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The Putative Terminase Subunit of Herpes Simplex Virus 1 Encoded by U_L28 Is Necessary and Sufficient To Mediate Interaction between pU_L15 and pU_L33

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Viral terminases play essential roles as components of molecular motors that package viral DNA into capsids. Previous results indicated that the putative terminase subunits of herpes simplex virus 1 (HSV-1) encoded by U_L15 and U_L28 (designated pU_L15 and pU_L28, respectively) coimmunoprecipitate with the U_L33 protein from lysates of infected cells. All three proteins are among six required for HSV-1 DNA packaging but dispensable for assembly of immature capsids. The current results show that in both infected- and uninfected-cell lysates, pU_L28 coimmunoprecipitates with either pU_L33 or pU_L15, whereas pU_L15 and pU_L33 do not coimmunoprecipitate unless pU_L28 is present. The U_L28 protein was sufficient to stabilize pU_L33 from proteasomal degradation in an engineered cell line and was necessary to stabilize pU_L33 in infected cells, whereas pU_L15 had no such effects. The presence of pU_L33 was dispensable for the pU_L15/pU_L28 interaction in lysates of both infected and uninfected cells but augmented the tendency for pU_L15 and pU_L28 to coimmunoprecipitate. These data suggest that pU_L28 and pU_L33 interact directly and that pU_L15 interacts directly with pU_L28 but only indirectly with pU_L33. It is logical to propose that the indirect interaction of pU_L15 and pU_L33 is mediated through the interaction of both proteins with pU_L28. The data also suggest that one function of pU_L33 is to optimize the pU_L15/pU_L28 interaction.

Late in infection with all herpesviruses, capsids lacking DNA and viral concatameric DNA accumulate in infected-cell nuclei. By analogy to double-stranded DNA bacteriophages, it is presumed that capsid assembly culminates when a viral terminase cleaves the concatameric DNA into genomic lengths and hydrolyzes ATP to drive the DNA through a unique structure within the capsid, termed the portal vertex. In the case of herpes simplex virus (HSV), the portal vertex is likely composed of a dodecameric ring of the U_L6 protein (pU_L6) (16, 21).

Terminases consist of at least two subunits in all viral systems studied to date (7). Although obtaining direct evidence for the identity of the terminase subunits in herpesviruses has been hampered by the lack of an in vitro packaging system, several lines of indirect evidence have implicated the products of U_L15 and U_L28 (pU_L15 and pU_L28, respectively) as terminase components as follows: (i) pU_L15 and pU_L28 interact in vitro and in vivo with one another and in vitro with the portal protein pU_L6 (1, 6, 11, 12, 22), (ii) pU_L28 has been shown to bind DNA sequences necessary for formation of genomic ends (2), (iii) pU_L15 contains a highly conserved Walker box motif that is essential for HSV DNA packaging and resembles motifs maintained in the ATPase domains of some bacteriophage terminases (9, 15, 23), and (iv) it is likely that the terminase functions are conserved, inasmuch as the homologs of pU_L15 and pU_L28 in human cytomegalovirus (hCMV), encoded by U_L89 and U_L56, respectively, which also interact, have been shown to form a complex with the hCMV portal protein and are required for DNA packaging (10, 13).

The approximately 19,000-M_r protein encoded by herpes simplex virus 1 (HSV-1) U_L33 has also been shown to interact with pU_L15 and pU_L28 by immunoprecipitation from lysates of HSV-infected cells (6). Although its exact function is not known, pU_L33, like pU_L15 and pU_L28, is required for DNA cleavage and packaging (3, 8). Thus, engineered mutations in any of these genes can cause empty capsids lacking DNA to accumulate in infected cells (3, 4, 20). Small amounts of pU_L33, pU_L15, and pU_L28 have also been shown to associate with HSV-1 capsids, suggesting that they maintain their interaction during packaging (5, 18, 24).

Because it would provide information about the HSV terminase, one goal of the present work was to characterize the roles of the individual proteins in the formation of the pU_L15/pU_L33/pU_L28 complex.

MATERIALS AND METHODS

Cells and virus. Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (growth medium). A U_L15 deletion virus was propagated on a cell line designated clone 17 as previously described (4). A previously described cell line designated D4 was used to propagate the U_L33 deletion virus (17). Clone 17 and D4 cell lines were maintained in growth medium supplemented with 500 μg G418 per ml. HSV-1(F) virus and the U_L15, U_L28, and U_L33 null viruses have been described previously (4, 8, 20). The CV28 cell line described herein was used to propagate the previously described U_L28 deletion virus (20).

Antibodies. Polyclonal rabbit antisera recognizing the first 35 amino acids of pU_L15 (designated U_L15N), the C terminus of pU_L15, and the entire U_L28- and U_L33-encoded proteins have been described previously (6, 17, 19). Actin antibody was purchased from Santa Cruz Biotechnology.

Plasmids. Plasmid pJB125 contained the U_L15 cDNA in vector pCDNA3 (Invitrogen), whereas plasmid pJB112 contained the U_L28 coding sequence

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cloned into this vector. To construct a shuttle plasmid (designated pJB401) to transfer U_L28 sequences into a defined site within CV1 cells, pJB112 was cleaved with BamHI and EcoRV, and a 2.36-kb DNA fragment containing the entire U_L28 open reading frame was isolated and cloned into pCDNA5/FRT vector (Invitrogen) at the BamHI and EcoRV sites. This vector contains the hygromycin resistance gene with an Flp recombinase targeting site (FRT site) embedded in the 5′ coding sequence. To construct pJB433, a PCR amplicon from HSV-1(F) DNA containing the entire U_L33 coding sequence was cloned into pCDNA3 at the HindIII and EcoRI sites. pJB433 was digested with HindIII and EcoRI, and the U_L33 coding sequences were gel purified and cloned into pCDNA5/FRT at HindIII and EcoRI sites. The resultant plasmid was designated pJB481. The genotype of each plasmid was confirmed by DNA sequencing.

Transfections for transient expression. Ninety-five-percent-confluent cells were transfected with the plasmids indicated in Results by use of Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Cells were harvested at 24 h posttransfection and subjected to either immunoprecipitation or immunoblot analysis as described below.

