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Deletions of the Carboxy Terminus of Herpes Simplex Virus Type 1 UL42 Define a Conserved Amino-Terminal Functional Domain

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The herpes simplex virus type 1 UL42 protein was synthesized in reticulocyte lysates and assayed for activity in vitro. Three functional assays were used to examine the properties of in vitro-synthesized UL42: (i) coimmunoprecipitation to detect stable complex formation with purified herpes simplex virus type 1 DNA polymerase (Pol), (ii) a simple gel-based assay for DNA binding, and (iii) a sensitive assay for the stimulation of Pol activity. UL42 synthesized in reticulocyte lysates formed a stable coimmunoprecipitable complex with Pol, bound to double-stranded DNA, and stimulated the activity of Pol in vitro. Carboxy-terminal truncations of the UL42 protein were synthesized from restriction enzyme-digested UL42 gene templates and gene templates made by polymerase chain reaction and assayed for in vitro activity. Truncations of the 488-amino-acid (aa) UL42 protein to aa 315 did not abolish its ability to bind to Pol and DNA or to stimulate Pol activity. Proteins terminating at aas 314 and 313 showed reduced levels of binding to Pol, but these and shorter proteins were unable to bind to DNA or to stimulate Pol activity. These results suggest that all three of the biochemical functions of UL42 colocalize entirely within the N-terminal 315 aas of the UL42 protein. Amino acid sequence alignment of alpha herpesvirus UL42 homologs revealed that the N-terminal functional domain corresponds to the most highly conserved region of the protein, while the dispensable C terminus is not conserved. Conservative aa changes at the C terminus of the 315-aa truncated protein were used to show that conserved residues were important for activity. These results suggest that 173 aa of UL42 can be deleted without a loss of activity and that DNA-binding and Pol-binding activities are correlated with the ability of UL42 to stimulate Pol activity.

The herpes simplex virus type 1 (HSV-1) double-stranded DNA genome is 152 kb long and encodes over 70 open reading frames (21). Three origins of replication within the genome are believed to direct replication by a rolling-circle mechanism (reviewed in reference 2). Seven virally encoded proteins have been found to be necessary and sufficient for the replication of HSV-1 origin of replication-containing plasmids within mammalian (31) or insect (26) cells and to be required for HSV replication and DNA synthesis in infected cells (reviewed in reference 30). These seven proteins include a DNA polymerase catalytic subunit, Pol or UL30; its accessory factor, UL42; a single-stranded DNA-binding protein, ICP8 or UL29; a complex of three proteins, UL5, UL8, and UL52, constituting a helicase-primase; and a sequence-specific origin-binding protein, UL9.

Early efforts to isolate Pol from infected cells resulted in the copurification of a two-subunit protein complex consisting of the catalytic subunit (Pol) and another protein (24, 29). Since that time, studies have shown the other protein to be the product of the UL42 gene (UL42), a previously recognized, abundant 65-kDa DNA-binding protein (8, 20). The Pol-UL42 heterodimer also has been isolated from insect cells coinfected with recombinant baculoviruses expressing each protein (10, 13). The activity (7, 28) and processivity (10, 13) of Pol were found to be increased by UL42 in vitro. UL42 protein homologs from other herpesviruses also have been demonstrated to be associated with and to stimulate the in vitro activity of their respective Pols (6, 15).

Although the UL42 protein has been shown to be absolutely required for HSV-1 DNA replication (14, 19), the structural interaction of Pol and UL42 is relatively undefined. Recent studies have sought to determine the sequences of Pol and UL42 required for complex formation and Pol activity stimulation. With UL42 and Pol proteins synthesized in reticulocyte lysates, the C-terminal region of Pol was found to be necessary and sufficient for the formation of a coimmunoprecipitable complex with UL42 (5). Mutations in the UL42-binding domain of HSV-1 Pol expressed in *Saccharomyces cerevisiae* and during infection have been used to identify sequences required for a functional interaction with UL42 produced in reticulocyte lysates and in vivo (4a, 28).

UL42 produced in reticulocyte lysates was used to demonstrate the stimulation of Pol activity in vitro (7). Additionally, large deletions within the C terminus (239 amino acids [aa]) and N terminus (245 aa) of the 488-aa UL42 protein abolished the ability to stimulate Pol activity (7). These studies revealed that the regions of UL42 responsible for Pol activity stimulation did not reside solely within the N-terminal or C-terminal halves of the protein.

Although the ability of UL42 to bind to Pol, to stimulate Pol activity, and to bind to DNA has been demonstrated in vitro, no thorough studies to date have attempted to determine the regions of UL42 that encode these functions. Here we describe experiments to map the sequences of the UL42

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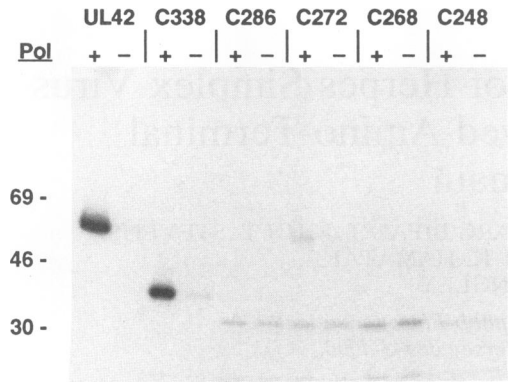

