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The Extreme C Terminus of Herpes Simplex Virus DNA Polymerase Is Crucial for Functional Interaction with Processivity Factor UL42 and for Viral Replication

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The herpes simplex virus DNA polymerase is composed of two subunits, a large catalytic subunit (Pol) and a smaller subunit (UL42) that increases the processivity of the holoenzyme. The interaction between the two polypeptides is of interest both for the mechanism by which it enables the enzyme to synthesize long stretches of DNA processively and as a possible target for the rational design of novel antiviral drugs. Here, we demonstrate a combination of insertion and deletion mutagenesis that the carboxy-terminal 35 amino acids of Pol are crucial for binding UL42. The functional importance of the interaction was confirmed by the finding that a pol mutant defective for UL42 binding retained polymerase activity, but did not synthesize longer DNA products in the presence of UL42. Moreover, several association-incompetent mutants failed to complement the replication of a pol null mutant in a transient transfection assay, confirming that the Pol-UL42 interaction is necessary for virus replication in vivo and therefore a valid target for directed drug design.

Replicative DNA polymerases generally function as multiprotein complexes, including a catalytic subunit and one or more accessory proteins that modify the properties of the core polypeptide. The mechanism by which such proteins interact to form an enzyme capable of replicating long stretches of DNA rapidly and processively is of considerable interest. In part because of the ease with which the viral genetic information can be modified, the herpes simplex virus type 1 (HSV-1) DNA polymerase provides an excellent model system for the study of the structure and function of polymerase complex association. The HSV catalytic subunit (Pol) also shares considerable sequence homology with a large family of eukaryotic and prokaryotic DNA polymerases, including the major eukaryotic replicative enzymes (16, 20, 44), suggesting a broad conservation of functional mechanisms, possibly extending to those depending on less well-conserved accessory subunits.

Herpes simplex virus is also a significant pathogen, causing considerable morbidity and some mortality. To date, the most successful anti-HSV drugs have been directed against catalytic sites of the polymerase (reviewed in reference 4), but especially in immunocompromised patients, resistance to the commonly used compounds is a growing problem (19). Therefore, the identification of alternative therapeutic targets is of interest, and as previously hypothesized, the interaction between Pol and its accessory subunit may well provide such a distinct target (10).

Pol normally associates with a 65-kDa double-stranded-DNA-binding protein encoded by the UL2 gene (14, 37, 42). One known function of UL42 is to increase the ability of Pol to synthesize longer DNA products (16, 18) through an increase in processivity (16). Both pol and UL42 are essential genes for viral growth (1, 22, 27, 29, 38), leading to the hypothesis that the interaction between the two polypeptides is also essential (10, 16).

Previously, we succeeded in reconstituting the Pol-UL42 complex in vitro by the translation in rabbit reticulocyte lysate of bacteriophage RNA polymerase-generated transcripts coding for the two proteins and demonstrated that UL42 bound to the carboxy-terminal 228 amino acids of Pol, a region of previously unknown function (10). Here, we present the results of experiments designed to define more clearly the nature of the Pol-UL42 interaction, through finer-scale mapping of the Pol residues involved in UL42 binding and analysis of the ability of these mutants to synthesize long DNA products in the presence of UL42. We also test the hypothesis that the Pol-UL42 interaction is necessary for viral growth by examining the ability of mutants deficient in UL42 binding to support replication in virus-infected cells.

MATERIALS AND METHODS

Linker insertion mutagenesis. A plasmid containing a full-length copy of the pol gene (pDP2A [28]) was pseudorandomly linearized by digestion with the 4-bp recognition sequence restriction enzyme HaeIII (New England Biolabs) or ThaI (Bethesda Research Laboratories). The digests were done in the presence of an empirically determined concentration of ethidium bromide sufficient to inhibit further digestion of linear plasmid molecules generated by the first endonuclease cut (36). The digested plasmid was then linker tagged by ligation in the presence of an excess of the unphosphorylated 12-bp palindromic oligonucleotide, TG CATCGATGCA, generating predominantly linear DNA containing a copy of the linker at both ends. To facilitate mapping of the insertion site, the 12-bp oligonucleotide was designed to contain a ClaI restriction endonuclease site, a sequence not found in the pol gene. The linker was also designed so as not to contain either termination or proline codons in any of the three possible reading frames. High-molecular-weight DNA was separated from noncovalently bound oligonucleotide, by centrifugation through a Centricon-100 filter (Amicon) in the presence of