Construction and maintenance of novel U_L33- and U_L28-expressing cell lines. Complementing cell lines were constructed by using the Flp-In-CV-1 system (Invitrogen) according to the manufacturer's protocol. Briefly, either pJB481 or pJB401 (see above) was cotransfected with a plasmid (pOG44) containing Flp recombinase under the control of a constitutive hCMV promoter/enhancer into an engineered cell line (Flp-CV1). This cell line was derived from CV1 cells (a derivative of Vero cells) and contains an Flp target sequence (FRT) at a single locus that also bears a *lacZ* gene fused to a gene encoding zeocin resistance. Transcription of the fused gene was driven by the simian virus 40 early promoter. The Flp recombination event was expected to cause insertion of the pCDNA5/FRT construct into the cellular genome at the integrated FRT site. Insertion of the pCDNA5/FRT construct at this site was expected to bring the simian virus 40 promoter and the ATG initiation codon in frame with the hygromycin resistance gene, with concomitant inactivation of the *lacZ*-Zeo^r fusion gene.

After recombination, cells resistant to hygromycin were selected by growth in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 200 μg/ml hygromycin B. Once hygromycin-resistant foci were identified, the cells were trypsinized and pooled. Monolayers of the entire population of cells containing either U_L33 or U_L28 were screened for the ability to complement the growth of the U_L33 or U_L28 null mutants, respectively. All tested cell populations were able to complement the replication of the corresponding viral null mutants (not shown), and the cells were designated CV33 and CV28, respectively. CV28 and CV33 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml hygromycin B.

Immunoprecipitation and immunoblotting. Cells were washed with cold phosphate-buffered saline (PBS) and resuspended in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM NaF, 0.1 mM Na_3VO_4). After incubation on ice for 30 min without sonication, the lysates (800 μl from 8.8×10^6 cells) were clarified at 14,000 rpm for 15 min at 4°C in a microcentrifuge. The supernatants of all lysates were precleared by reaction with preimmune rabbit serum and 30 μl of a 50% slurry of Gammabind G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C with constant rotation. After the beads were pelleted by centrifugation, the supernatants were incubated with rabbit antibodies directed against pU_L15, pU_L28, or pU_L33 for 2 h at 4°C. Thirty microliters of a 50% slurry of Gammabind G-Sepharose beads was then added. The mixture was incubated overnight at 4°C with constant rotation. The beads were washed four times with excess radioimmunoprecipitation assay buffer, and immune complexes were eluted in loading buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 12.5% glycerol) and boiled for 10 min. The immunoprecipitated material was electrophoretically separated on 12% SDS-polyacrylamide gels, and proteins were transferred electrically to nitrocellulose. In some experiments, a portion of the lysates was denatured in loading buffer, electrophoretically separated, and transferred to nitrocellulose.

Nitrocellulose sheets were washed twice in PBS and blocked overnight in PBS supplemented with 10% nonfat dry milk (Carnation). Primary rabbit polyclonal antibodies directed against the C terminus of pU_L15 or pU_L28 were diluted 1:1,000 in PBS supplemented with 2% bovine serum albumin, whereas anti-pU_L33 rabbit polyclonal antibody was diluted 1:400, as previously described (17). Actin-specific antibody was diluted 1:200 according to the manufacturer's protocol. The diluted antibodies were reacted with the blocked nitrocellulose sheets for 2 h at room temperature and washed, and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G diluted 1:5,000 in PBS plus 2% bovine serum albumin was added for 2 h at room temperature. The bound immunoglobulins were revealed by enhanced chemiluminescence (Amersham Pharmacia Biotech). Where applicable, the image intensities of bands on immunoblots were quantified with a Molecular Dynamics PhosphorImager before exposure to radiographic film.

To strip and reprobe the immunoblots, developed blots were incubated in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 min as described in the ECL manual (Amersham), followed by immunoblotting as described above.

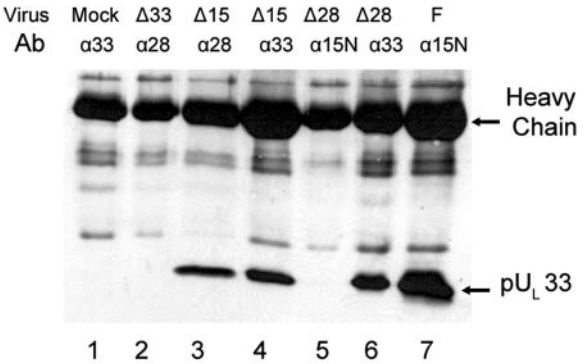

FIG. 1. Immunoblot probed with anti-pU_L33 polyclonal antibody. Vero cells were mock infected (Mock, lane 1) or infected with U_L15 null (Δ15, lanes 3 and 4), U_L28 null (Δ28, lanes 5 and 6), U_L33 null (Δ33, lane 2) or wild-type HSV-1(F) (F, lane 7) virus at a multiplicity of infection of 5 PFU/cell. At 18 h p.i., lysates of the cells were subjected to immunoprecipitations with anti-pU_L15N (α15N, lanes 5 and 7), anti-pU_L28 (α28, lanes 2 and 3), or anti-pU_L33 (α33, lanes 1, 4, and 6) polyclonal antibody. The immunoprecipitates were electrophoretically separated on a 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with anti-pU_L33 polyclonal antibody. Bound immunoglobulin was revealed by enhanced chemiluminescence. Virus and Ab, respectively, indicate the infecting virus and the antibody used for immunoprecipitation for that particular experiment.

RESULTS

pU_L33 interacts directly with pU_L28 and indirectly with pU_L15. To investigate the roles of individual proteins in complex formation, cells were mock infected or were infected with wild-type HSV-1(F) or viral deletion mutants lacking U_L15, U_L28, or U_L33. Cells were then lysed, and the pU_L15, pU_L33, and pU_L28 proteins were separately immunoprecipitated from the lysates with appropriate antibodies. Whether pU_L33 was present in immunoprecipitated material was then determined by immunoblotting. The results indicated that pU_L33 was immunoprecipitated with the pU_L33-specific antibody from lysates of cells infected with wild-type HSV-1(F) (not shown) and the U_L28 and U_L15 deletion viruses (Fig. 1, lanes 4 and 6). This indicated that the absence of pU_L15 or pU_L28 did not preclude expression of pU_L33 within infected cells. On the other hand, levels of pU_L33 were consistently lower in immunoprecipitations from cells infected with the U_L28 and U_L15 deletion viruses than in immunoprecipitations from cells infected with wild-type virus (not shown).