FIG. 1. Coimmunoprecipitation of complexes of Pol and UL42 or C-terminally truncated UL42. Equivalent counts of radiolabeled UL42 or UL42 truncation mutants were incubated with (+) or without (−) rPol, immunoprecipitated with polyclonal rabbit anti-Pol antisera and protein A-Sepharose, and analyzed by PAGE. C-terminal truncations of UL42 are denoted by a C followed by the number of N-terminal aa encoded. The migrations of molecular mass standards (Amersham), run in parallel, are indicated in kilodaltons. The Pol-independent background coimmunoprecipitation of the mutant proteins that failed to bind to Pol is seen as bands migrating at about 30 kDa. The larger protein coimmunoprecipitated from the C272 mutant resulted from a partial digest of the DNA template, while the authentic C272 mutant protein was not coimmunoprecipitated.

protein that are responsible for these biochemical functions and to determine whether these functions map to separate or common domains of the protein. We used reticulocyte lysates to express UL42 and UL42 mutants and assayed their ability to functionally interact with Pol by using three assays: binding to Pol, stimulation of Pol activity, and binding to DNA. We showed that a full one-third of the 488-aa protein sequence at the C terminus is not required for activity in all the in vitro assays and that DNA binding and Pol binding are correlated with the ability to stimulate Pol activity. Additionally, sequences required for activity correlate with those conserved among the known alpha herpesvirus UL42 homologs, suggesting that this boundary is the limit of a conserved functional region.

MATERIALS AND METHODS

Plasmid construction. The HSV-1 strain KOS UL42 gene was excised from pNN4 (31), modified by insertion of a *Bam*HI site and four adenosine residues upstream of the ATG by use of a linker that extended to the *Mlu*I site at nucleotide 287 of the HSV-1 strain 17 UL42 gene (22; GenBank-EMBL accession number 19121; A of ATG = nucleotide 241), and cloned as a *Bam*HI-*Nsi*I (nucleotide 2518) fragment into the *Bam*HI site of pVL1393 to form pRH107. The UL42 gene was further subcloned as a *Bam*HI fragment from pRH107 into the *Bam*HI and *Bgl*II sites of pTZ18U to create pAS600.

Recombinant baculovirus-expressed proteins. Recombinant HSV-1 Pol (rPol) was expressed and purified from recombinant baculovirus-infected cells as described previously (13), except that DNA-cellulose was used in place of heparin-agarose. A recombinant baculovirus containing the UL42 gene was isolated after homologous recombination with plasmid pRH107 (11). The purification and characterization of the recombinant UL42 protein are described elsewhere (11).

Protein expression in reticulocyte lysates. Plasmid pAS600 was linearized with various restriction enzymes, processed, and transcribed by use of T7 RNA polymerase to yield capped runoff RNA as described previously (18). Alternatively, the T7 RNA polymerase promoter was incorporated into 5′ polymerase chain reaction (PCR) primers and used to synthesize transcription templates from the pRH107 UL42 DNA (100 ng) by use of *Taq* polymerase and various 3′ primers. C-terminal truncations of the UL42 protein were produced by use of the following restriction enzymes and yielded the indicated proteins: *Bss*HII, resulting in a protein terminating at aa 402 (C402); *Sfi*I, C338; *Bsa*I, C331; *Eco*57I, C326; *Age*I, C303; *Mbo*II, C286; *Sph*I, C272; *Sal*I, C268; *Not*I, C248; *Hpa*I, C206; *Sty*I, C185; *Nhe*I, C151; and *Nde*I,

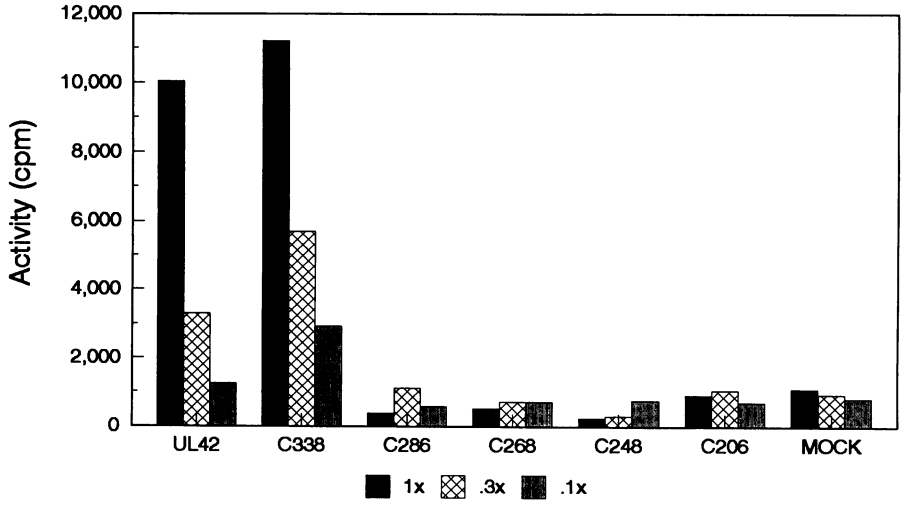

FIG. 2. HSV-1 Pol activity stimulation by UL42 or C-terminally truncated UL42. Threefold dilutions of equimolar amounts of radiolabeled UL42 or UL42 truncation mutants were assayed for stimulation of the activity of 30 ng of rPol with a poly(dA) · oligo$(dT)_{20}$ template. One microliter of UL42 was used as 1×. MOCK, mock translation in the absence of added RNA. The values presented are Cerenkov determinations of [^{32}P]dTTP incorporated.