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10% dimethyl sulfoxide (25). Linear unit-length plasmid was gel purified from a 1% low-melting-point agarose gel, allowed to reanneal, and transformed into Escherichia coli DH5α (Bethesda Research Laboratories). Colonies were screened for intact plasmids containing a single insertion within the gene of interest by standard minipreparation and restriction enzyme digest procedures. Mutants were designated according to the codon position disrupted by the insertion or, in in-frame insertions, the codon immediately 5’. One mutant, designated F-1208, contained a linker insertion at codon 1208 from which the final base pair of the linker had been deleted. This resulted in the expression of a polypeptide in which the final 28 amino acids had been replaced by an unrelated sequence of 30 residues, thus maintaining the approximate size of the polypeptide, but not the primary structure. For in vitro expression and immunoprecipitation analysis, the central 2,350-bp BglII-PstI fragment of the pol gene was removed from these plasmids and replaced with a BglII-PstI fragment from pT730AN960 (see next section) containing a bacteriophage T7 RNA polymerase promoter upstream of amino acid codon 960 of the pol gene. The resulting plasmids could be transcribed in vitro to produce mRNA encoding the carboxy-terminal 276 amino acids of Pol plus the desired insertion. In some cases, a 900-bp PstI-XbaI fragment encoding the C terminus of the mutant Pol was excised from the pDP2A or the pT730 construct and ligated into similarly cut p911. p911 contains the entire pol gene cloned into pBluescript KSII+ (Stratagene) under the control of a bacteriophage T3 promoter. The insertion mutant I-1216 was generated by linearizing plasmid pT730AN960 (see below) with the enzyme Nael and then linker tailing as described above. The precise locations of insertion mutants that could not be unambiguously located by restriction mapping were determined by dideoxy nucleotide sequencing, as were those of insertions that altered UL42 binding.

Deletion and nonsense mutagenesis of pol. Plasmid pT730AN960, encoding the final 276 amino acids of Pol under the control of a bacteriophage T7 promoter, was constructed by using an FspI site at nucleotide 3458 of the pol gene (15) and a downstream EcoRV site in the polylinker of plasmid pNG30 (10) to excise a 1.1-kb DNA fragment, which was then ligated into the SmaI site of plasmid pT7-7 (40). In this construct, an in-frame ATG codon is supplied by the vector. An internal deletion of nucleotides 118 and 119 from 3’ to the first residue of the full-length protein present. Plasmid pT730AN1075 was constructed in a similar fashion, except that an RsaI site at nucleotide 3791 of the pol gene was used. Plasmids pT730AN960 (Δ1060-1078), -Δ(1078-1115), -Δ(1115-1131), -Δ(1131-1175), and -Δ(1175-1203), encoding the indicated deletions in the background of ΔN960, were constructed by using the ClaI sites in the corresponding linker insertion mutants (therefore, these constructs also encode a four-amino-acid insertion from the linker). Briefly, the pT730AN960 family of constructs was digested with PstI and ClaI to divide the plasmid into a vector fragment containing the T7 promoter and pol sequences 3’ to the insertion, and a small fragment containing pol sequences 5’ to the insertion, but downstream of the PstI site. These D fragments were then purified by agarose gel electrophoresis and religated in the appropriate combinations to generate the internal deletions. Mutants ΔN960ΔCl207 and ΔN960ΔCl 1216 (numbered according to the final Pol-specific residue present) were generated by linearizing plasmids pT730ΔN960(-1208) and -(I-1216), respectively, with Clal before in vitro transcription to generate mRNAs lacking pol-specific coding capacity downstream of the numbered amino acid. The corresponding truncations of the full-length Pol polypeptide, n-1207 and n-1216, were constructed by linearizing the appropriate pT330 construct with ClaI and then linker tailing with the partially self-complementary oligonucleotide CGCTAGTCTAGACTAG as described above. The linker contains stop codons in all three translational reading frames and an XbaI restriction enzyme site. The four mutants described above also contain additional non-Pol-specific amino acids at their C termini arising from the linkers; in single-letter code, n-1207 contains an additional VH sequence; n-1216, CI207, VH; ΔCI216, CI.

