The herpes simplex virus (HSV) genome replicates in the nucleus of an infected host cell, resulting in the production of longer-than-unit-length head-to-tail concatamers of viral DNA. Production of infectious virus requires the processing of concatameric DNA into unit-length genomes by the packaging machinery followed by encapsidation into preassembled capsids (63). Several lines of evidence suggest that recombination plays a role in concatamer formation (69). Genomic inversions, proposed to occur through recombination, can be detected very early during infection, and these inversions require sequence homology (2, 23, 56). In addition, replication intermediates in HSV type 1 (HSV-1)-infected cells adopt a complex nonlinear structure that does not migrate in a pulsed-field gel, even after digestion with an enzyme that cuts once per unit length of the genome (1, 33, 51, 71). Electron micrographs have revealed that replication intermediates are branched and contain Y- and X-shaped junctions (19, 52). Furthermore, high levels of recombination have been reported not only between coinfecting viral strains (4, 17, 50, 62, 66) but also in plasmids containing repeated sequence elements (10, 11). These observations, taken together, are not consistent with a simple rolling circle mechanism of replication and suggest that, reminiscent of the bacteriophages T4 and lambda, HSV-1 utilizes a recombination-dependent replication mechanism to generate concatameric viral DNA.

We have previously reported that virus-encoded proteins are capable of participating in recombination events in vitro. The viral 5′–3′ alkaline exonuclease (UL12) and the single-strand binding protein (ICP8) together mediate strand exchange in vitro, and we have proposed that UL12 and ICP8 function as a two-component recombinase similar to the well-studied phage lambda Red α/β recombinase system (48). ICP8 and UL12 also interact in vivo during HSV-1 infection (61). The lambda exonuclease (Red α) and the single-strand binding protein (Red B) mediate strand exchange and recombination (57). In the absence of host recombination proteins, Red α/β is capable of generating head-to-tail concatamers by strand annealing, and in the presence of host recombination factors, the viral recombinase participates in strand invasion reactions (44, 57).

The phenotype of AN-1, a UL12 null virus, is complex (see Discussion for more detail), suggesting that UL12 plays a role either in the initiation of recombination or in processing of recombination intermediates (or both) (33, 43, 53, 64). By analogy with lambda, the viral recombinase may also function in conjunction with components of the cellular DNA damage response machinery responsible for the homologous recombination repair (HRR) pathway.

During HSV-1 infection, the nucleus is dramatically reorganized, resulting in the formation of globular replication compartments (RCs) in which gene expression, DNA replication, and encapsidation occur (7, 23, 45). Several cellular proteins known to participate in host damage responses are recruited to replication compartments (15, 30, 55, 60, 65, 68) and are required for efficient virus production (29, 30, 37, 60). The MRN complex, consisting of Mre11, Rad50, and Nbs1, is particularly important in the normal cellular damage response, as it acts as a sensor of double-strand breaks (DSBs) and is recruited to the site of DNA damage, thereby signaling activation of at least one of the phosphatidylinositol 3-kinase (PI3 kinase)-like kinases (PIKKs), ATM (ataxia-telangiectasia mutated) (24, 30,
TABLE 1. Sequences of oligonucleotides used in the construction of the various truncation mutants of UL12

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5′-3′)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP1</td>
<td>AAGCTTGAATTCGCCACCATGGAGG</td>
</tr>
<tr>
<td>RP104</td>
<td>GGAGATCTCTAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>RP27</td>
<td>GCAGATGGATCTCCAGGGGAGGAGGAGG</td>
</tr>
<tr>
<td>RP30</td>
<td>GCAGATGGATCTCCACAGGTGAGGAGGAGG</td>
</tr>
<tr>
<td>RP421</td>
<td>GCAGATGGATCTTCAGGGGACAGGGGACAGGGG</td>
</tr>
<tr>
<td>RP490</td>
<td>GGAGATCTCTCCAGGGGACAGGGGACAGGGG</td>
</tr>
<tr>
<td>RP2</td>
<td>TGAAAGATCCATGGTGTGAGGGGAGGAGGAGG</td>
</tr>
<tr>
<td>RP2</td>
<td>ATGGGATCTCCAGGGGACAGGGGACAGGGG</td>
</tr>
<tr>
<td>RP3</td>
<td>ATAGAAGCTCATGAGGACAGGGGACAGGGG</td>
</tr>
<tr>
<td>RP50</td>
<td>ATGGGATCGGAGCGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>RP75</td>
<td>ATGGGATCTCATGAGGACAGGGGACAGGGG</td>
</tr>
<tr>
<td>RP100</td>
<td>ATGGGATCGGAGCGAGGAGGAGGAGGAGG</td>
</tr>
</tbody>
</table>