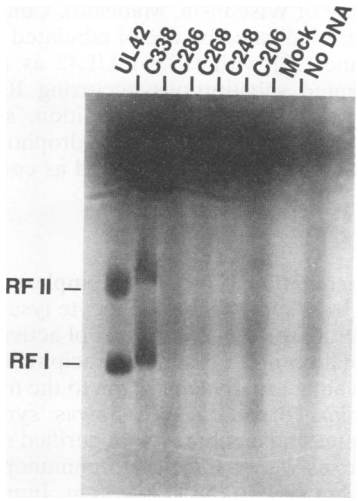

FIG. 3. DNA binding by UL42 or C-terminally truncated UL42. Equivalent counts of radiolabeled UL42 or UL42 truncation mutants in reticulocyte lysates were assayed for binding to φX174 RF DNA. Following incubation of the protein and DNA, the mixture was assayed by agarose gel electrophoresis. The positions of ethidium bromide-stained supercoiled (RFI) and relaxed (RFII) forms of DNA are indicated. Mock, mock translation in the absence of added RNA. No DNA, radiolabeled UL42 in the absence of added φX174 RF DNA.

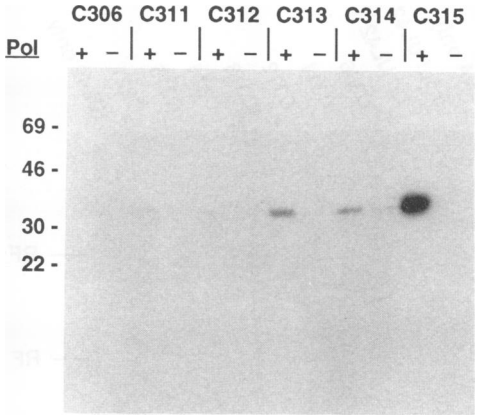

FIG. 4. Coimmunoprecipitation of complexes of Pol and UL42 or C-terminally truncated UL42 made with PCR-generated templates. Coimmunoprecipitation of equivalent counts of radiolabeled UL42 proteins made with PCR-generated transcription templates was assayed as described in the legend to Fig. 1.

C139. The presence in the KOS strain DNA used in these studies of all relevant restriction sites in the sequenced HSV-1 strain 17 DNA extending from the *Hpa*I site to the *Sfi*I site (see above) was confirmed (data not shown). Templates that were generated with enzymes producing 3′ overhangs were made blunt by use of the Klenow fragment of *Escherichia coli* DNA polymerase I. All other truncations were made by use of the PCR with 5′ UL42 primers containing the T7 RNA polymerase promoter 5′-TAATAC GACTCACTATAGGGAGA-3′, as detailed by Sarkar and Sommer (25), followed by a consensus eukaryotic translation initiation signal (CCACCATG; 17) and the first 18 nucleotides of the UL42 gene. For in vitro synthesis of UL42, 4 to 10 μl of RNA was translated in 50 μl of rabbit reticulocyte lysates (Promega) containing 40 μCi of [^{35}S]methionine for 90 min at 30°C.

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the addition of 2× protein sample buffer (6% SDS, 10% β-mercaptoethanol, 100 mM Tris-HCl [pH 6.8], 0.02% bromphenol blue, 20% glycerol), boiling for 3 min, and electrophoresis in precast SDS-polyacrylamide gels (ISS, Hyde Park, Mass.). Following electrophoresis, the gels were fixed in 5% methanol–7.5% acetic acid (15 min), rinsed in H_2O (10 min), soaked in 1 M salicylate (15 min), dried, and autoradiographed. Proteins were analyzed by PAGE and quantitated on a Betascope 603 blot analyzer (Betagen, Waltham, Mass.). Equivalent amounts of each UL42 mutant were then added to functional assays.

Coimmunoprecipitation of rPol and radiolabeled UL42. Complexes between UL42 synthesized in vitro and 30 ng of rPol were formed for 90 min on ice in the presence of an approximately 15-μl total reaction mixture volume (50 mM NaCl, 500 μg of bovine serum albumin [BSA] per ml, 50 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM dithiothreitol). rPol was used in molar excess to ensure quantitative coimmunoprecipitation of UL42 that was able to bind to rPol. Complexes were immunoprecipitated by the addition of 100 μl of IP buffer (0.1% Nonidet P-40, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.6], 0.02% Na azide) and 2 μl of polyclonal rabbit anti-Pol antisera. The tubes were incubated on ice for 60 min, 35 μl of protein A-Sepharose (10% [wt/vol]; Pharmacia) was added, and the samples were rocked at 4°C for 15 min. The precipitates were washed once with 750 μl of IP buffer and twice with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl [pH 7.5]) and analyzed by SDS-PAGE. The maximum level of complex formation was achieved after 90 min of coincubation of rPol and radiolabeled UL42 and was not increased by further incubation to 24 h (data not shown). Increasing either the amount of [^{35}S]methionine in the UL42 translations or the amount of rPol in the coincubations did not result in a significant augmentation of complex coimmunoprecipitation.

DNA binding by radiolabeled UL42. Equivalent counts of UL42 protein were added to 0.5 μg of φX174 replicative-form (RF) DNA (Bethesda Research Laboratories) in 1.4 μl of 10 mM Tris (pH 7.4)–1 mM EDTA. The mixture was incubated on ice for 30 min to allow binding to occur. One microliter of 6× DNA loading buffer (Ficoll [15%, wt/vol], bromphenol blue [0.25%, wt/vol], xylene cyanol [0.25%, wt/vol]) was added, and the samples were electrophoresed in 0.8% agarose–TBE (90 mM Tris, 90 mM boric acid, 1 mM EDTA) gels at 70 V. The migration of the supercoiled (RFI) and relaxed (RFII) forms of DNA was determined by ethidium bromide staining. The gels were washed briefly in H_2O, dried, and autoradiographed.