In vitro transcription, translation, and immunoprecipitation. Plasmids were transcribed in vitro after linearization with the appropriate restriction enzyme, and the resulting mRNA was translated in rabbit reticulocyte lysate as previously described (10). Mutant Pol proteins were tested for their ability to bind cotranslated UL42 by immunoprecipitation with antisera specific to either polypeptide. Briefly, mRNAs encoding mutant Pol proteins or UL42 were translated either singly to provide controls for the specificity of the immunoprecipitation reactions, or together to allow the polypeptides to associate. Aliquots of the in vitro translations were then analyzed by gel electrophoresis before and after immunoprecipitation with antisera directed against either polymerase or UL42, as previously described (10), except that siliconized microcentrifuge tubes (National Scientific Supply Co.) were used to reduce the background of nonspecific precipitation, and antiseraum 13809 (32; generously provided by H. Marsden) was used to precipitate UL42.

Transient complementation of a pol null mutant. Vero cells at approximately 80% confluence were transfected with 300 ng of the indicated pDP2A or pT730 plasmid by the DEAE-dextran method. Sixteen hours later, the cells were infected with the HSV DNA Pol null mutant HP66 at a multiplicity of infection of 3. HP66 has a 2.2-kb deletion of the pol gene replaced by a lacZ gene (29). After virus adsorption for 1.5 h, the cells were washed twice with serum-free medium before and after treatment for 2 min with an acid-glycine-saline wash (3) to remove extracellular virus, before the addition of fresh medium. Twenty-four hours later, virus was harvested (5) and titers were determined on the DP6 cell line, which contains a resident copy of the pol gene and is therefore permissive for HP66 growth (29). Titer determinations were also performed on Vero cells to ensure that they did not arise from recombination events. Under the conditions used, 300 ng of DNA was saturating for transfection efficiency, therefore minimizing the effect of small errors in DNA quantitation.

Overexpression and purification of Pol and UL42. Both polypeptides were purified from lysates of SP9 cells infected with recombinant baculoviruses containing either the pol (BP58 [28]) or UL42 (AcNPV/UL42, generously supplied by J. Gottlieb and M. Chalberg [16]) gene. Protein was purified essentially by published methods (16, 29), but with modifications for Pol that will be reported elsewhere (43).

Polymerase activity assays. Polymerase assays were done by a modification of the protocol reported in reference 15. Briefly, 5 μl of reticulocyte lysate or, in some reactions, 15 fmol of purified Pol was diluted in 50 μl of buffer containing 100 mM (NH₄)₂SO₄, 20 mM Tris-Cl (pH 7.5), 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 40 μg of bovine serum albumin per ml, 60 μM dATP, dGTP, and dTTP, 10 μM [α-³²P]dCTP (2 μCi), and 50 fmol of a singly primed M13 or φX174 single-stranded template prepared as
FIG. 1. Deletion analysis of the UL42-binding domain. The indicated Pol mutants were synthesized in rabbit reticulocyte lysate containing [35S]methionine both without (−) and with (+) UL42 mRNA and then analyzed by gel electrophoresis before (lanes T) and after immunoprecipitation with anti-Pol sera (lanes 1) or anti-UL42 sera (lanes 2). Arrowheads indicate the migration of UL42 and the Pol mutants (ΔPol).

The UL42-binding domain of Pol was synthesized in rabbit reticulocyte lysate containing [35S]methionine both without (−) and with (+) UL42 mRNA and then analyzed by gel electrophoresis before (lanes T) and after immunoprecipitation with anti-Pol sera (lanes 1) or anti-UL42 sera (lanes 2). Arrowheads indicate the migration of UL42 and the Pol mutants (ΔPol).