* The restriction sites in the primer sequences are indicated in bold.

41, 42, 70). In 2004, we first demonstrated that HSV-1 infection results in the phosphorylation of Nbs1 (68), and other ATM targets were also identified, including Chk2, P53, 53BP1, and phospho-ATM (30, 55, 59). The activation of MRN and the ATM pathway in infected cells is consistent with a role for HRR during HSV-1 infection.

To test the hypothesis that viral and cellular proteins participate in recombination-dependent DNA synthesis in HSV-1-infected cells, we examined interactions between the viral recombinase and cellular HRR pathway proteins. It has been previously reported that the viral single-strand binding protein ICP8 interacts with various cellular DNA repair proteins, including Rad50, Mre11, DNA-PKcs, RPA, Ku86, BLM, BRG1, and WRN (60); however, the question of whether some of these interactions are direct or are mediated through other viral and cellular proteins has never been addressed. In this study we focus on identification of cellular proteins that interact with UL12, the nuclease component of the viral recombinase. We report a direct and specific interaction between UL12 and components of the MRN complex.

MATERIALS AND METHODS

Cell culture and viruses. African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum. Cells were grown in the presence of 0.1 mg/ml of streptomycin and 100 U/ml of penicillin at 37°C and 5% CO2. The KO strain of HSV-1 was used as the wild-type virus, and it produces both full-length UL12 (residues 1 to 626) and a subgenic protein, UL12.5 (residues 127 to 626). The UL12 null virus AN-1 does not express either UL12 or UL12.5, while the UL12 mutant virus AN-1 produces only UL12.5. These were propagated in UL12-complementing 6-5 cells (53). ICP8 mutant virus HD-2 was provided by David Knipe (Harvard Medical School) (12). Baculoviruses expressing His-Mre11, His-Rad50, and glutathione S-transferase (GST)-Nbs1 were provided by Tanya Paul (University of Texas at Austin) (27).

Antibodies. Anti-Rad50 (13B3) and anti-Mre11 (12D7) monoclonal antibodies were purchased from Genetex. Anti-Nbs1 monoclonal antibody (IC3) was purchased from Novus Biologicals. Antitubulin antibody (Tib657) was purchased from Sigma. Anti-UL12 antibody (BWPUL12), which detects both UL12 and UL12.5, was a gift from Joel Bronstein and Peter Weber (Parke-Davis Pharmaceutical Research). Anti-ICP8 monoclonal antibody (39-S) was provided by David Knipe (Harvard Medical School), and the polyclonal antibody (ICD) was provided by William Rueczan (The State University of New York) (54).

Plasmids. pSAKUL12.5 expresses both full-length UL12 and UL12.5. pSAKUL12.5 expresses only UL12.5, the pSAK N terminal expresses only the first 126 amino acids of UL12, and pSAK was used as the empty vector (47). The C-terminal truncations of UL12 were constructed by cloning PCR-amplified fragments of UL12 using pSAKUL12.5 as the template into EcoRI and BamHI-digested pSAK vector to derive pBN104, pBN271, pBN390, pBN421, and pBN490. Table 1 lists the oligonucleotides used in the construction of the various constructs in this report. FP1 was used as the forward primer along with the reverse primers RP104, RP271, RP30, RP421, and RP490 to PCR amplify the corresponding UL12 fragments. To construct the internal deletions of UL12, the fragments corresponding to residues 127 to 626 of UL12 were PCR amplified using FP2 as the forward primer and RP2 as the reverse primer. The PCR product was digested with EcoRI and BamH1 and cloned into pSAK to derive pBNUL12.5. The fragments corresponding to the first 50, 75, and 100 residues of UL12 were PCR amplified using the forward primer FP3 and the respective reverse primers RP50, RP75, and RP100. The PCR fragments were digested with HindIII and EcoRI and cloned into pBNUL12.5 to yield pBNUL12.5/H9262, pBNUL12.5/H9004, and pBNUL12.5/H9004. Table 1 lists the oligonucleotides used in the construction of the various truncation mutants of UL12.