Pol activity stimulation. Equimolar amounts of radiolabeled UL42 or truncated UL42 were mixed with 30 ng of rPol and incubated for 15 min on ice in a 15-μl total reaction mixture volume (50 mM NaCl, 500 μg of BSA per ml, 50 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM dithiothreitol), and then 35 μl of reaction buffer {10 μg of poly(dA) · oligo(dT)$_{20}$ per ml, 100 mM NH_4SO_4, 10 μM [^{3}H]dTTP (540 cpm/pmol) or [^{32}P]dTTP (3,000 Ci/mmol), 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of BSA per ml, 50 mM Tris-HCl (pH 8.0)} was added. Poly(dA) · oligo(dT)$_{20}$ (Pharmacia) was used at a 10:1 weight ratio, resulting in approximately one primer per 200 bases of template. After 45 min of incubation at 37°C, the

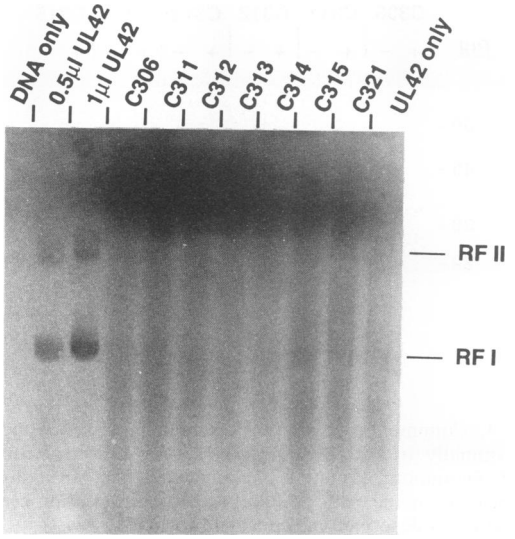

FIG. 5. DNA binding by UL42 or C-terminally truncated UL42 made with PCR-generated templates. Equivalent counts of UL42 truncation mutants were assayed for DNA binding as described in the legend to Fig. 3. The positions of ethidium bromide-stained supercoiled (RFI) and relaxed (RFII) forms of DNA are indicated. DNA only, ϕX174 RF DNA in the absence of added protein. UL42 only, radiolabeled UL42 in the absence of added ϕX174 RF DNA.

reaction was quenched by the addition of 50 μl of 10% ice-cold trichloroacetic acid, and the reaction mixture was incubated on ice for 10 min, filtered through GFC filters, washed twice with 1 N HCl and once with absolute ethanol, and counted with scintillation fluor.

Alignment of alpha herpesvirus UL42 protein homologs. The sequences of alpha herpesvirus UL42 protein homologs were obtained by translation of sequences available in the GenBank-EMBL data base (HSV-1 UL42 [22], varicella-zoster virus gene 16 protein [4], equine herpesvirus open reading frame 18 protein [27]). Protein sequences were aligned by use of the PileUp program (Genetics Computer Group, University of Wisconsin, Madison). Conservative aa substitutions were indicated by use of tabulated values from Harlow and Lane (12) with HSV-1 UL42 as the parental sequence. Accepted substitutions occurring 10% or more were indicated as conservative. In addition, substitutions involving an interchange of the bulky hydrophobic residues M, F, L, I, V, and Y were also indicated as conservative.

RESULTS

Coimmunoprecipitation of Pol-UL42 complexes. Coexpression of HSV-1 UL42 and Pol in reticulocyte lysates has been used to demonstrate the stimulation of Pol activity by UL42 (7) and the formation of a coimmunoprecipitable Pol-UL42 complex (5). As an alternative approach to the measurement of stable complex formation, UL42 was synthesized in reticulocyte lysates and incubated with purified rPol, and the resulting complexes were subjected to immunoprecipitation with polyclonal antisera to the Pol protein. Immunoprecipitation of radiolabeled UL42 was dependent on the presence of Pol in the reaction (Fig. 1), was unaffected by the presence of a heterologous protein (500 μg of BSA per ml), and could be inhibited by competition with excess unlabeled UL42 (data not shown). Pol-dependent coimmunoprecipitation of UL42, therefore, was indicative of a specific interaction between the two proteins.

To define the C-terminal limits of the region of UL42 responsible for binding to HSV-1 Pol, we assayed UL42 mutants synthesized from truncated gene templates for the ability to be coimmunoprecipitated with Pol. Figure 1 shows representative results for some of the truncation mutants. Truncation to aa 338 (C338) or aa 402 (data not shown) did not affect the ability of UL42 to form complexes with rPol; however, more extensive truncation of the protein (C286, C272, C268, or C248) prevented the formation of a stable complex (Fig. 1). The long autoradiographic exposure shown in Fig. 1 was used to demonstrate that the same background levels of coimmunoprecipitation were apparent in both the presence and the absence of Pol with proteins that were unable to bind to Pol. These same background levels of coimmunoprecipitation were observed with protein