RESULTS

Deletion mutagenesis of pol to refine the N-terminal boundary of the UL42-binding domain. Previously, the carboxy-terminal 228 amino acids (ΔN1008) of Pol had been shown to be sufficient to bind UL42, although with lower efficiency than the intact protein (10). Therefore, initially we tested the ability of both slightly larger and smaller carboxy-terminal (C-terminal) Pol fragments to bind UL42 when expressed in rabbit reticulocyte lysate. Figure 1 shows the results obtained with polypeptides containing the final 276 and 161 amino acids of Pol (ΔN960 and ΔN1075, respectively). In Fig. 1A, it can be seen that both UL42 and ΔN960 were immunoprecipitated by antisera directed against either protein when cotranslated, but were only precipitated by the cognate serum when translated independently (Fig. 1A, ΔN960, and C, UL42). As previously demonstrated, this specific coprecipitation provides evidence of a stable interaction between the two polypeptides. In Fig. 1B, it can be seen that although both UL42 and ΔN1075 were precipitated by anti-Pol serum when cotranslated, the converse did not seem to be true, in that the Pol polypeptide was not coprecipitated by anti-UL42 serum when UL42 was present. Nevertheless, the consistent one-sided cross-precipitation of the polypeptides does indicate an interaction, although most probably a less stable one than that observed between UL42 and larger Pol fragments.

Linker insertion mutations of UL42-binding domain. The above experiments suggested that ΔN960 would provide a suitable background for testing the effect of the linker insertion mutations on UL42 binding, as this fragment of Pol bound UL42 in vitro with an apparently equal efficiency to the full-length protein (8), but unlike full-length protein, it contained a number of methionine residues similar to UL42, so providing an approximately equal autoradiographic intensity when labelling with [35S]methionine. Therefore, 10 linker insertion mutations and 1 frameshift mutation which had been mapped to the C terminus of the pol gene were cloned into plasmid pT730ΔN960 and then tested for their ability to bind UL42 as described above. The locations and identities of the inserted amino acids are described in Table 1. Figure 2 shows the results obtained with five of the mutants, in which it can be seen that three of the altered polypeptides, containing insertions at amino acids 1115, 1131, and 1175, respectively, behaved like the “wild-type” ΔN960, in that both UL42 and the mutant Pol protein could be immunoprecipitated by either antisera when cotranslated. However, a four-amino-acid insertion at amino acid 1216 (I-1216) severely impaired this interaction, as only slightly larger than background quantities of UL42 and the mutant Pol were cross-precipitated by the heterologous antisera (Fig. 2D). In addition, a frameshift mutation, F-1208, which replaced the final 27 amino acids of Pol with an unrelated sequence of approximately the same length, displayed even less ability to associate detectably with UL42 in the immunoprecipitation assay (Fig. 2E).

Of the 1 frameshift and 11 insertion mutants examined, eight bound UL42 indistinguishably from wild-type Pol, while the others either did not detectably bind UL42 (I-1208 and F-1208) or did so at significantly reduced levels (I-1203 and I-1216). Figure 3 summarizes the locations of these mutations and the effect they had on complex formation. The three insertions and one frameshift affecting UL42 binding were all located at the very C terminus of the protein, while

<table>
<thead>
<tr>
<th>pol mutant</th>
<th>Sequence change</th>
</tr>
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<tbody>
<tr>
<td>I-1012</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1060</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1078</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1126</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1091</td>
<td>A→VHRC</td>
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<td>I-1115</td>
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</tr>
<tr>
<td>I-1175</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1203</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1208</td>
<td>A→VHRC</td>
</tr>
<tr>
<td>I-1216</td>
<td>CID</td>
</tr>
</tbody>
</table>

* The insertion mutants are numbered according to the amino acid codon disrupted by the linker insertion, except for the in-frame insertion I-1216, which is numbered according to the codon immediately 5' to the insertion.

† The inserted amino acid sequences are given in standard single-letter code. Also indicated where appropriate is the identity of the wild-type codon destroyed by the insertion.
FIG. 2. Linker insertion mutagenesis of the UL42-binding domain. The indicated Pol mutants were expressed in reticulocyte lysate and analyzed for their ability to bind UL42 as described in the text and the legend to Fig. 1.

the eight insertions upstream of these had no discernible effect. Therefore, the extreme C terminus of the polypeptide is critical for the Pol-UL42 interaction.

**Internal deletion and nonsense mutagenesis of the UL42-binding domain.** Fortuitously, all but the most C terminal of the insertions were in the same reading frame (Table 1) and could therefore be used to create a series of small in-frame deletions spanning the C terminus of Pol. Such deletions would help delineate amino acid residues not necessary for UL42 binding and could potentially indicate whether the disruptive effect of the three C-terminal insertions was direct or indirect. Accordingly, a set of deletions was created that removed most residues between amino acids 1060 and 1235 of Pol in steps ranging in size from 17 to 45 amino acids, in the background of the large amino-terminal deletion ΔN960. The mutant polypeptides were then tested for their ability to bind UL42 as described above.