As expected, pU_L33 was not immunoprecipitated from lysates of mock- or U_L33 deletion virus-infected cells (Fig. 1, lanes 1 and 2). Reaction of HSV-1(F)-infected-cell lysates with antibody directed against the N terminus of pU_L15 caused coimmunoprecipitation of pU_L33 (Fig. 1, lane 7). Similarly, the pU_L28-specific antiserum caused coimmunoprecipitation of

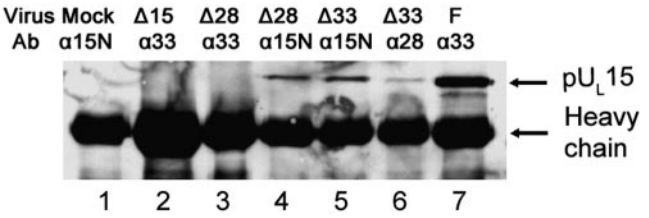

FIG. 2. Immunoblot probed with anti-pU$_L$15 antibody. Vero cells were mock infected (Mock, lane 1) or infected with U$_L$15 null (Δ15, lane 2), U$_L$28 null (Δ28, lanes 3 and 4), U$_L$33 null (Δ33, lanes 5 and 6), or HSV-1(F) (F, lane 7) virus at a multiplicity of infection of 5 PFU/cell. At 18 h p.i., the cells were lysed and subjected to immunoprecipitation with antibodies against the N terminus of pU$_L$15 (α15N, lanes 1, 4, and 5), pU$_L$28 (α28, lane 6), or pU$_L$33 (α33, lanes 2, 3, and 7). The immunoprecipitates were probed for the presence of pU$_L$15 by immunoblotting with an antibody directed against the C terminus of pU$_L$15. Bound immunoglobulin was revealed by enhanced chemiluminescence. Virus and Ab indicate the infecting virus and the immunoprecipitating antibody, respectively.

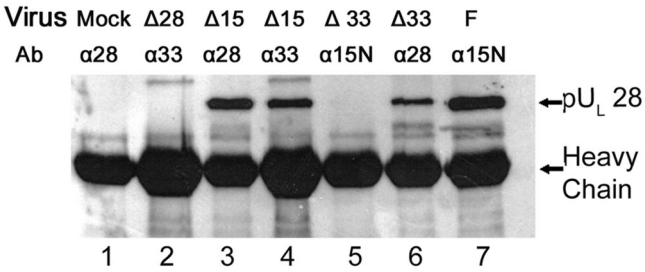

FIG. 3. Immunoblot probed with anti-pU$_L$28 antibody. Vero cells were mock infected (Mock, lane 1) or infected with U$_L$15 null (Δ15, lanes 3 and 4), U$_L$28 null (Δ28, lane 2), U$_L$33 null (Δ33, lanes 5 and 6), or HSV-1(F) (F, lane 7) virus. At 18 h p.i., immunoprecipitations were performed with antibodies against the N terminus of pU$_L$15 (α15N, lanes 5 and 7), pU$_L$28 (α28, lanes 1, 3, and 6), or pU$_L$33 (α33, lanes 2 and 4). The immunoprecipitates were denatured, separated on denaturing polyacrylamide gels, and transferred onto a nitrocellulose membrane, followed by immunoblotting with pU$_L$28-specific polyclonal antibody. Virus and Ab indicate the infecting virus and the immunoprecipitating antibody, respectively.

pU$_L$33 from lysates of cells infected with the U$_L$15 deletion virus (Fig. 1, lane 3), indicating that U$_L$15 was not necessary for the interaction between pU$_L$33 and pU$_L$28. Surprisingly, however, pU$_L$33 was not coimmunoprecipitated with pU$_L$15 N-terminal-specific antibody from lysates of cells infected with the U$_L$28 deletion mutant (Fig. 1, lane 5), despite the presence of ample pU$_L$33 within the lysate (Fig. 1, lane 6). These data indicate that pU$_L$28 is necessary for pU$_L$33 to interact with pU$_L$15 in infected cells and suggest that the pU$_L$15/pU$_L$33 interaction is indirect and normally mediated through pU$_L$28.

The presence of pU$_L$33 enhances the pU$_L$15/pU$_L$28 interaction. Cells were mock infected or were infected with wild-type HSV-1(F) or viral deletion mutants lacking U$_L$15, U$_L$28, or U$_L$33. Lysates were prepared, clarified, and reacted with antibodies against pU$_L$15N, pU$_L$28, or pU$_L$33, and the presence of pU$_L$15 in the immunoprecipitations was monitored by immunoblotting. The anti-C-terminal pU$_L$15 antiserum was used for immunoblotting throughout this study due to its high sensitivity and specificity in this assay. The U$_L$15 protein was readily immunoprecipitated with the pU$_L$15N-specific antibody from lysates of cells infected with wild-type virus (not shown) and the U$_L$28 and U$_L$33 deletion viruses (Fig. 2, lanes 4 and 5) but was not immunoprecipitated from lysates of mock- or U$_L$15 deletion mutant-infected cells (Fig. 2, lanes 1 and 2). Reaction of HSV-1(F)-infected-cell lysates with pU$_L$33-specific antibody caused coimmunoprecipitation of pU$_L$15 (Fig. 2, lane 7), whereas pU$_L$15/pU$_L$33 coimmunoprecipitation did not occur in lysates of cells infected with the U$_L$28 deletion virus (Fig. 2, lane 3). These observations were consistent with previous data (Fig. 1) demonstrating that pU$_L$28 was necessary for the pU$_L$15/pU$_L$33 interaction. Importantly, antibody against pU$_L$28 reacted with lysates of cells infected with the U$_L$33 deletion virus immunoprecipitated only a portion of the pU$_L$15 that was immunoprecipitated with its cognate antibody (compare Fig. 2, lanes 5 and 6). These data indicate that while pU$_L$33 is ultimately dispensable for the pU$_L$15/pU$_L$28 interaction, it also acts in some way to enhance the interaction in infected-cell lysates.

