the faster-migrating bands corresponding to released oligonucleotide (Fig. 4) indi-

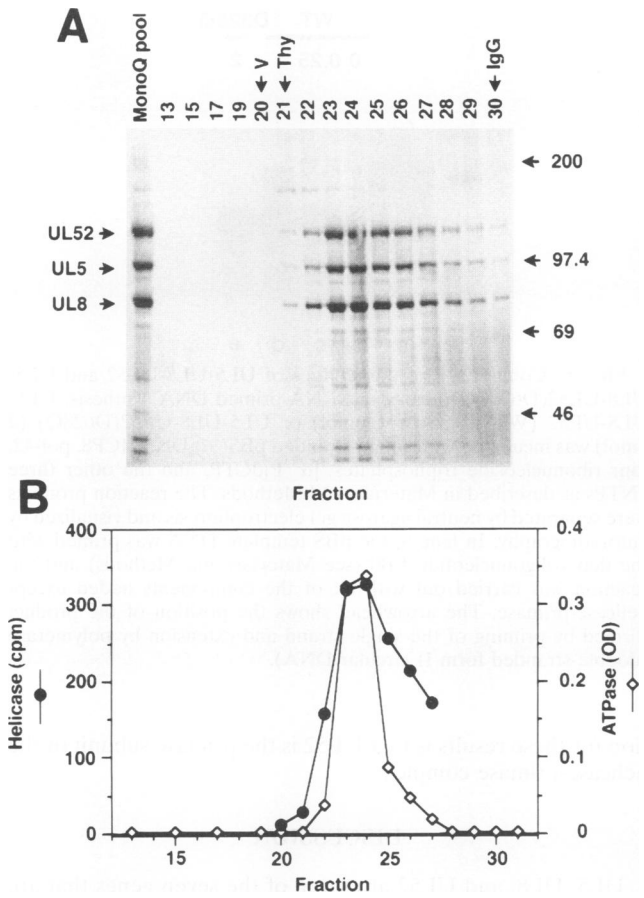

FIG. 3. Gel filtration chromatography of UL5-UL8-UL52(D628Q) protein. (A) The indicated fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins in the gel were visualized by silver stain. The void volume of the column (V) and the positions of elution of thyroglobulin (Thy) with a molecular mass of 670 kDa and immunoglobulin G (IgG) with a molecular mass of 158 kDa were determined by elution of protein standards using the same conditions. (B) ATPase activities and helicase activities of the indicated fractions were determined as described in Materials and Methods. ATPase activity is expressed in relative optical density (OD) units. After electrophoretic separation of the products, helicase activity was quantitated on the Betagen Betascope 603 and is expressed as counts per minute of the displaced oligonucleotide.

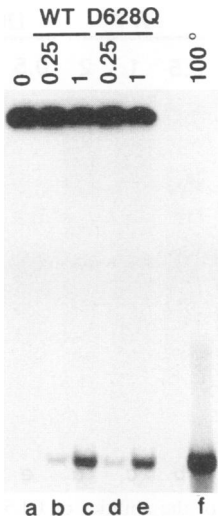

FIG. 4. Comparison of the helicase activities of wild-type UL5-UL8-UL52 (WT) and UL5-UL8-UL52(D628Q). UL5-UL8-UL52 (0.25 or 1 pmol) or UL5-UL8-UL52(D628Q) (0.25 or 1 pmol) was incubated at 37°C for 1 h in the presence of single-stranded M13mp18 DNA that had been annealed to a ^{32}P-labeled oligonucleotide as described in Materials and Methods. Following electrophoresis through a 15% nondenaturing polyacrylamide gel, the products were visualized by autoradiography. The reaction mixture in lane a was incubated without added protein, and that in lane f was heated to 100°C just prior to electrophoresis.

cated that the UL5-UL8-UL52(D628Q) complex exhibited 90% of the helicase activity of the wild-type complex (Table 2).