Figure 4 shows the results obtained with four such deletions located between amino acids 1059 and 1204. The two more amino-terminal deletions Δ1060-1078 (Fig. 4A) and Δ1078-1115 (Fig. 4B) still coprecipitated with anti-UL42 in amounts not significantly different from the parental polypeptide ΔN960. However, a more C-terminal deletion, Δ1131-1175 (Fig. 4C), indirectly precipitated UL42 with anti-Pol antisera at wild-type levels, but was coprecipitated by anti-UL42 sera in lower amounts, suggesting that it was altered but not totally deficient in binding activity for UL42. Similarly, the next deletion, Δ1175-1203, was also impaired in its ability to bind UL42, but still evidenced an interaction with UL42, as it was reproducibly precipitated by anti-UL42 serum in the presence of UL42, although it did not always reproducibly coprecipitate UL42 with anti-Pol sera (Fig. 4D). However, two C-terminal truncations created by linearizing the plasmid template at the ClaI site of the linker in I-1208 and I-1216 (ΔC1217 and Δ1216, respectively) totally failed to bind UL42 (Fig. 4E, and data not shown).

We also tested the effect of the disruptive C-terminal mutations on UL42 binding when set in the background of full-length polymerase, because although ΔN960 bound UL42 with an efficiency similar to that of the wild-type protein, it was possible that deletion of the amino terminus of the protein increased the apparent severity of the C-terminal mutations. In addition, we wished to assay for the effect of these mutations on polymerase function in vitro and in transfected Vero cells, which required the background of full-length Pol (see below). Therefore, we subcloned the 3' end of the mutant pol genes into a plasmid containing the entire HSV Pol gene under the control of a bacteriophage T3 promoter (pT300 series), to allow the expression of full-length Pol proteins containing the desired mutation by in vitro transcription and translation as described above (8). Mutants equivalent to ΔC1207 and ΔC1216 (n-1207 and n-1216, respectively) were created by linearizing the appropriate insertion mutant with ClaI and then linker tailing with an oligonucleotide containing stop codons in all three reading frames as described in the Materials and Methods. When
DIGARD described in binding ability, the tested, from reduced when greatest the UL42 mutants, insertion formation, culminating downstream had results also To both we ratio, polymerase-to-template in activity primed singly for those seen in the background of ΔN960, in that I-1208, I-1216, F-1208, n-1207, and n-1216 showed very little UL42-binding ability, while I-1203 was less impaired, but clearly reduced from wild-type levels (8).

The effects and locations of the complete set of deletions are summarized in Fig. 3, where it can be seen that like the insertion mutants, the more carboxy-terminal mutations had the greatest effect. The very large amino-terminal deletion ΔN1075 retained some UL42-binding function, while Pol mutants lacking amino acids between 1059 and 1116 bound UL42 at apparently wild-type levels. Deletions further downstream had a progressively greater impact on complex formation, culminating in C-terminal truncations as short as 19 amino acids which totally failed to bind UL42. These results also show that the C terminus of Pol is crucial for binding UL42.

**DNA polymerase activity on singly primed M13 templates.** To attempt to correlate UL42 binding with polymerase function, we next tested selected mutant Pol proteins for both intrinsic DNA polymerase activity and polymerase activity in the presence of UL42. Previous work has shown that Pol alone synthesizes relatively short products on a singly primed single-strand DNA template when at a low polymerase-to-template ratio, but will synthesize much longer products in the presence of UL42, and that this can be taken as an indication of the processivity of the enzyme (16, 18). Therefore, based on a previous observation that in vitro-translated Pol is enzymatically active (12), the mutant Pol polypeptides were expressed in rabbit reticulocyte lysate and then tested for DNA polymerase activity in the presence or absence of UL42 on a uniquely primed single-strand circular DNA template. Newly synthesized single-strand DNA was monitored by autoradiography after fractionation on an alkaline agarose gel. Figure 5 shows the results obtained with wild-type Pol, either purified from an overexpression system or expressed in reticulocyte lysate, and the mutants I-1131 and F-1208. Both purified Pol and the reticulocyte lysate expressing wild-type Pol synthesized predominantly short-length DNA of around 100 to 300 nucleotides in the absence of UL42 (lanes 1 and 5), but synthesized significantly longer products, including a small amount of full-length M13, when 80 fmol of purified UL42 was added to the reactions (Fig. 5, lanes 2 and 6). Therefore, the assay displays a clear distinction between the size of DNA products synthesized by Pol with and without UL42. Consistent with its apparently wild-type behavior in the heterodimerization assay, the Pol mutant containing an insertion at