To confirm some of the above results, lysates of mock-infected cells or cells infected with HSV-1(F) or the U$_L$15, U$_L$33, or U$_L$28 deletion virus were reacted with antibodies to these proteins, and the immunoprecipitated material was probed for the presence of pU$_L$28 by immunoblotting. The U$_L$28 protein was not immunoprecipitated from lysates of mock-infected cells or cells infected with the U$_L$28 deletion virus (Fig. 3, lanes 1 and 2). In contrast, pU$_L$28 was readily immunoprecipitated from lysates of cells infected with wild-type virus (not shown) and deletion viruses lacking either U$_L$15 or U$_L$33 (Fig. 3, lanes 3 and 6). Of interest was the observation that despite the presence of pU$_L$28 in the lysates of cells infected with the U$_L$33 deletion mutant, antibody to the pU$_L$15 N terminus coimmunoprecipitated only barely detectable levels of pU$_L$28 (Fig. 3, lane 5). This observation further indicated that the pU$_L$15/pU$_L$28 interaction was significantly augmented by the presence of pU$_L$33. Moreover, antibody directed against pU$_L$33 coimmunoprecipitated pU$_L$28 from lysates of cells infected with the U$_L$15 deletion mutant (Fig. 3, lane 4), indicating that the absence of pU$_L$15 did not preclude an interaction between pU$_L$33 and pU$_L$28.

pU$_L$28 is necessary and sufficient to mediate the pU$_L$15/pU$_L$33 interaction in mammalian cells. To determine if pU$_L$28 was sufficient to mediate the interaction between pU$_L$33 and pU$_L$15, a cell line expressing pU$_L$33 was constructed as described in Materials and Methods. This cell line (CV33) was able to support replication of the U$_L$33 deletion mutant (data not shown). CV33 cells were transfected with plasmids expressing U$_L$15, U$_L$28, or both. Twenty-four hours later, lysates were prepared and either electrophoretically separated or immunoprecipitated with pU$_L$33-specific antibody, followed by electrophoretic separation on denaturing polyacrylamide gels. In both cases, separated material was transferred to nitrocellulose and analyzed for the presence of pU$_L$15 and pU$_L$28 by immunoblotting. Transfection of plasmids containing U$_L$15 and U$_L$28 into CV33 cells caused production of detectable levels of the U$_L$15- and U$_L$28-encoded proteins (Fig. 4, lanes 2 to 4). The pU$_L$33-specific antibody readily coimmunoprecipitated pU$_L$28 from lysates of CV33 cells expressing pU$_L$28 or coexpressing pU$_L$28 and pU$_L$15 (Fig. 4A, lanes 6 and 8), indicating that pU$_L$28 and pU$_L$33 can interact in the absence of other HSV proteins. In contrast, the pU$_L$33-specific antibody coimmuno-

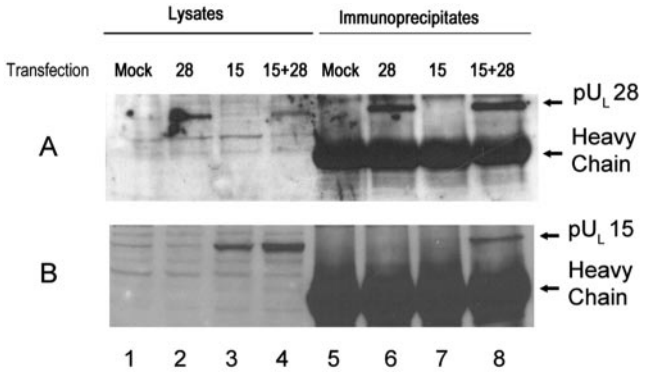

FIG. 4. Immunoblots of CV33 cells (CV1 cells expressing pU_L 33) transiently expressing pU_L28 and/or pU_L15. CV33 cells were mock transfected (Mock) or transfected with plasmids expressing the genes indicated above each lane (28, pU_L28; 15, pU_L15; 15+28, pU_L15 and pU_L28). Lysates were prepared 24 h later, and lysates (lanes 1 to 4) or immunoprecipitations from these lysates obtained using pU_L33-specific antibody (lanes 5 to 8) were electrophoretically separated, transferred to nitrocellulose, and probed with the pU_L28-specific antisera (A) or antisera against the C terminus of pU_L15 (B).

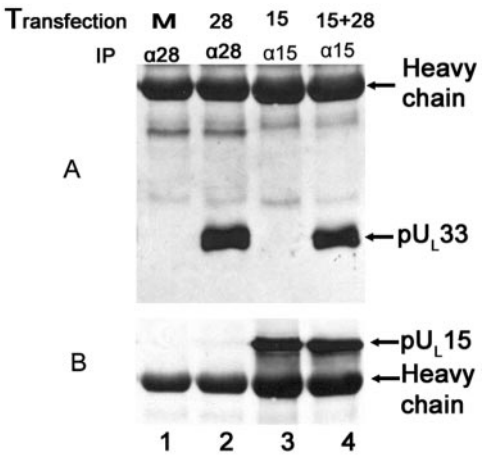

FIG. 5. Immunoblots of CV33 cell lysates immunoprecipitated with pU_L15N- and pU_L28-specific antibodies. CV33 cells were mock transfected (M, lane 1) or transfected with plasmids expressing U_L28 (28, lane 2), U_L15 (15, lane 3), or U_L15 and U_L28 (15+28, lane 4). Twenty-four hours later, lysates were prepared and immunoprecipitations (IP) were performed with antibodies directed against pU_L28 (α28, lanes 1 and 2) or the N terminus of pU_L15 (α15, lanes 3 and 4). Immunoblots of the immunoprecipitated material were probed with antibody directed against pU_L33 (A). The same blots were stripped as described in Materials and Methods and reprobed with antibody against the C terminus of pU_L15 (B).

precipitated pU_L15 only from lysates of CV33 cells expressing both pU_L28 and pU_L15 (Fig. 4B, lane 8), whereas pU_L15 was not coimmunoprecipitated by the pU_L33-specific antibody when pU_L15 was expressed in the absence of pU_L28 (Fig. 4B, lane 7). These data therefore indicate that pU_L28 is both necessary and sufficient to mediate the interaction between pU_L15 and pU_L33.