Each of these assays measures a property of the helicase-primase that is independent of the requirement for other HSV-1 replication proteins. The results indicate that UL5-UL8-UL52(D628Q) is capable of catalyzing these reactions to the same extent as the wild-type enzyme. It has been shown previously that the UL5-UL52 complex, lacking UL8, also carries out these two core reactions as well as the wild-type heterotrimer. A more complex reaction that depends on other HSV proteins in addition to UL5 and UL52 is an assay for leading-strand synthesis utilizing a preformed fork substrate (25a). This assay requires, in addition to the helicase functions measured by the simpler reactions, the ability of the helicase-primase complex to unwind long stretches of duplex DNA and, very likely, the ability to participate in protein-protein interactions that have not yet been characterized in detail. Unlike the simpler ATPase and helicase assays, the leading-strand assay requires UL8. To determine the extent to which UL5-UL8-UL52(D628Q) could participate in this complex reaction, 0.25 or 1 pmol of UL5-UL8-UL52 or UL5-UL8-UL52(D628Q) was incubated with the preformed fork substrate in the presence of pol-42 and ICP8 as detailed in Materials and Methods, and the products of the reaction were separated on a 0.8% alkaline agarose gel. The wild-type helicase-primase was capable of supporting rolling-circle replication, as evidenced by the formation of long DNA single strands exceeding 20 kb in length (Fig. 5, lane d). A reaction containing only ICP8 and the polymerase holoenzyme showed only unit-length molecules (Fig. 5, lane a). The products of reactions containing the UL5-UL8-UL52(D628Q) complex were essentially identical to those with the wild-type complex, showing the same range of sizes and the same distribution of products. Thus, these results indicate that the UL5-UL8-UL52(D628Q) complex can carry out all of the functions required for leading-strand replication as efficiently as UL5-UL8-UL52.

TABLE 2. ATPase and helicase activities of UL5-UL8-UL52 and UL5-UL8-UL52(D628Q)

Enzyme	Activity of:		Ratio of mutant to wild type
	UL5-UL8-UL52	UL5-UL8-UL52(D628Q)	
Helicase[a]	1.16 fmol/pmol/h	1.04 fmol/pmol/h	0.90
ATPase[b]	2.9 nmol/pmol/h	2.86 nmol/pmol/h	0.98

[a] Femtomoles of oligomer released per picomole of enzyme per hour.
[b] Nanomoles of P_i released per picomole of enzyme per hour.

The D628Q mutation eliminates primase activity. The primase activity of the UL5-UL8-UL52(D628Q) complex was compared with that of the wild-type complex by two different assays. In the first assay, primers synthesized by the helicase-

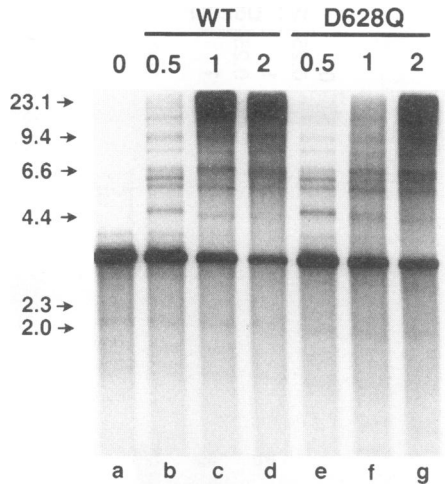

FIG. 5. Comparison of the abilities of UL5-UL8-UL52 and UL5-UL8-UL52(D628Q) to support leading strand replication. UL5-UL8-UL52 (WT) or UL5-UL8-UL52(D628Q) (0.5 to 2 pmol) was incubated with a preformed fork substrate [single-stranded pBS(+) singly primed with oligonucleotide PB32 and extended with pol-42 to yield double-stranded pBS with a free 5′ tail] in the presence of pol-42, ICP8, [α-^{32}P]dCTP, the other three dNTPs, and the four ribonucleoside triphosphates for 1.5 h at 30°C as detailed in Materials and Methods. Following alkaline agarose gel electrophoresis, the single-stranded products were visualized by autoradiography. The positions of molecular size markers (in kilobases) are indicated to the left.