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**FIG. 4.** Internal deletion analysis of the UL42-binding domain. The indicated Pol deletions were tested for their UL42-binding ability as described in the text and the legend to Fig. 1. Panels D and E are taken from higher-percentage gels than panels A to C.

**FIG. 5.** DNA polymerase activity of Pol mutants containing lesions in the UL42-binding domain. Purified Pol (lanes 1 and 2), or reticulocyte lysate programmed with water (lanes 3 and 4), or mRNAs encoding the indicated Pol proteins (lanes 5 to 10) were tested for polymerase activity on a singly primed M13 single-stranded DNA template in either the presence (+) or absence (−) of 80 fmol of purified UL42. Newly synthesized single-stranded DNA was monitored by autoradiography after fractionation on an 1.3% alkaline agarose gel. The migration of full-length M13 is indicated.
TABLE 2. Ability of selected Pol mutants to complement a pol null mutant virus in a transfection assay

<table>
<thead>
<tr>
<th>Pol mutant</th>
<th>% Complementation</th>
</tr>
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<tbody>
<tr>
<td>I-1060</td>
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</tr>
<tr>
<td>I-1078</td>
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</tr>
<tr>
<td>I-1088</td>
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</tr>
<tr>
<td>I-1091</td>
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</tr>
<tr>
<td>I-1115</td>
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</tr>
<tr>
<td>I-1175</td>
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</tr>
<tr>
<td>I-1203</td>
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<tr>
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</tr>
<tr>
<td>I-1216</td>
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</tr>
<tr>
<td>F-1208</td>
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</tr>
<tr>
<td>n-1207</td>
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<tr>
<td>n-1216</td>
<td>0.67</td>
</tr>
<tr>
<td>No plasmid</td>
<td>0.67</td>
</tr>
</tbody>
</table>

* Values are calculated as a percentage relative to the complementation seen with the wild-type gene. All results are the average of at least three separate experiments.

**: Figures in boldface are considered to be significantly lower than wild-type complementation.

amino acid 1131 also synthesized longer DNA products in the presence of UL42 (Fig. 5, lanes 7 and 8). However, the mutant F-1208 only synthesized short products irrespective of added UL42 (Fig. 5, lanes 11 and 12), which is consistent with its failure to bind UL42, and indicative of the importance of a functional interaction between the two proteins. To control for possible instability of the F-1208 polypeptide, aliquots of the Pol assays were analyzed by SDS-polyacrylamide gel electrophoresis after DNA synthesis had been terminated, but no degradation of the expressed polypeptide was detected (8).

**Transient complementation of a pol null mutant.** The hypothesis that the Pol-UL42 interaction is necessary for viral replication predicts that mutants deficient in UL42 binding should also be impaired for growth. Therefore, we examined the effects of some of the pol mutations described above in virus-infected cells by testing their ability to complement the replication of a virus lacking an intact pol gene. Accordingly, Vero cells were transfected with plasmids containing the full-length mutant pol genes and superinfected with a defective virus containing a deleted pol gene (HP66 [29]), and the titers of the resulting progeny were determined on a cell line permissive for growth of the defective virus. HP66 cannot replicate without an exogenously supplied Pol (29), so any resultant progeny in this assay resulted from functional Pol supplied in trans by the transfected plasmids. The results obtained with 10 insertion mutants and 3 of the deletion mutants are presented in Table 2. In general, the data are consistent with the in vitro analysis of UL42 interaction. The majority of the linker insertion mutants which had no observable defect in UL42 binding also significantly complemented the growth of HP66. Conversely, the insertion mutation at amino acid 1208 failed to complement replication of the defective virus to wild-type levels, which is consistent with its greatly reduced ability to bind UL42. Similarly, both of the small C-terminal truncations, n-1207 and n-1216, and the frameshift mutant F-1208 failed to complement the pol null mutant. This is also consistent with the inability of F-1208 to synthesize longer DNA products in the presence of UL42 (Fig. 5). In addition, immunofluorescence experiments showed that both n-1207 and F-1208 were expressed and localized to the nucleus (8), indicating that their replication incompetence was not attributable to instability or incorrect cellular localization. Therefore, these data support the hypothesis that the Pol-UL42 interaction is necessary for viral replication. However, the insertion mutants I-1203 and I-1216, which were at least partially defective in interacting with UL42, did complement viral growth, while two insertion mutants, at positions 1059 and 1175, with no apparent defect in heterodimer formation failed to complement HP66. These results will be discussed below.