In the reciprocal reaction, lysates of CV33 cells that were mock transfected or transfected with plasmids expressing either U_L15 or U_L28 or both were subjected to immunoprecipitation with antibodies directed against either the N terminus of pU_L15 (Fig. 5, lanes 3 and 4) or pU_L28 (Fig. 5, lanes 1 and 2). The presence of pU_L33 and pU_L15 in the immunoprecipitated material was then determined by immunoblotting. The U_L33-encoded protein was coimmunoprecipitated with the pU_L28-specific antibody when pU_L28 was expressed in the absence of pU_L15 (Fig. 5A, lane 2). In contrast, and despite the presence of ample amounts of pU_L15 in U_L15N antibody-immunoprecipitated material (Fig. 5B, lane 3), pU_L33 was coimmunoprecipitated with the pU_L15N-specific antibody only when pU_L28 was coexpressed with pU_L15 (Fig. 5A, lane 4). These data further support the conclusion that pU_L28 is not only necessary for the pU_L33/pU_L15 interaction but also sufficient to mediate the interaction in the absence of other HSV proteins.

pU_L28 can protect pU_L33 from efficient degradation by the proteosome. Preliminary evidence indicated that pU_L33 in CV33 cells was difficult to detect by immunoblotting unless the cells were infected with wild-type HSV-1 (not shown). To determine whether the decreased amounts of pU_L33 in uninfected CV33 cells were a consequence of its proteasomal degradation or poor overall expression, CV33 cells were untreated or treated with the proteosome inhibitor MG132 or lactacystin or with a similar amount of dimethyl sulfoxide (DMSO), which was used as a carrier in the MG132 treatment. As shown in Fig. 6A, treatment with various amounts of MG132 significantly increased the amount of detectable pU_L33 in CV33 cells over levels obtained after mock treatment or treatment with DMSO. This suggested that pU_L33 within CV33 cells was efficiently degraded by the proteosome. To confirm the results, we repeated this experiment with the proteosome inhibitor lactacystin and, as controls, chloroquine (an inhibitor of the lysosomal degradation pathway) and calpain inhibitor 2 (which blocks calpain proteases). As shown in Fig. 6B, both MG132 and lactacystin substantially increased the amount of detectable pU_L33 above levels obtained in cells that were not treated, whereas chloroquine, DMSO, or calpain inhibitor 2 did not dramatically increase the levels of pU_L33. We therefore conclude that pU_L33 is efficiently degraded by the proteosome in uninfected CV33 cells.

Because the evidence presented above indicated that pU_L28 and pU_L33 interacted directly, we hypothesized that the interaction of pU_L28 with pU_L33 might increase the stability of the latter. To test this possibility, CV33 cells were transfected with expression plasmids containing U_L28, U_L15, or both, the cells were lysed 24 h later, and the presence of pU_L33 and actin (as a loading control) in the lysates was determined by immunoblotting.

As shown in Fig. 7, the presence of pU_L28 correlated with a greatly increased level of pU_L33-specific immunoreactivity in the CV33 cells, whereas expression of pU_L15 did not increase the amount of detectable pU_L33 above that obtained upon mock transfection (compare Fig. 7A, lanes 1 to 3). Coexpression of pU_L15 and pU_L28 also increased levels of pU_L33 immunoreactivity (Fig. 7A, lane 4). These data indicate that U_L28 causes increased amounts of pU_L33 to accumulate in the CV33 cell line, whereas U_L15 did not induce such effects. Taken together with the knowledge that pU_L33 is normally degraded by the proteosome in CV33 cells, these data suggest that the increased stability of pU_L33 conferred by expression of pU_L28 is a consequence of the interaction of these proteins.

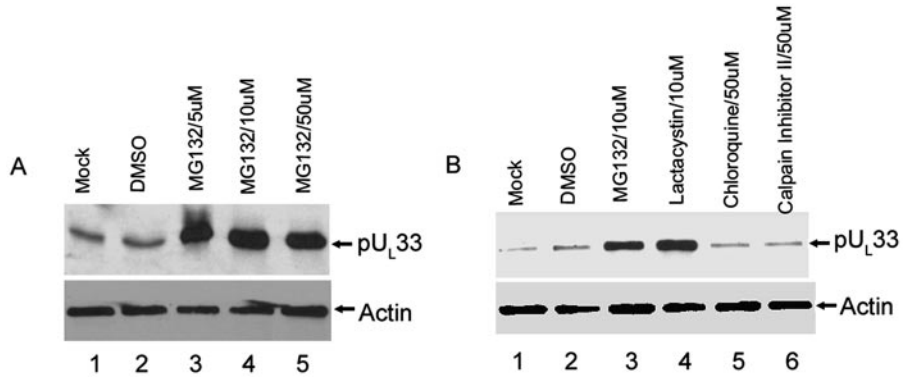

FIG. 6. Immunoblots of CV33 cells treated with proteosome inhibitors. CV33 cells (CV1 cells engineered to express pU$_L$33) were treated for 6 h with the indicated compounds, at which time cell lysates were prepared and separated by SDS-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, immunoblotting was performed using antibodies against pU$_L$33 (upper panels) or actin as a loading control (lower panels).

To determine whether pU$_L$28 or pU$_L$15 could increase the stability of pU$_L$33 in infected cells, Vero cells were mock infected or were infected with wild-type virus or deletion viruses lacking U$_L$28 or U$_L$15. At 12 h postinfection (p.i.), the infected cells were left untreated or were treated with 10 μM lactacystin or the equivalent concentration of the DMSO carrier, and lysates of the cells were prepared at 18 h p.i. Immunoblots of the lysates were then probed with pU$_L$33 antibody. As shown in Fig. 7E, less pU$_L$33 was detected in cells infected with the U$_L$28 deletion mutant (Fig. 7E, lanes 2 and 3) than in cells infected with either the wild-type virus (Fig. 7E, lanes 8 and 9) or the U$_L$15 deletion virus (Fig. 7E, lanes 5 and 6). Moreover, lactacystin treatment increased the accumulation of pU$_L$33 in cells infected with the U$_L$28 deletion mutant (Fig. 7E, lane 4) but did not affect accumulation of pU$_L$33 in cells infected with wild-type HSV-1(F) (Fig. 7E, lane 9) or the U$_L$15 deletion