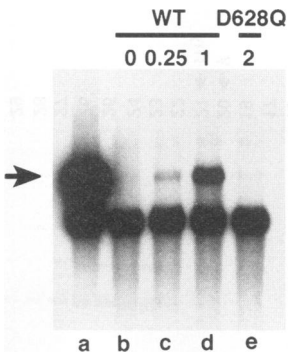

FIG. 6. Comparison of the abilities of UL5-UL8-UL52 and UL5-UL8-UL52(D628Q) to carry out RNA-primed DNA synthesis. UL5-UL8-UL52 (WT) (0.25 or 1 pmol) or UL5-UL8-UL52(D628Q) (2 pmol) was incubated with single-stranded pBS(+) DNA, ICP8, pol-42, four ribonucleoside triphosphates, [α^{32}P]dCTP, and the other three dNTPs as described in Materials and Methods. The reaction products were separated by neutral agarose gel electrophoresis and visualized by autoradiography. In lane a, the pBS template DNA was primed with the deoxyoligonucleotide PB0 (see Materials and Methods) and the reaction was carried out with all of the components added except helicase-primase. The arrowhead shows the position of the product formed by priming of the single strand and extension by polymerase (double-stranded form II circular DNA).

primase complex on ICP8-coated single-stranded DNA are elongated by the HSV-encoded pol-42 complex to result in fully duplex circular molecules (46). In the second assay, RNA primer synthesis was analyzed directly.

To assess the ability of the D628Q complex to participate with pol-42 and ICP8 in RNA-primed DNA synthesis (lagging-strand assay), single-stranded pBS(+) DNA was incubated with pol-42, ICP8, and UL5-UL8-UL52 or UL5-UL8-UL52(D628Q) and the resultant products were analyzed by electrophoresis through a 1% neutral agarose gel and autoradiography (Fig. 6). With 0.25 and 1 pmol of the wild-type complex (Fig. 6, lanes c and d), a dose-dependent production of double-stranded circular molecules was evident, while 2 pmol of the D628Q complex (lane e) resulted in only a background level of double-stranded circles. These results indicated that while the wild-type enzyme was able to synthesize primers that could be elongated by the HSV-1 DNA polymerase, the mutant enzyme was completely incapable of performing this function. This result would be obtained if the UL5-UL8-UL52(D628Q) complex did not synthesize primers or if it synthesized primers that could not be elongated by the DNA polymerase.

To assay directly for the capacity of the mutant enzyme to carry out primer synthesis, RNA primers were synthesized in the presence of [α-^{32}P]UTP and visualized by autoradiography after separation on a sequencing-type denaturing polyacrylamide gel. The results are shown in Fig. 7. The wild-type enzyme produced primers approximately 5 to 14 nucleotides in length (predominantly 8 to 9 nucleotides) in a dose-dependent manner. In contrast, there was no detectable synthesis of RNA primers with the UL5-UL8-UL52(D628Q) enzyme.

In both the direct and the coupled assay, the mutant enzyme complex exhibited less than 12% of the activity of wild-type UL5-UL8-UL52, while in the three assays for helicase activity, the two complexes were equivalent. The most likely explanation for these results is that UL52 is the primase subunit of the helicase-primase complex.

DISCUSSION

UL5, UL8, and UL52 are three of the seven genes that are essential for HSV-1 DNA replication. The products of these three genes form a heterotrimeric complex with helicase and primase activity (13, 15). Given its known roles, it is likely that the complex functions in both leading- and lagging-strand replication. The helicase presumably acts to unwind duplex DNA ahead of the progressing replication fork, thereby producing the open configuration needed for both continuous- and discontinuous-strand synthesis. By analogy to other primases, the primase of HSV-1 presumably initiates both continuous DNA synthesis near the origin of replication and discontinuous DNA synthesis on the lagging strand by providing the oligoribonucleotide primers that are elongated by the polymerase.