**DISCUSSION**

The C-terminal 35 amino acids of Pol are critical for UL42 binding. Previously, we had shown that UL42 stably bound to the C-terminal 228 amino acids of the HSV DNA polymerase (10). Here, we present experiments that further delineate the residues of Pol involved in association with UL42. The analysis of 11 separate four-amino-acid insertions within this region suggested that the area downstream of amino acid 1203 was especially crucial for UL42 binding. In confirmation of this, individual small deletions of all but the C-terminal 35 amino acids of Pol were tolerated without totally abolishing complex formation. Sequences upstream of these 35 amino acids clearly play a role in UL42 binding, as indicated by the lessened coprecipitation efficiencies shown by ΔN1075 and Δ1114-1132, for example. However, as upstream sequences seem insensitive to insertion mutagenesis and display no residual binding activity in the absence of the extreme C terminus, we suggest they are involved indirectly, possibly inducing the correct folding or presentation of the actual protein-protein contact area. In contrast to the relatively small effect of the more-upstream deletions, the loss of as few as 19 amino acids from the C terminus totally abolished the ability to bind UL42.

The interpretation of results obtained with the C-terminally truncated mutants is complicated by their construction from insertion mutants which were themselves defective for UL42 binding. However, we note that while I-1216, ΔC1216, and n-1216 were all impaired for UL42 binding in the complementation assay, ΔC1216 appeared more defective for UL42 binding than the parental I-1216, and similarly, n-1216 was clearly more severely affected than I-1216 as it failed to complement replication of a pol null mutant. Therefore, the overall results suggest that UL42 binds to the extreme C terminus of Pol, with the last 35 or so amino acids strongly implicated as containing the actual site of interaction.

**Possible structures for the C terminus of Pol.** The Robson-Garnier secondary structure prediction algorithm predicted a predominantly alpha-helical structure for the final 35 amino acids of Pol (Fig. 6A), interrupted by a poorly predicted region centered on four glycine residues (residues 1210 to 1217). In addition, the last 35 amino acids also contain a heptad hydrophobic repeat (shaded in Fig. 3), composed of three leucines and two alanines. Residues at the i + 3 position are also largely hydrophobic, which, taken with the secondary structure prediction, is suggestive of an amphipathic helix. Figure 6B shows the amino acid sequence plotted out on a helix-wheel diagram, where it can be seen that one face of the putative helix is mainly hydrophilic, while the other face contains several polar and charged residues. Also indicated are five potential helix-stabilizing salt bridges between oppositely charged residues in i, i + 3, or i + 4 positions (24, 30). Therefore, it is possible Pol and UL42 associate through a coiled-coil hydrophobic interaction, similar to that observed in fibrous proteins such as...
myosin (6), or more recently in the leucine zipper class of DNA-binding proteins (24, 34, 35). One prediction of such a hypothesis is that a corresponding amphipathic helix must be present in UL42, and indeed, a region of predicted alpha-helix with a 3-4 hydrophobic repeat exists between amino acids 239 and 256 of the protein. Alternatively, the postulated amphipathic helix on Pol may bind to a hydrophobic patch or pocket on UL42, as proposed for the interaction between calmodulin and a variety of peptide and protein ligands (33). A third possibility of a kinked alpha-helix, perhaps involved in a conformational change upon binding UL42, is raised by the four glycine residues between two well-predicted helices. In this respect, it is interesting that the HSV-2 Pol contains a proline residue within the region containing the glycine residues (41). At an extreme, the two helices could form a hairpin structure with each other stabilized by a hydrophobic interface, as proposed for the C terminus of bacteriophage T4 gene 32 protein (2). This would suggest that the interaction with UL42 itself might be hydrophobic, based on the exterior surface of the amphipathic helix. Further mutagenesis of Pol and UL42 should be able to test these possibilities (9).