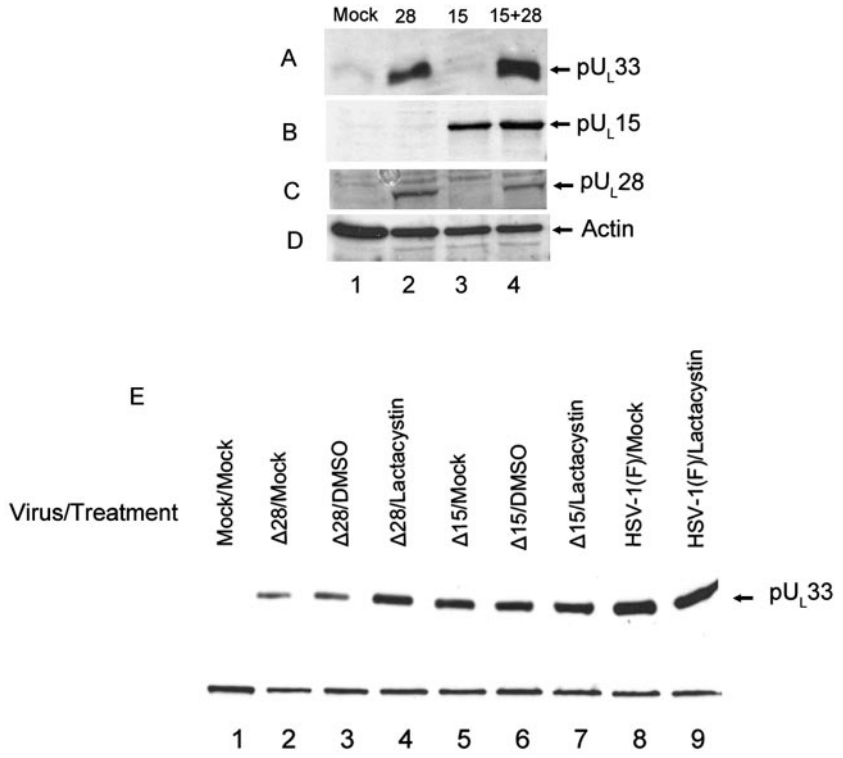

FIG. 7. Immunoblots of pU$_L$33 in the presence and absence of U$_L$28 and U$_L$15. CV33 cells were mock transfected (Mock) or transfected with plasmids expressing the indicated open reading frames (28, U$_L$28; 15, U$_L$15; 15+28, U$_L$15 and U$_L$28). Twenty-four hours later, cell lysates were prepared and separated by SDS-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane, immunoblotting was performed using the polyclonal antibody against pU$_L$33 (A), the C terminus of pU$_L$15 (B), or pU$_L$28 (C) or actin as a loading control (D). (E) Vero cells were infected with the indicated virus (Δ15, U$_L$15 null virus; Δ28, U$_L$28 null virus) or mock infected (Mock) and were incubated in the presence or absence of 10 mm lactacystin or the DMSO carrier from 12 to 18 h p.i. Immunoblots of lysates harvested at 18 h p.i. were probed with the antibody against pU$_L$33. A cellular protein recognized by the pU$_L$33 antibody served as a loading control.

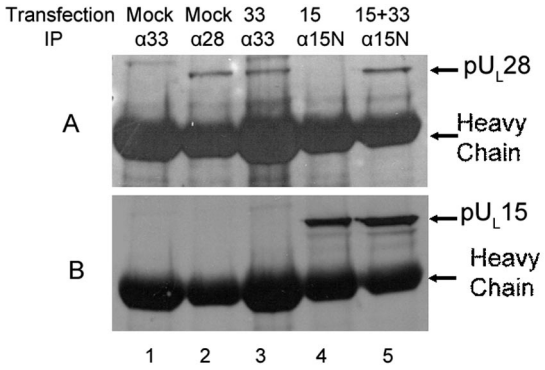

FIG. 8. Immunoblots of proteins immunoprecipitated from a U_L28-expressing cell line (CV28). CV28 cells were mock transfected (Mock, lanes 1 and 2) or transfected with plasmids expressing U_L33 (33, lane 3), U_L15 (15, lane 4), or U_L15 and U_L33 (15+33, lane 5). Twenty-four hours later, lysates of the cells were prepared and subjected to immunoprecipitation (IP) with antibodies directed against pU_L33 (α33, lanes 1 and 3), pU_L28 (α28, lane 2), or the N terminus of pU_L15 (α15N, lanes 4 and 5). An immunoblot of electrophorectically separated material was probed with antibody to pU_L28 (A). The same immunoblot was stripped as described in Materials and Methods and reprobed with antibodies against the C terminus of pU_L15 (B).

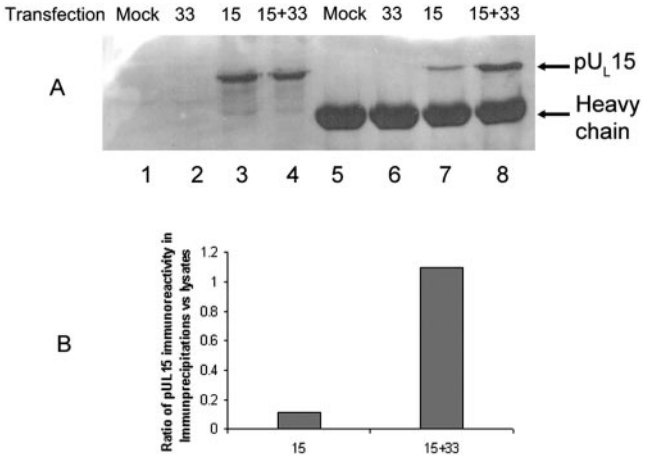

FIG. 9. Immunoblots of lysates and immunoprecipitates of CV28 cells expressing putative terminase proteins probed with pU_L15-specific antibody. (A) CV28 cells (CV1 cells expressing pU_L28) were mock transfected (Mock, lanes 1 and 5) or were transfected with U_L33 (33, lanes 2 and 6), U_L15 (15, lanes 3 and 7), or U_L15 and U_L33 (15+33, lanes 4 and 8). Lysates were prepared 24 h posttransfection. Fifteen microliters of the lysates (lanes 1 to 4) or material immunoprecipitated with the pU_L28-specific antibody (lanes 5 to 8) was electrophoretically separated, and immunoblots of the separated proteins were probed with antibody directed against the C terminus of pU_L15. pU_L15-specific immunoreactivity in the blot was quantified with a Molecular Dynamics PhosphorImager before exposure to radiographic film. (B, left histogram) The amount of pU_L15 immunoreactivity in lane 7 was divided by the amount in lane 3. (B, right histogram) The amount of pU_L15 immunoreactivity in lane 8 was divided by the amount in lane 4. These ratios differed by approximately 9.5-fold.