Transfection and infection. Vero cells at 70 to 80% confluence plated the previous day were transfected with plasmids expressing the indicated proteins and using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. For immunoprecipitation, cells in 100-mm dishes were transfected with 4 μg of DNA. At 20 h posttransfection the cell monolayer was washed three times in phosphate-buffered saline (PBS) with 1 mM MgCl2 and 0.1 mM CaCl2, and processed either for immunofluorescence or immunoprecipitation as described below. Infections and viral growth analyses were carried out as described in reference 53 with minor modifications.

Immunofluorescence. Infected or transfected cells grown on glass coverslips were washed three times in PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed three times in PBS and were permeabilized for 10 min in 1% Triton X-100 in PBS. Fixed and permeabilized cells were washed three times in PBS and blocked overnight at 4°C using 3% normal goat serum (NGS) in PBS. Soluble proteins were removed with cold Cytoskeleton (CSK) buffer (10 mM HEPES-KOH [pH 7.4], 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and protease inhibitors) containing 0.5% Triton X-100 as described in reference 9. Preextracted cells were washed with PBS and then fixed with 4% paraformaldehyde. The fixed cells were processed for immunofluorescence as described in reference 32 and using the primary antibodies (anti-ICP8 antibody 39-S, 1:500; anti-UL12 antibody BWPUL12, 1:300; anti-Rad50 antibody 13B3, 1:100) diluted in 3% NGS for a period from 2 h to overnight. AlexaFluor anti-mouse and anti-rabbit antibodies (Molecular Probes) conjugated to fluorophores that excited at wavelengths of 488 and 647 nm and diluted in 3% NGS were used as the secondary antibodies. TO-PRO-3 (Molecular Probes) was used as a nuclear marker at a 1:500 dilution, along with the secondary antibody mix. Mounting of the coverslips, imaging on a Zeiss LSM 510 Meta confocal
microscope, and processing of images were performed as described in refer-
ence 32.

**Immunoprecipitation.** Infected or transfected cells were washed twice with
cold PBS at the indicated times. The cells were scraped into cold PBS containing
protease inhibitors and transferred into 15-ml tubes. Scraped cells were pelleted
for 10 min at 1,500 rpm at 4°C and frozen at −80°C. Frozen cells were thawed
on ice and resuspended in 450 μl of the IP buffer (1× PBS with 1% NP-40, 0.5%
sodium deoxycholate [SDC], and 0.1% SDS) with protease inhibitors and trans-
ferred to 1.5-ml tubes. The cells were further disrupted using a 1-ml syringe with
a 25-gauge 5/8 needle and incubated on ice for 1.5 h for complete lysis. Cell
extracts were clarified by centrifugation at 10,000 rpm for 10 min at 4°C, and
the supernatant was collected as the cell lysate. The following steps were carried out
at 4°C with constant agitation in the presence of protease inhibitors. Cell lysates
were precleared with 5 μl normal rabbit IgG (Santa Cruz Biotechnology) for
1.5 h, followed by the addition of 40 μl protein A-agarose beads (Santa Cruz
Biotechnology) for 1.5 h. Preleared lysates were collected after centrifugation
at 2,500 rpm for 10 min. A small portion of the precleared lysates (10 μl) was mixed
with SDS sample loading buffer and saved as the input sample. The remaining
precleared lysate was immunoprecipitated with 5 μl of UL12 antibody
(BWpUL12) overnight followed by the addition of 40 μl protein A-agarose
beads. After 24 h beads were collected by centrifugation at 2,500 rpm for 10 min
and washed in PBS with protease inhibitors. For samples that were subjected to
DNase treatment, the beads were incubated in a 50-μl buffer containing 5U of
DNase (Invitrogen) and 10 mM MgCl2 in PBS with protease inhibitors for 15 min
at room temperature. The DNase activity was tested on an identical preparation
to that used in the IP experiment to verify that the DNase was active (data not
shown). The beads were washed five times with 500 μl IP buffer prior to
centrifugation for 8 min at 2,500 rpm. After washing, the beads were resuspended
in 40 μl SDS sample buffer and stored at −20°C until SDS-PAGE separation
and immunoblotting.