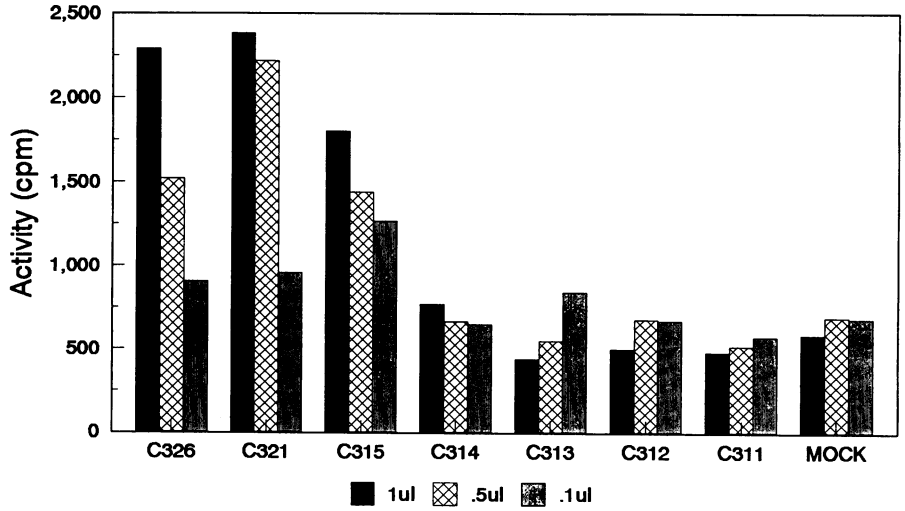

FIG. 6. HSV-1 Pol activity stimulation by UL42 truncation mutants made with PCR-generated templates. Pol assays were carried out as described in the legend to Fig. 2, except that proteins were unlabeled, [^{3}H]dTTP (540 cpm/pmol) was used for labeling instead of [^{32}P]dTTP, and samples were counted with scintillation fluor.

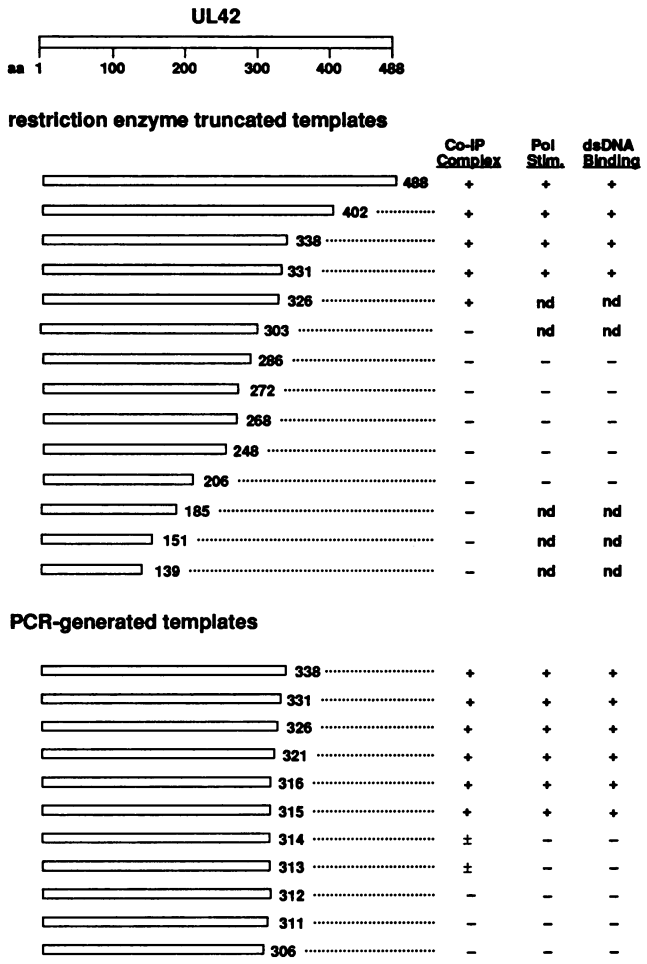

FIG. 7. Results of the mapping of UL42 functional domains in vitro. Shown is a summary of the results of in vitro experiments with C-terminally truncated mutants of UL42 made by restriction enzyme digestion of UL42 gene templates (top) or made by the PCR (bottom). Positive (+) or negative (−) results or positive activity at levels markedly reduced from that of full-length UL42 (±) is indicated. nd, not determined. Co-IP, coimmunoprecipitated; Stim., stimulation; ds, double stranded.

A-Sepharose alone, in the absence of antisera (data not shown). Quantitation of specifically coimmunoprecipitated UL42 proteins revealed that the same percentages of wild-type and C338 proteins coimmunoprecipitated with Pol, suggesting that both proteins have similar binding affinities for Pol.

Pol binding is correlated with the ability to stimulate Pol activity. To determine whether the stimulation of Pol activity by UL42 correlates with Pol binding and whether the regions encoding these activities colocalize within the protein, we assayed for the stimulation of Pol activity in vitro. Pol activity on short stretches of single-stranded, primed templates, such as activated calf thymus DNA, can be stimulated three- to fivefold by UL42 (7, 28). Templates on which more processive Pol activity can occur, such as singly primed M13 phage DNA (10, 13) and synthetic templates with a high ratio of single-stranded to double-stranded DNA, can be used to demonstrate 10- to 100-fold stimulation of Pol activity by UL42. We have observed up to 100-fold stimulation of Pol activity by UL42 by using proteins purified from recombinant baculovirus-infected cells and a poly(dA) · oligo(dT)$_{20}$ template DNA (11). We therefore used this template in a sensitive assay for Pol activity stimulation by UL42 mutants made in vitro.

Full-length UL42 synthesized in vitro stimulated the activity of 30 ng of rPol in a concentration-dependent fashion at least 10-fold on poly(dA) · oligo(dT)$_{20}$ DNA (Fig. 2). Higher levels of stimulation could not be achieved because of the inhibition of Pol activity by the addition of more than 1 to 2 μl of reticulocyte lysate to the reaction. No stimulation was observed when a reticulocyte lysate not containing UL42 was added.