The helicase activity of the UL5-UL52 subcomplex is as efficient as that of the UL5-UL8-UL52 complex when measured by an assay in which relatively short 3′-tailed oligonucleotides are displaced from single-stranded DNA (6, 18, 46). In addition, the UL5-UL52 subcomplex can also synthesize oligoribonucleotide primers (18, 46). Therefore, UL8 is not required for the core helicase and primase activities of the complex. A requirement for UL8 in both leading- and lagging-strand replication has, however, been demonstrated. UL8 appears to be required for the efficient utilization of primers by polymerase, thereby increasing the efficiency of lagging-strand synthesis (46). Additionally, it has been shown that UL8 stimulates leading-strand synthesis in vitro by a mechanism that is distinct from any possible role in primase activity. UL8 is required for the synthesis of long (>20-kb) DNA single strands in a reaction utilizing a circular preformed fork substrate and requiring six of the seven essential replication proteins of HSV (no UL9) (25a). The role of UL8 in this latter assay is unclear; one possibility is that UL8 is required to

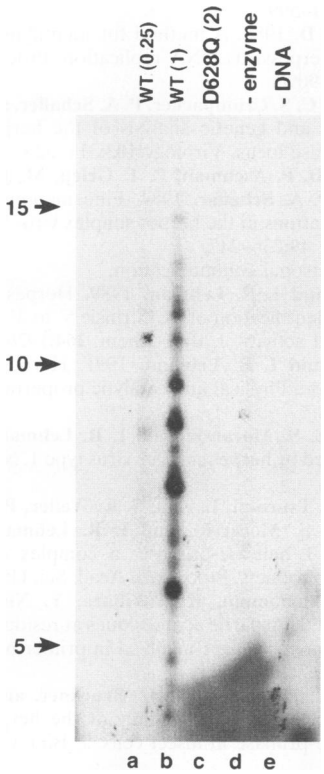

FIG. 7. Comparison of the abilities of UL5-UL8-UL52 and UL5-UL8-UL52(D628Q) to synthesize primers. UL5-UL8-UL52 (WT) (0.25 or 1 pmol) or UL5-UL8-UL52(D628Q) (2 pmol) was incubated with single-stranded pBS DNA in the presence of [α-^{32}P]UTP and the other three ribonucleoside triphosphates as detailed in Materials and Methods. The products were isolated by NENsorb 20 chromatography, separated on an 18% Hydrolink Long Ranger gel with 7 M urea, and visualized by autoradiography. The positions of 5′ end-labeled oligonucleotides U_5, U_{10}, and U_{15} used as molecular size markers are indicated to the left and were determined in a separate experiment with the products of wild-type primase run in a parallel lane. Lanes d and e show the effects of omitting enzyme or template DNA, respectively.

increase the processivity of the helicase on long regions of duplex DNA.

Examination of the amino acid sequence of UL5 strongly suggests that it is the helicase of the complex, as it shares conserved sequence motifs with other known helicases (25, 29) and mutations within these motifs abolish the ability of UL5 to support replication of an ori$_s$-containing plasmid in an in vivo assay (56). Formally, however, the question of whether UL5 is the helicase remains open, as UL5 alone has not demonstrated convincing levels of helicase activity in the absence of UL52 (6, 46). If UL5 is, as seems likely, the helicase, then an important question that remains to be understood is how UL52 stimulates its helicase activity in the formation of the UL5-UL52 complex. One possibility is that UL5 undergoes an activating conformational change upon UL52 binding that results in an increased affinity for DNA or nucleoside triphosphate (NTP). Another possibility is that UL52 contributes essential amino acids to the structure of the catalytic site. In favor of the first possibility is the fact that UL5 itself contains all six motifs associated with other helicases.

The association of helicase with primase is not unique to HSV, although this pairing of activities is more reminiscent of prokaryotic than eukaryotic systems. In phage T4, gene 61 primase and gene 41 helicase each stimulate the activity of the other in vitro assays (51). Therefore, these proteins have been shown to be functionally, if not physically, associated. In phage T7, the helicase and primase activities are encoded by a single gene (28, 45, 49). The products of phage T7 gene 4 exist in a longer (63-kDa) form with both activities (38) and a shorter (56-kDa) form with only helicase activity (4, 38). These two forms can be isolated as a complex in a 1:1 ratio (38), or they may also be expressed individually, in which case the 56-kDa form stimulates the primase activity of the 63-kDa form up to 100-fold (38). In *Escherichia coli*, the helicase activity of DnaB and the primase activity of DnaG also appear coordinated (1, 33). The mechanisms responsible for the mutual stimulation of the activities of the helicase and primase have not been determined for any of these systems.