**Immunoblotting.** Cell monolayers washed in PBS were lysed in buffer containing
0.5% (wt/vol) SDS and 0.05 M Tris, pH 8.0. Lysates were freeze-
thawed once and sonicated, and the cell debris was removed by centrifugation
before adding SDS sample buffer and processing for immunoblotting. Samples
boiled in SDS sample buffer were separated on a 4-to-20% SDS-PAGE gel and
transferred to a polyvinylidene difluoride membrane. Membranes were cut into
horizontal strips according to the molecular weight of the protein of interest
before probing with primary antibody. The membranes were immunoblotted and
developed as described in reference 46. The membranes were probed with primary antibodies (anti-ICP8 antibody [367] at 1:1,000; anti-UL12 antibody
[BWpUL12] at 1:5,000; anti-ultrabaculovirus UL12 antibody [BWpUL12] at 1:5,000; anti-Mre11 antibody [12D7] at 1:500; anti-Rad50 [13B3]; anti-Nbs1 at 1:800) diluted in
Tris-buffered saline with Tween 20 (TBST). Appropriate horseradish peroxidase-
conjugated secondary antibodies were used, and Super Signal West Pico chemi-
luminescence (Pierce) was used to detect the antigen-antibody complexes. For
probing proteins of similar molecular weights, membranes were either stripped
(25 mM glycine, 1% SDS with HCl; pH 2.0) or quenched (10 mM sodium azide
followed by centrifugation (1× PBS with 1% NP-40, 0.5% sodium deoxycholate [SDC], and 0.1% SDS) with protease inhibitors and transferred
inhibit the activity of SDC and contribute to that used in the IP experiment to verify that the DNase was active (data not
shown). The beads were washed five times with 500 μl IP buffer for 15 min
followed by centrifugation for 8 min at 2,500 rpm. After washing, the beads were resuspended in 40 μl SDS sample buffer and stored at −20°C until SDS-PAGE separation and immunoblotting.

**Protein purification.** UL12 was expressed in insect cells infected with recom-
binant baculovirus and purified to over 90% purity as described in reference 47. It has long been recognized that ICP8 localizes to replication compartments in infected
cells; however, initial immunofluorescence (IF) studies indi-
cated that UL12 localizes diffusely in the infected cell nucleus (47, 61). In these experiments, the diffuse staining made it
difficult to determine whether some UL12 colocalized with ICP8. In order to more easily visualize UL12 in replication
compartments, we preextracted infected cells with detergent
prior to fixation, as described previously (9, 67). Detergent
extraction has been reported to remove soluble proteins and
proteins that are tethered by protein-protein interactions while
leaving insoluble any chromatin-associated or matrix-associ-
ated proteins (9, 67). It has been reported that UL12 binds to
viral DNA (5), and we hypothesized that a subpopulation of
UL12 would be resistant to detergent extraction. In the top
panel of Fig. 1 (unextracted), infected cells were fixed and
permeabilized, and we observed that UL12 localized diffusely
throughout the nucleus, although some staining could be de-
tected in RCs stained with ICP8 antibodies. The bottom panel
shows infected cells that were preextracted with a cytoskeletal
buffer prior to fixation, and we observed that most of the
pan-nuclear UL12 staining was removed, leaving behind a sub-
population of UL12 that localized in replication compartments
stained with ICP8 antibodies.