virus (Fig. 7E, lane 7). These data indicate that pU_L28 is necessary for protection of pU_L33 from proteasomal degradation in infected cells, whereas the presence of pU_L15 does not affect pU_L33 accumulation.

pU_L33 augments coimmunoprecipitation of pU_L15 and pU_L28 in a pU_L28-expressing cell line. To determine if pU_L33 could enhance the pU_L15/pU_L28 interaction in the absence of other HSV proteins, a U_L28-expressing cell line (CV28) was mock transfected or transfected with expression plasmids containing U_L33, U_L15, or both. Twenty-four hours after transfection, cell lysates were prepared and reacted with antibody against pU_L33, pU_L15, or pU_L28. The presence of pU_L15 and pU_L28 in the immunoprecipitated material was determined by immunoblotting.

The constitutively expressed U_L28 protein was immunoprecipitated from lysates of mock-transfected CV28 cells by the pU_L28-specific antibody (Fig. 8A, lane 2) and was coimmunoprecipitated with the pU_L33-specific antibody from lysates of CV28 cells expressing pU_L33 (Fig. 8A, lane 3). The U_L28 protein of CV28 cells was not immunoprecipitated with the pU_L33-specific antibody in the absence of pU_L33 expression (Fig. 8A, lane 1). Surprisingly, expression of U_L15 in CV28 cells followed by reaction with the pU_L15N antibody immunoprecipitated pU_L15 (Fig. 8B, lane 4) but not pU_L28 (Fig. 8A, lane 4) from CV28 cells. These data suggest that in this cell line pU_L33 augments the interaction of pU_L15 and pU_L28 in the absence of other viral proteins.

To further investigate this conclusion, a reciprocal experiment was performed. Specifically, lysates of CV28 cells that were mock transfected or transfected with expression plasmids bearing U_L33, U_L15, or both were subjected to immunoprecipitation with pU_L28-specific antibody, and the presence of pU_L15 in the immunoprecipitated material was monitored by immunoblotting. As shown in Fig. 9A, lanes 7 and 8, pU_L15 was coimmunoprecipitated with pU_L28 antibody in CV28 cells whether or not pU_L33 was expressed in the lysate; however, the presence of pU_L33 significantly augmented the coimmunoprecipitation of pU_L28 and pU_L15. To quantify the extent of this augmentation, the amounts of pU_L15 in the lysates of CV28 cells transfected with pU_L15 or pU_L15 and pU_L33 together were determined using a Molecular Dynamics PhosphorImager. The level of pU_L15 immunoreactivity in the immunoprecipitates (Fig. 9A, lanes 7 and 8) was then compared to that obtained from the lysates (Fig. 9A, lanes 3 and 4), and a ratio of the two values was calculated (Fig. 9B). These calculations revealed that expression of pU_L33 enhanced coimmunoprecipitation of pU_L15 and pU_L28 approximately 9.5-fold.

DISCUSSION

The current results confirm previous results showing that pU_L33 coimmmunoprecipitates with pU_L15 and pU_L28 in lysates of infected cells (6). Using pU_L15, pU_L28, and pU_L33 overexpressed in insect cells, previous experiments demonstrated interactions between U_L15 and U_L33, pU_L28 and pU_L15, and pU_L33 and pU_L28 (1, 6). In contrast to some of the previous results, the current results with HSV-infected cells demonstrate that pU_L15 interacts with pU_L33 only upon coexpression of pU_L28. It was also of interest to note that this coexpression greatly stabilized pU_L33. This observation is consistent with the notion that the pU_L33/pU_L28 interaction precludes aberrant folding of pU_L33, thereby protecting it from targeted degradation by the proteosome. We cannot exclude the possibility that pU_L15 and pU_L33 interact directly but view

it likely that such an interaction is considerably weaker than that of pU$_L$28 with pU$_L$33. Weaker interactions may be revealed more readily when proteins are highly expressed, as might have occurred in the previous study when the very strong baculovirus polyhedron promoter was used to drive gene expression (6). Because the current study investigated the putative terminase proteins under conditions of their native environment, stoichiometry, and levels of expression, we give more credence to the current results.

Because all of the interactions in both studies were identified in the context of a cell lysate, a further caveat is that other proteins may affect the interactions. Because few viral proteins other than pU$_L$15 and pU$_L$33 coimmunoprecipitate with pU$_L$28 (6), it seems most likely that any augmentation of the interaction would be mediated by cellular proteins or perhaps transiently by viral proteins yet to be identified.

It was of interest to find that pU$_L$33 augmented the interaction of pU$_L$15 and pU$_L$28 both in infected cells and when transiently expressed in uninfected mammalian cells. This represents the first identification of an activity of pU$_L$33 that might be relevant to its role in the HSV DNA cleavage/packaging reaction. The relatively small pU$_L$33 has no obvious DNA or ATP binding motifs that might be expected of a terminase subunit (14), and this is consistent conceptually with its primary role as an adapter to augment interaction between the DNA binding (likely pU$_L$28) and ATPase (likely pU$_L$15) subunits of the terminase. Because pU$_L$33 interacts primarily with pU$_L$28, it seems most likely that pU$_L$33 enhances the pU$_L$15/pU$_L$28 interaction by optimizing pU$_L$28's capacity to bind pU$_L$15, perhaps by optimizing pU$_L$28 folding. On the other hand, given the multifunctionality of most HSV proteins it also seems likely that pU$_L$33 will exhibit other interesting activities upon more extensive analyses.