Pol activity stimulation by UL42 proteins was directly correlated with the ability to coimmunoprecipitate with Pol (Fig. 2); whereas C338 retained activity equivalent to that of full-length UL42, C286 was inactive. These results suggest that the Pol-binding and Pol activity-stimulating activities colocalize within the N-terminal 338 aa of UL42.

DNA-binding activity of amino-terminal sequences of UL42. Other DNA polymerase accessory proteins that serve as processivity factors, such as the beta subunit for *E. coli* DNA polymerase III or proliferating cell nuclear antigen for human delta polymerase, are thought to form oligomeric structures that bind to polymerase and encircle the DNA, thereby acting as a sliding clamp during DNA synthesis (16). Unlike UL42, however, these proteins do not possess intrinsic DNA-binding activity. Nevertheless, the DNA-binding activity of UL42 may contribute to its function in the stimulation of Pol processivity. To determine whether the sequences of UL42 required for binding to DNA could be separated from those required for a functional interaction with Pol, we determined DNA binding by the UL42 mutants in a novel gel-based assay. When full-length UL42 synthesized in vitro was incubated with ϕX174 RF DNA, a DNA-UL42 complex was demonstrable by coelectrophoresis of the radiolabeled UL42 with the DNA (Fig. 3). UL42 was electrophoresed in the absence of DNA, and mutants unable to bind to DNA did not migrate as distinct species but rather migrated as diffuse smears within the agarose gel. Only the UL42 mutants that bound to Pol and stimulated Pol activity were found to bind to DNA (Fig. 3). As with the coimmunoprecipitation and stimulation results, the level of DNA binding by C338 was equivalent to that obtained with an equimolar amount of the full-length protein (data not shown).

The N-terminal 338 aa of UL42 were fully functional in vitro, while the C-terminal 150 aa were dispensable for activity. Truncation of UL42 to aa 286 abolished all three in vitro UL42 activities, indicating that sequences between aa 338 and 286 were required for these activities. Stimulation of Pol activity by the UL42 mutants therefore correlated directly with binding to both DNA and Pol.

Precise definition of the amino-terminal functional domain of UL42. Further experiments with UL42 gene templates truncated by restriction enzyme digestion revealed that C326 complexed with rPol but that C303 and smaller N-terminal deletion proteins did not. To map more precisely the C-terminal limits of the region of UL42 responsible for binding to HSV-1 Pol and to attempt to separate further the three functional activities, we adopted a strategy for synthesizing transcription templates independently of the location of restriction enzyme cleavage sites. UL42 gene truncations were synthesized by the PCR with a 5′ PCR primer containing the T7 RNA polymerase promoter sequence (25) and various 3′ primers within the gene to generate proteins with

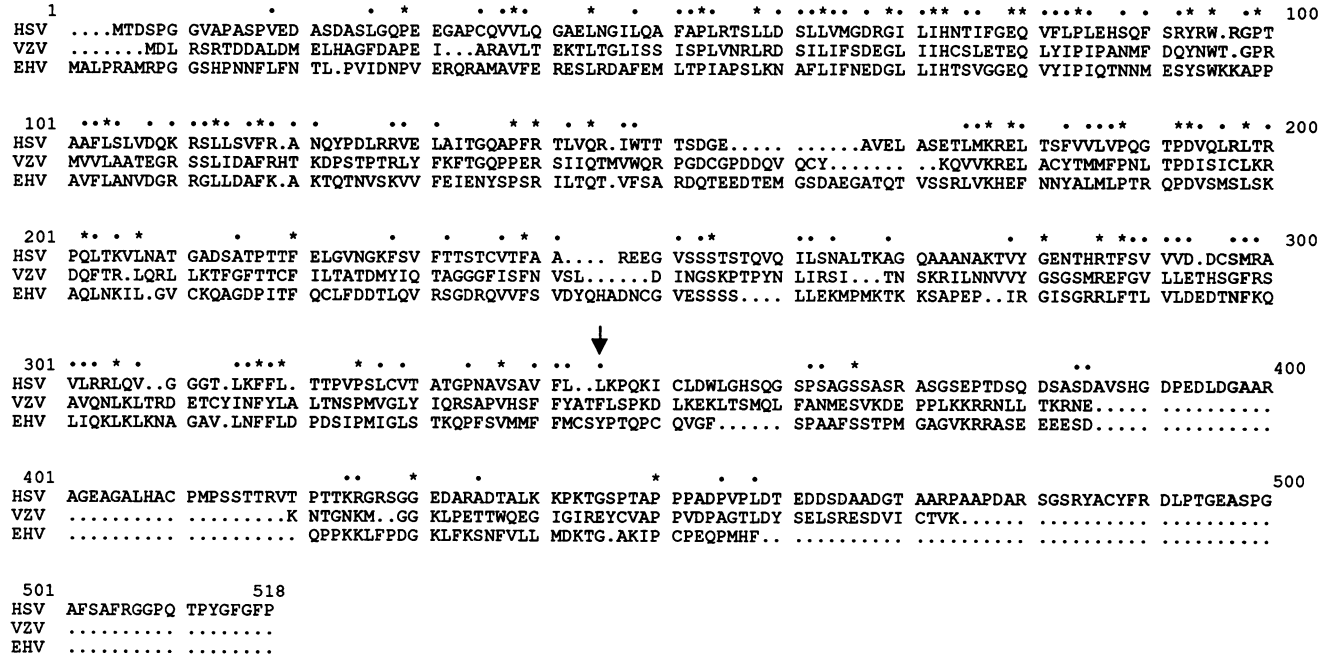

FIG. 8. Alignment of DNA polymerase accessory protein sequences of alphaherpesviruses. Shown are the aa sequences of HSV-1 UL42 (22) and the homologous varicella-zoster virus gene 16 protein (VZV [4]) and equine herpesvirus open reading frame 18 protein (EHV [27]). Protein sequences were aligned by use of the PileUp program. Identical aa are indicated by an asterisk over the sequence, while conservative substitutions in three homologs, as defined in Materials and Methods, are noted by a dot. The vertical arrow marks the position of aa 315 of HSV-1 UL42.