The HSV-1 alkaline nuclease UL12 interacts with compo-
ments of the cellular MRN complex during infection. Several HRR proteins, including Rad51, RPA, Ku86, ATM, and
MRN, have been reported to localize to RCs (15, 60, 65, 68); however, their precise roles in the virus life cycle have not been
defined, nor is it clear how they are recruited. To address these
questions, we sought to identify cellular interaction partners of
UL12. UL12 was immunoprecipitated from mock-infected and
wild-type- and mutant HSV-1-infected Vero cells, and copre-
pititating proteins were analyzed by immunoblotting for DNA
repair and recombination factors. Figure 2A (input panel)
demonstrates that approximately equal levels of cellular pro-
teins were present in all the infected cell lysates. In lane 4,
coommunoprecipitation with anti-UL12 antibody shows that
MRN components Mre11, Rad50, and Nbs1 associate with
UL12 in KOS-infected cell extracts. Immunoblots were incu-
bated with antisera specific for other cellular factors involved
in HRR, such as Brca2, Rad51, ATM, and RPA32; however,
these proteins did not appear to communoprecipitate with
UL12 under these conditions (data not shown) and are not
considered further in this paper.

ICP8 has been reported to interact with Rad50 and Mre11
during HSV-1 infection (60). In order to determine whether
the interaction between the MRN components and UL12 seen
in Fig. 2 reflected a direct association with UL12 or were
mediated by ICP8, cells were infected with the ICP8 null virus
(HD-2). Figure 2A, lane 6, shows that Mre11, Rad50, and
Nbs1 still precipitated with UL12 even in lysates from cells
infected with the ICP8 null virus (HD-2), indicating that the
interaction between UL12 and MRN is independent of ICP8.
MRN components were not precipitated from cell lysates of
mock-infected or UL12 null virus (AN-1)-infected control
samples (Fig. 2A, lanes 2 and 3). Furthermore, lysates from
cells infected with the wild-type virus incubated with a nonsp-
fail to synthesize viral DNA, such as the viral polymerase null
Nbs1 is not phosphorylated in cells infected with viruses that
PAGE. These results are consistent with the observation that
which cell lysates were resolved for a longer time by SDS-
UL12 mutant virus can be more clearly seen in Fig. 2C, for
The Nbs1 mobility shift in cells infected with wild-type or
UL12 mutant viruses but not in mock- or HD-2-infected cells.
68). In Fig. 2A and 3A, Nbs1 mobility is shifted (indicating
and its downstream targets, such as Nbs1 and Chk2 (30, 55,
interact with MRN components. MRN proteins were not
precipitated in cells infected with wild-type and UL12 mutant viruses but
not in mock- or HD-2-infected cells.

The UL12 gene is encoded by a 2.3-kb mRNA, and embed-
ded within this RNA is a subgenic 1.9-kb mRNA encoding an
N-terminally truncated version of UL12, designated UL12.5
(34). In order to map the MRN interaction domain within
UL12, we first asked whether MRN proteins could be precip-
itated by full-length UL12 and UL12.5. We previously re-
ported the isolation of a mutant virus, ANF-1, which produces
only UL12.5 (34). Immunoprecipitation of lysates from ANF-
1-infected cells indicated that UL12.5 alone does not appear to
interact with MRN (Fig. 2A, lane 1). It is possible that UL12.5
does not interact with MRN because of its cytoplasmic local-
ization; however, experiments presented below indicate that
UL12.5 that has been directed to the nucleus is also unable to
interact with MRN components.

HSV-1 infection results in the activation of the ATM kinase
and its downstream targets, such as Nbs1 and Chk2 (30, 55,
68). In Fig. 2A and 3A, Nbs1 mobility is shifted (indicating phosphorylation) only in cells infected with wild-type and
UL12 mutant viruses but not in mock- or HD-2-infected cells.
The Nbs1 mobility shift in cells infected with wild-type or
UL12 mutant virus can be more clearly seen in Fig. 2C, for
which cell lysates were resolved for a longer time by SDS-
PAGE. These results are consistent with the observation that
Nbs1 is not phosphorylated in cells infected with viruses that
fail to synthesize viral DNA, such as the viral polymerase null
virus (HP66) (15, 68). Viral DNA synthesis would be expected
in cells infected with wild-type and UL12 mutant viruses but
not in mock- or HD-2-infected cells.