Implications for development of antiviral drugs. Despite the efficacy of a variety of nucleoside analog-based antiviral drugs, most notably acyclovir, there is growing interest in the possibility of rationally designed inhibitors aimed at blocking or interfering with protein-protein interactions. Such a strategy is generally initiated with peptides designed to mimic an interface involved in the association of the target polypeptides. Examples of this approach include peptide-based inhibitors of the two-subunit HSV ribonucleotide reductase (7, 13), the M-NP association in influenza virus (45), and the Oct-1–VP16 complex of HSV (17). If a particular protein complex is to provide a suitable target for this sort of intervention, the interaction must perform an essential function. With the HSV Pol–UL42 interaction, both polypeptides have previously been shown to be required for viral growth, and UL42 had been shown to alter the catalytic properties of Pol, so it seemed likely that formation of the heterodimer was essential. Here, we present experiments which strongly support this hypothesis. While several Pol C-terminal insertion mutants with little or no defect in UL42 binding could also complement a pol null mutant in trans to close to wild-type levels, the four mutants most severely impaired for UL42 binding did not (1-1208, F-1208, n-1207, and n-1216). We also demonstrated that the mutant F-1208 retained polymerase activity in vitro, but could not synthesize longer DNA products in the presence of UL42. In addition, the failure of F-1208 to complement replication of the pol-deficient virus was unlikely to have resulted from instability or incorrect intracellular localization of the protein, as immunofluorescence experiments revealed nuclear staining (8). Therefore, as the only demonstrated defect in F-1208 is its failure to interact correctly with UL42, it is likely that this accounts for its inviability in vivo. This lends credence to the hypothesis that the Pol-UL42 interface is a valid target for the design of novel therapeutic agents. In support of this, preliminary experiments have shown that peptides corresponding to the C terminus of Pol inhibit processive synthesis by the holoenzyme (11), and similarly, UL42-based peptides also inhibit DNA synthesis (31).

In general, the ability of the mutants to bind UL42 in the
immunoprecipitation assay correlated well with their phenotype in the in vivo complementation assay. However, two of the insertion mutants, I-1203 and I-1216, significantly complemented infected replication of a J subunit mutant despite a markedly decreased ability to bind UL42 in vitro. These mutants also synthesized some longer DNA products in the presence of UL42 on a primed single-stranded DNA template (8). We feel that this apparent contradiction in phenotype most likely reflects the degree of impairment seen and the relatively high stringency of the immunoprecipitation assay. Although impaired, I-1203, and to a lesser extent I-1216, retained some ability to form a complex with UL42, while during the immunoprecipitation assay, the association between the two polypeptides has to be of sufficient avidity to survive the presence of antibody, detergents, and a low overall protein concentration. In fact, some components of the bacteriophage T4 DNA replication complex have been shown to associate stably only in the presence of a high macromolecular concentration (21).

Is UL42 binding sufficient to confer processivity? Interestingly, two of the insertion mutants (I-1060 and I-1175) failed to complement replication of the pol-defective virus even though they bound UL42 in vitro at apparently wild-type levels. This may result simply from instability of the mutant proteins in vivo or from defects in Pol function not assayed for in this study. However, although the experiments presented here provide evidence that the Pol-UL42 association is essential for polymerase function in vitro and in vivo, they do not directly address whether merely binding UL42 is sufficient to increase the processivity of the enzyme. That UL42 binds to double-stranded DNA suggests the attractive hypothesis that it increases the processivity of Pol by clamping it to the primer template (16) in a manner analogous to but mechanistically distinct from that of the β-subunit of *Escherichia coli* Pol III (the β-subunit has no intrinsic affinity for DNA [23, 39]). Nevertheless, it is possible that UL42 also induces an alteration in how Pol itself contacts DNA (or Pol induces an alteration in UL42) which contributes to the processivity of the enzyme, and that simple addition of the UL42 DNA-binding activity is not sufficient. The recent finding that the C terminus of Pol contains a DNA-binding site (43) provides a functional basis for this proposal.

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