precise C termini. Truncation mutants C331, C326, C321, and C316 formed stable complexes with Pol, while C311, C306, or C301 failed to be coimmunoprecipitated (data not shown). Further analysis with truncations at each aa within aa 315 to 311 revealed that C315 was efficiently coimmunoprecipitated, while shorter mutants (C314 and C313) were abrogated in their ability to bind to Pol (Fig. 4), exhibiting approximately one-third of the binding capacity of full-length UL42 (data not shown). UL42 truncated at aa 312 (C312) or shorter proteins were not coimmunoprecipitated with Pol (Fig. 4).

The results of DNA-binding assays with the PCR-generated UL42 truncations were similar to those of the coimmunoprecipitations. C321 and C315 bound to DNA, as indicated by comigration of radiolabeled protein with ϕX174 DNA RFI and RFII. DNA binding could not be detected, however, with equivalent amounts of C314 or shorter proteins (Fig. 5).

Results from Pol activity stimulation assays also indicated that C315 was the shortest functional UL42 mutant protein (Fig. 6). The stimulation of Pol activity as well as DNA binding (see above) obtained with these mutants was reduced compared with that obtained with UL42 synthesized from restriction enzyme-digested templates. This result was due to the lower yields of UL42 synthesized in the reticulocyte lysates following transcription of PCR-generated templates and limitations as to the amount of reticulocyte lysate that can be assayed in the absence of interference. Appreciable amounts of full-length UL42 protein could not be synthesized by the PCR method; therefore, a direct comparison with these shorter proteins was not possible. In two separate experiments, however, C315 resulted in approximately 75% the stimulation of Pol activity that was seen with C321 generated by the PCR method (e.g., Fig. 6).

The PCR was also used to generate N-terminal truncations of the UL42 active domain (ending at aa 315) in vitro for the assay of Pol-binding activity. Although the N-terminal 18 aa of UL42 could be deleted without a loss of binding to Pol, further deletion of the N-terminal 31, 61, or 91 aa resulted in proteins that did not coimmunoprecipitate (data not shown). Larger N-terminal deletions could not be analyzed in the coimmunoprecipitation assay because of unacceptable levels of nonspecific binding by the proteins (data not shown). Therefore, although a large portion of the C terminus of UL42 is dispensable for Pol-binding activity, very little of the N terminus can be deleted.

The results of functional analyses of the UL42 mutant proteins synthesized in vitro are summarized in Fig. 7. The three activities of UL42 are encoded in the N-terminal 315 aa of the protein, and 173 aa at the C terminus are dispensable for in vitro activity. The reduced activity of UL42 aa 1 to 313 and 1 to 314 in coimmunoprecipitation assays was accompanied by a complete loss of detectable activity in the DNA-binding and Pol activity stimulation assays.

The sequence near aa 315 is important for UL42 activity. Alignment of the available sequences of UL42 homologs from other alphaherpesviruses was performed to ascertain whether the functional region of UL42 contained conserved residues. The sequence alignment (Fig. 8) revealed a significant amount of sequence conservation in the N-terminal two-thirds of the proteins, whereas a marked lack of homology was observed for the C-terminal region, distal to aa 313 to 315 in HSV-1 UL42. Notably, the most highly conserved sequences of UL42 also represent the functional region of the protein and may represent a distinct structural domain. The pseudorabies virus UL42 homolog also displays a high level of homology with HSV-1 UL42 in the N-terminal functional domain (1). The N-terminal portions of the beta-

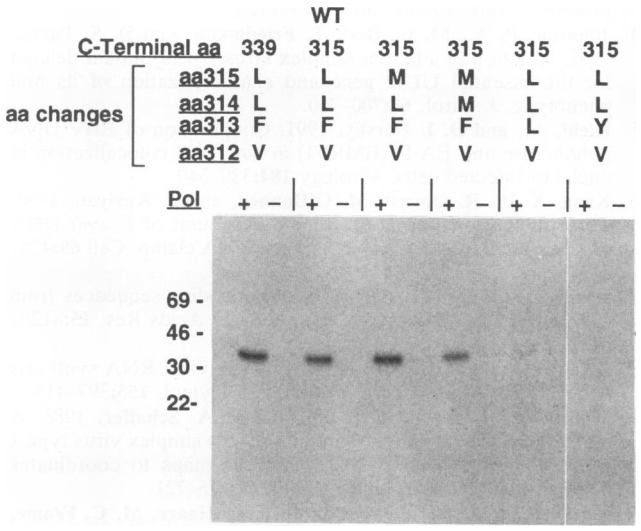

FIG. 9. Coimmunoprecipitation of UL42 truncation mutants with terminal substitutions. Coimmunoprecipitation of equivalent counts of radiolabeled UL42 or UL42 truncation mutants made with PCR-generated transcription templates was assayed as described in the legend to Fig. 1. The row marked C-terminal aa indicates the number of aa synthesized, beginning at aa 1. The rows marked aa changes show the aa sequences at residues 312 to 315 (M, methionine; L, leucine; V, valine; F, phenylalanine; Y, tyrosine). WT, wild type.

herpesvirus UL42 homologs in human cytomegalovirus and human herpesvirus 6 also show sequence conservation (6).