The UL12- MRN complex interaction is independent of
DNA and does not require other viral proteins. Both UL12 and
the MRN complex are known to interact with DNA, and we
next asked whether the observed interaction is mediated
through protein-protein interactions or indirectly through
DNA. In Fig. 3A, UL12 was precipitated from KOS-infected
cell lysates as described above except that the sample shown in
lane 3 was subjected to DNase treatment as described in Ma-
terials and Methods. Beads containing the immunoprecipitates
were incubated in the presence of DNase prior to the final
washes. If MRN components were precipitated with the UL12
antibody by virtue of their ability to interact with DNA, they
would be removed during the washing steps. The results shown
in Fig. 3A confirm that MRN proteins did not precipitate in
the absence of UL12, as MRN components were not present in
immunoprecipitates from mock-infected (lane 1) or AN-1-infec-
ted (lane 2) cell lysates. Figure 3A, lane 3, demonstrates that
the interaction between UL12 and components of the
MRN complex were not affected by DNase treatment, indicat-
ing that this interaction is not mediated by DNA. UL12 has
also been reported to interact with ICP8 (47, 60, 61); however,
the question of whether this interaction is dependent on DNA
has never been addressed. Figure 3A, lane 3, demonstrates that
UL12 interacts with ICP8 in a DNA-independent fashion.

Next we asked whether ICP8 or any other viral protein is
required for UL12 to interact with the components of the
MRN complex. Vero cells were transfected with plasmids that
express full-length UL12 and UL12.5 (pSAK UL12/UL12.5). 
Transfected cell lysates were immunoprecipitated with anti-
UL12 antibody. Figure 3B (lane 1) demonstrates that expres-
sion of UL12 and UL12.5 is sufficient to coimmunoprecipitate
the components of the MRN complex. MRN proteins were not
observed in the precipitates from cells transfected with an

![Image](http://jvi.asm.org/DownloadedFrom/vol84/issue8/12507/F1.png)
empty vector (Fig. 3B, lane 2). Taken together, these observations indicate that the interaction between UL12 and MRN is not mediated by other viral proteins or DNA.

UL12 directly interacts with the MRN complex in vitro. The experiments described above indicate that the UL12-MRN interaction is independent of DNA and viral proteins, but the experiments did not address whether the interaction could be mediated through other cellular proteins. We next asked if this interaction could be demonstrated in vitro with purified recombinant proteins in SPR assays. In Fig. 4, purified UL12 was immobilized on a biosensor chip and protein analytes of known concentrations were injected over the sensor as described in Materials and Methods. The binding of MRN at multiple concentrations (6, 12.5, 25, 50, and 100 nM) to immobilized UL12 is shown. Purified BSA at 100 nM did not bind to the MRN complex (data not shown), suggesting that the MRN-UL12 interaction is specific. These observations show that UL12 can bind directly to the MRN components even in the absence of other viral and cellular factors. The kinetics of the MRN-UL12 interaction at various concentrations was analyzed, and the rate of association (\(K_a = 4.266E+4 \text{ per ms}\)) and the rate of dissociation (\(K_d = 0.001323 \text{ per s}\)) was determined. Based on these values the apparent equilibrium dissociation constant (\(K_D\)) for the UL12-MRN interaction was determined to be 31.1 nM, which represents a fairly high-affinity interaction. To put this number in perspective, it is similar to other reported interactions between cellular DNA repair factors MutSβ and the WRN complex (8.8 nM) and between MutSβ and the WRN complex (35 nM) (49). In Saccharomyces cerevisiae, the interaction between MutSβ and the yeast ortholog of WRN, Sgs1, is required for Sgs1 function during DNA repair in vivo (13, 58).