That the pU$_L$15/pU$_L$28/pU$_L$33 complex can form independently of the capsid or portal under physiological conditions in infected cells is supported by the observation here (not shown) and elsewhere that pU$_L$6 does not coimmunoprecipitate with pU$_L$15, pU$_L$28, or pU$_L$33 from infected-cell lysates (6). Although antibodies can interfere with coimmunoprecipitations, it seems unlikely that three different antibodies to three different proteins would all fail to pull down the portal if the terminase/portal protein complex was soluble and intact. On the other hand, the lysates employed in these studies would not be expected to contain abundant amounts of nuclear proteins. If the terminase requires the portal vertex within an intranuclear procapsid for binding, as seems likely, the current studies would not be expected to detect a portal/terminase interaction because the lysates should not contain intact procapsids. That pU$_L$6 and putative terminase components pU$_L$15 and pU$_L$28 can interact is supported by studies using transient-expression systems (22). Clearly, further studies of the distributions of the relevant protein complexes in HSV-infected cells are necessary to determine the sites of assembly and interaction of the putative terminase and portal encoded by pU$_L$6.

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REFERENCES

1. **Abbotts, A. P., V. G. Preston, M. Hughes, A. H. Patel, and N. D. Stow.** 2000. Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. J. Gen. Virol. **81:**2999–3009.
2. **Adelman, K., B. Salmon, and J. D. Baines.** 2001. Herpes simplex DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc. Natl. Acad. Sci. USA **98:**3086–3091.
3. **Al-Kobaisi, M. F., F. J. Rixon, I. McDougall, and V. G. Preston.** 1991. The herpes simplex virus UL33 gene product is required for the assembly of full capsids. Virology **180:**380–388.
4. **Baines, J. D., C. Cunningham, D. Nalwanga, and A. J. Davison.** 1997. The U$_L$15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the U$_L$15 gene product. J. Virol. **71:**2666–2673.
5. **Beard, P. M., and J. D. Baines.** 2004. The DNA cleavage and packaging protein encoded by the UL33 gene of herpes simplex virus 1 associates with capsids. Virology **324:**475–482.
6. **Beard, P. M., N. S. Taus, and J. D. Baines.** 2002. DNA cleavage and packaging proteins encoded by genes U$_L$28, U$_L$15, and U$_L$33 of herpes simplex virus type 1 form a complex in infected cells. J. Virol. **76:**4785–4791.
7. **Black, L. W.** 1989. DNA packaging in dsDNA bacteriophages. Annu. Rev. Microbiol. **43:**267–292.
8. **Cunningham, C., and A. J. Davison.** 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology **197:**116–124.
9. **Davison, A. J.** 1992. Channel catfish virus: a new type of herpesvirus. Virology **186:**9–14.
10. **Dittmer, A., J. C. Drach, L. B. Townsend, A. Fischer, and E. Bogner.** 2005. Interaction of the putative human cytomegalovirus portal protein pUL104 with the large terminase subunit pUL56 and its inhibition by benzimidazole-D-RIBONUCLEOSIDES. J. Virol. **79:**14660–14667.
11. **Koslowski, K. M., P. R. Shaver, J. T. I. Casey, T. Wilson, G. Yamanaka, A. K. Sheaffer, D. J. Tenny, and N. E. Pedersen.** 1999. Physical and functional interactions between the herpes simplex virus UL15 and UL28 DNA cleavage and packaging proteins. J. Virol. **73:**1704–1707.
12. **Koslowski, K. M., P. R. Shaver, X.-Y. Wang, D. J. Tenny, and N. Pedersen.** 1997. The pseudorabies virus UL28 protein enters the nucleus after coexpression with the herpes simplex virus UL15 protein. J. Virol. **71:**9118–9123.
13. **Krosky, P. M., M. R. Underwood, S. R. Turk, K. W. Feng, R. K. Jain, R. G. Ptak, A. C. Westerman, K. K. Biron, L. B. Townsend, and J. C. Drach.** 1998. Resistance of human cytomegalovirus to benzimidazole ribonucleosides maps to two open reading frames: UL89 and UL56. J. Virol. **72:**4721–4728.
14. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
15. **Mitchell, M. S., S. Matsuzaki, S. Imai, and V. B. Rao.** 2002. Sequence analysis of bacteriophage T4 DNA packaging/terminase genes 16 and 17 reveals a common ATPase center in the large subunit of viral terminases. Nucleic Acids Res. **30:**4009–4021.
16. **Newcomb, W. W., R. M. Juhas, D. R. Thomsen, F. L. Homa, A. D. Burch, S. K. Weller, and J. C. Brown.** 2001. The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. J. Virol. **75:**10923–10932.
17. **Reynolds, A. E., Y. Fan, and J. D. Baines.** 2000. Characterization of the U$_L$33 gene product of herpes simplex virus 1. Virology **266:**310–318.
18. **Salmon, B., and J. D. Baines.** 1998. Herpes simplex virus DNA cleavage and packaging: association of multiple forms of U$_L$15-encoded proteins with B capsids requires at least the U$_L$6, U$_L$17, and U$_L$28 genes. J. Virol. **72:**3045–3050.
19. **Salmon, B., D. Nalwanga, Y. Fan, and J. D. Baines.** 1999. Proteolytic cleavage of the amino terminus of the U$_L$15 gene product of herpes simplex virus type 1 is coupled with maturation of viral DNA into unit-length genomes. J. Virol. **73:**8338–8348.
20. **Tengelsen, L. A., N. E. Pedersen, P. R. Shaver, M. W. Wathen, and F. L. Homa.** 1993. Herpes simplex virus type 1 DNA cleavage and capsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J. Virol. **67:**3470–3480.
21. **Trus, B. L., N. Cheng, W. W. Newcomb, F. L. Homa, J. C. Brown, and A. C. Steven.** 2004. Structure and polymorphism of the UL6 portal protein of herpes simplex virus type 1. J. Virol. **78:**12668–12671.
22. **White, C. A., N. D. Stow, A. H. Patel, M. Hughes, and V. G. Preston.** 2003. Herpes simplex virus type 1 portal protein UL6 interacts with the putative terminase subunits UL15 and UL28. J. Virol. **77:**6351–6358.
23. **Yu, D., and S. K. Weller.** 1998. Genetic analysis of the UL 15 gene locus for the putative terminase of herpes simplex virus type 1. Virology **243:**32–44.
24. **Yu, D., and S. K. Weller.** 1998. Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. J. Virol. **72:**7428–7439.