The data in this paper provide the first experimental evidence that UL52 functions as a primase. A single-amino-acid mutation (changing the aspartate at position 628 to glutamine) completely abolished the ability of the UL5-UL8-UL52 complex to support replication of an origin-containing plasmid in vivo, to support lagging-strand DNA synthesis in vitro, and to synthesize oligoribonucleotide primers in vitro. In contrast, the mutation had no effect on any measurable aspect of leading-strand replication: ATPase, helicase, or displacement DNA synthesis from a preformed fork in conjunction with ICP8, DNA polymerase, and UL42.

Although the most straightforward explanation for our results is that UL52 contains the catalytic site for primase activity, there are other possible explanations. One possibility is that UL5 contains both helicase and primase activities and that UL52 is required to stimulate both of these activities but itself does not play a role in primer synthesis. Another alternative possibility is that the UL52 subunit contains some but not all of the sequences required for primase activity and that both UL5 and UL52 contain domains that must physically interact in such a way as to create the primase catalytic sites. If this were the case, the mutation at amino acid 628 could either interfere with this interaction or form part of a defective catalytic site. No direct evidence exists to distinguish among these possibilities as yet. Biochemical characterization of other UL52 mutants and of UL5 mutants may help to clarify this issue. Recently, partially purified preparations of UL52 alone have been shown to retain primase activity (11a). These results suggest that UL52 contains all of the elements comprising the primase active site.

In the attempt to identify short sequence similarities between UL52 and known primases, only one potential conserved motif, (IL)(VIM)(LF)DhD, was found. This motif is located within a conserved region of the small subunit of mouse primase and yeast primase 1, as well as in phage T7 primase. The significance of this motif is underscored by the fact that similar sequences are also found in most classes of nucleic acid polymerases, including DNA-dependent DNA polymerases, reverse transcriptases, and RNA-dependent RNA polymerases (2), and in other prokaryotic primases, including *E. coli* and *Salmonella typhimurium* DnaG, *Bacillus subtilis* DnaE, T4 gp61, T3 gp4 (30), and the primases of some conjugative plasmids (48). Among these various classes of polymerases, the only invariant residues are DD or DXD. However, the DNA and RNA polymerases seem to have in common a secondary structure in which the aspartates are flanked by hydrophobic residues that form a β hairpin structure, leaving the aspartates on an exposed loop (2). The protein secondary structure near the aspartates in the primases is less clear. There is a tendency toward hydrophobicity

proximal to the motif, but the C-terminal flanking region is somewhat less hydrophobic than in the other classes of polymerases.

The DD or DXD motif in the polymerases has been proposed to play a role in Mg^{2+} or NTP binding or possibly to play a direct role at the polymerase catalytic site (2). Mutations have been made in the proposed hairpin loop of several polymerases, with a variety of results. Poliovirus RNA polymerase mutants with single-amino-acid substitutions for the glycine in the TGDD motif had reduced or undetectable polymerase activity (31). A similar mutation that changed the glycine to alanine at the YGDTD sequence of HSV-1 DNA polymerase was lethal in the context of the virus (35). Mutations within the sequence YCDTD of *B. subtilis* ϕ29 DNA polymerase had effects both on protein-primed initiation of DNA synthesis and on elongation, depending on the specific mutation and its position (3). Finally, conservative aspartate-to-glutamate mutations at either position of the sequence GDMD of rat DNA polymerase β caused diminished binding of the polymerase to the primer/template, leading to drastically reduced DNA polymerase activity (16).

Our results indicate that mutation of the first aspartate in the sequence IILDLD of HSV-1 UL52 eliminates any detectable primer synthesis by the UL5-UL8-UL52(D628Q) complex. Further biochemical characterization of this and other UL52 mutants may yield information concerning the mechanism of the defect in primer synthesis, which could be in metal binding, NTP binding, template binding, or a direct defect in catalysis. In this regard, conservative mutations of the aspartates at positions 628 and 630, such as D→E mutations, might exhibit diminished but measurable primase activity, thereby allowing kinetic analyses of various steps of the primase reaction. Likewise, partially defective mutants in other regions of the protein, such as K677E, might permit the localization of the domains of the UL52 protein that are responsible for various steps in the reaction.

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