Residues in the N terminus of UL12 are responsible for the interaction with the MRN complex. In order to more finely map the region of UL12 responsible for the MRN interaction, cells were transfected with a series of C-terminal truncation mutants of UL12, generated as described in Materials and Methods. To minimize problems associated with expression of globally misfolded proteins from truncation mutants, the end points of the truncations were selected based on secondary structure predictions and were chosen at positions predicted to be either unstructured or in loop regions between domains.
expected sizes were expressed by each of the truncations. A components in all lysates. Figure 5D confirms that proteins of the shows the presence of equal levels of the cellular MRN com-

ponents in all lysates. Figure 5D confirms that proteins of the MRN complex (Fig. 7C), confirming that specific residues re-

quired for interaction reside within the first 126 amino acids of UL12.

FIG. 3. The UL12-MRN interaction does not require DNA or other viral proteins. (A) Vero cells mock infected or infected with UL12 null virus AN-1 or the wild-type (WT) KOS virus, as indicated, were collected 6 hpi. Cell lysates were immunoprecipitated with anti-UL12 antibody. In lane 3 (+), the sample was subjected to DNase treatment during immunoprecipitation, while in lanes 1, 2, and 4 (−), immunoprecipitation was carried out in the absence of DNase. (B) Vero cells were transfected with either pSAK UL12/12.5 expressing the wild-type UL12 under the cytomegalovirus promoter (lane 1) or with pSAK empty vector (lane 2). Cells were collected 20 h posttransfection, and cell lysates were immunoprecipitated using anti-UL12 antibody and subjected to DNase treatment as described in Materials and Methods.

predicted to have structure. Figure 5A shows a diagram of the full-length UL12, UL12.5, and the series of truncations used in this experiment. In Fig. 5B, the truncations were tested for their expression and localization by IF. The fragment 1-104 was localized primarily to the nucleus with some diffuse cytoplasmic localization. All other C-terminal truncations, including 1-126, showed predominantly nuclear localization, confirming the presence of an NLS within the first 126 residues of UL12 (47).

In Fig. 5C, the immunoblot results with whole-cell lysates of Vero cells transfected with the various C-terminal truncations created in this study indicate that UL12 fragments of the expected size were synthesized from the respective truncation plasmids. The shortest fragment of 1-104 was detected around the expected size of 11.5 kDa (lane 2), and the full-length UL12 was detected around 75 kDa (lane 1). The other C-terminal truncations also produced fragments of expected sizes (lanes 3 to 6). Vero cells were transfected with the various constructs described above, and lysates were immunoprecipitated using anti-UL12 antibody and probed with antibodies for each of the components of the MRN complex. The input panel shows the presence of equal levels of the cellular MRN components in all lysates. Figure 5D confirms that proteins of the expected sizes were expressed by each of the truncations. A band of around 50 kDa was detected in all the lanes, likely due to nonspecific cross-reactions between IgG and the secondary antibody. The three bottom panels of Fig. 5D demonstrate that all constructs, with the exception of fragment 1-104, UL12.5, and the empty vector (lanes 2, 7, and 9) produced a fragment that could interact with components of the MRN complex. The observation that fragment 1-126 and larger fragments, but not fragment 1-104, interact with MRN components indicates that the interaction domain resides within the first 125 amino acids of UL12.

To map the interaction more finely, we used a series of N-terminal truncations provided by James Smiley (Fig. 6A) (6). In Fig. 6B, cell lysates of Vero cells transfected with the full-length and mutant constructs were precipitated using the anti-UL12 antibody. The input panel shows the presence of equal levels of the cellular MRN components in all lanes. The IP panel shows that the truncation mutants expressed proteins of the expected sizes (Fig. 6B, lanes 1 to 6) (6). As seen in Fig. 5D, a nonspecific band resulting from cross-reaction of the secondary antibody with the IgG was observed in all lanes. MRN components were not precipitated from lysates of cells transfected with the empty vector or plasmids expressing UL12.5, Δ100, Δ125, or M185 (Fig. 6B, lanes 3 to 6 and 8). On the other hand, full-length UL12 or fragments lacking the first 25 or first 50 residues of UL12 were able to precipitate similar levels of MRN (Fig. 6B, lanes 1, 2, and 7), indicating that the first 50 residues of UL12 are dispensable for the MRN interaction. The lack of apparent interaction between MRN and UL12.5 (lane 8) could be due to the fact that UL12.5 resides primarily in the cytoplasm and therefore may not be in direct contact with the nuclear MRN complex (47). A UL12.5 fragment fused with the SV40 NLS still failed to interact with the MRN complex (Fig. 7C), confirming that specific residues required for interaction reside within the first 126 residues of UL12.