A series of discrete point mutations were introduced at the terminus of the C315 protein, the boundary of the functional domain, to examine the importance of conserved residues. A change in the moderately conserved leucine residue at position 314 or 315 to a similarly hydrophobic methionine residue did not reduce the interaction with Pol (Fig. 9). Conservative substitution of the absolutely preserved phenylalanine residue at position 313 (Fig. 8) to a similarly large nonpolar leucine residue or to a large tyrosine residue with intermediate polarity significantly reduced the binding to Pol. These results suggest that the conserved phenylalanine residue at position 313 is important for the formation of a protein able to bind to Pol, while residues at positions 314 and 315 are less critical.

DISCUSSION

The HSV-1 UL42 protein is essential in HSV-1 infection and in the replication of viral DNA (14, 19). UL42 forms a complex with Pol (3, 8, 10, 24, 29) and increases the activity and processivity of Pol in vitro (7, 10, 13). Altogether, these findings have identified a role for UL42 as the HSV-1 DNA Pol accessory protein.

In previous studies, Pol and UL42 expressed in reticulocyte lysates (5, 28) were used to study the regions of Pol required for the formation of the Pol-UL42 complex. UL42 synthesized in reticulocyte lysates was also shown to stimulate the activity of Pol similarly synthesized on an activated DNA template (7). We used UL42 synthesized in reticulocyte lysates to measure all three functions of UL42 in vitro. The use of rPol allowed the sensitive detection of complexes formed between Pol and radiolabeled UL42 by coimmunoprecipitation and, combined with the use of a stimulation-sensitive poly(dA) · oligo(dT)$_{20}$ template, enabled us to readily achieve much higher levels of stimulation by UL42 produced in vitro.

The combined use of three assays for the function of UL42 mutants in vitro enabled us to colocalize the functional regions of UL42 to the N-terminal 315 aa of the protein. Deletion of regions N terminal to aa 315 severely affected each function of UL42 in vitro. Our results are consistent with the hypothesis that Pol activity stimulation requires the combined functions of Pol binding and DNA binding and that these functions are encoded in a single structural domain.

It is interesting to note that limited proteolysis of UL42 with several different proteases has revealed a protease-resistant domain that retains the ability to bind to DNA and to Pol and to stimulate Pol activity (11). The protease-resistant domain encompassed the amino-terminal two-thirds of the protein, its boundaries corresponding closely to those of the functional domain that we have defined in this study. These observations reinforce the hypothesis that a distinct structural domain constitutes the active N-terminal region. The existence of such a discrete structural domain within UL42 may explain the simultaneous loss of both DNA binding and Pol binding as a result of small deletions and point mutations at the C-terminal boundary of the functional domain. It is plausible that deletions that encroach on this domain may result in destabilization of its structural integrity. While proteins with deletions at the C-terminal boundary of the domain were not notably altered in their stability or solubility, deletions of more than 18 aa at the N terminus resulted in proteins showing high levels of nonspecific binding to protein A-Sepharose beads in coimmunoprecipitation experiments, a feature most likely consistent with global misfolding of the protein. The general loss of function associated with deletions that remove aa at positions 313 to 315 may therefore indicate that these residues play a critical role in structural stabilization of the functional domain. Nevertheless, a more direct role in binding to both Pol and DNA cannot be ruled out until discrete mutations within the functional domain that affect one function without affecting another are identified.

Analysis of the aa sequence alignment of UL42 with its homologs in other alphaherpesviruses (Fig. 8) revealed significantly higher levels of similarity in the region corresponding to the N-terminal functional domain of the protein defined in this work. Marked divergence was observed in the C-terminal region that was dispensable for UL42 activity in vitro. Similarly, the first 20 aa of UL42 were not conserved, and we demonstrated that the N-terminal 18 aa could be deleted without affecting binding to Pol. Therefore, our results suggest that nonconserved regions at both the N- and C-terminal ends of UL42 are dispensable for in vitro activity.

Despite the lack of sequence conservation in the C-terminal region of the UL42 homologs, the region is maintained in all herpesviruses for which sequence information is available. Therefore, although it is dispensable for the in vitro activity that we examined, it may have additional functions in infected cells. The higher level of sequence divergence in this region may indicate that it encodes a species-specific function, such as an interaction with other viral replication proteins or specific host factors. Alternatively, this region may participate in protein stability, nuclear localization, the establishment of or reactivation from latency, or HSV-1 gene expression. Consistent with a possible role for UL42 in gene expression is the finding that the temporal kinetics of HSV-1 gene expression are advanced approximately 2 h in a cell line expressing UL42 (14). Previously, the HSV ICP8

protein was implicated in late viral gene expression in addition to DNA replication (9, 23).

A further mutational analysis of the N-terminal functional domain of UL42 will be required to identify more precisely the DNA-binding and Pol-binding regions of the protein and to determine the extent to which these functions might be separated within the protein. In vivo studies will be needed to establish whether UL42 mutant proteins that are functional in vitro can support lytic replication of HSV in infected cells and to determine whether other functions of UL42 can be realized.

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