The UL12-MRN interaction domain thus appears to reside between residues 50 and 125 of UL12. To more finely map the required residues, three UL12 constructs with internal deletions in the region between residues 50 and 125 (Fig. 7A) were constructed, and they all localized efficiently to the nucleus (Fig. 7B), indicating that residues 1 to 50 of UL12 are sufficient.
for nuclear localization. Vero cell lysates transfected with the indicated constructs were immunoprecipitated using anti-UL12 antibody, and the precipitates were immunoblotted with antibodies to each of the MRN components. The input panels demonstrate that equal amounts of the cellular MRN components were present in all lanes. The IP panel shows that equal levels of UL12 or the various fragments of UL12 were precipitated, and the bottom three panels indicate that the MRN complex can only be precipitated from cells transfected with full-length UL12 (lane 5). These results indicate that the region between residues 50 and 126 of UL12 contains the interaction domain for MRN, and even a relatively small deletion of 25 residues between residues 100 and 126 is sufficient to disrupt this interaction.

**DISCUSSION**

In this paper we report a direct interaction between UL12 and the components of the MRN complex as early as 2 hpi, and we have identified the region between residues 50 and 125 of UL12 to be necessary for this interaction. This observation suggests that viral and cellular recombination factors may act together during HSV-1 replication. Furthermore, we show that this interaction is not dependent on the presence of ICP8 or any other viral or cellular proteins, nor is it mediated by DNA, suggesting that this is a direct protein-protein interaction. Using SPR analysis we determined the apparent equilibrium dissociation constant ($K_D$) to be 31.1 nM.

Potential role of UL12 in the HSV-1 life cycle. The precise role of UL12 in viral infection is not known, and the analysis of UL12 mutant viruses has revealed a complex phenotype. UL12 null virus (AN-1) yield is decreased significantly (100- to 1,000-fold) in Vero cells, but viral DNA synthesis is only moderately affected (64). In AN-1-infected cells, some DNA is encapsidated; however, these capsids show a tendency to disgorge their contents, forming “A” or empty capsids, and are defective in nuclear egress (33, 53). DNA packaged in cells infected with UL12 mutant viruses is not infectious (43). One model that is
consistent with this complex phenotype is that UL12 is essential for the production of viral DNA that can be packaged productively into virions. Thus, accumulation of fragile genomes and capsids that fail to leave the nucleus in AN-1-infected cells could result from improperly initiated or improperly processed viral replication products (14, 33, 53). Interestingly, baculovirus encodes a related exonuclease, and null mutants in this gene display similar defects to those of AN-1 in DNA processing.

FIG. 6. The N-terminal 50 amino acids of UL12 are not required for the UL12-MRN interaction. (A) Schematic representation of the N-terminal deletion constructs used for the experiment shown in panel B. (B) Vero cells were transfected with plasmids expressing N-terminal truncations of UL12 as indicated (lanes 1 to 5), empty vector (lane 6), full-length UL12 (lane 7), or UL12.5 (lane 8). Cells were collected at 20 h posttransfection, and cell lysates were immunoprecipitated using anti-UL12 antibody as described in the legend for Fig. 5. The asterisk marks a nonspecific band arising due to the cross-reaction between the secondary antibody and IgG.

FIG. 7. The N-terminal amino acids 100 to 126 of UL12 are essential for the UL12 interaction with the components of the MRN complex. (A) Schematic representation of UL12 constructs used in the experiments shown in panels B and C. (B) Vero cells were transfected with plasmids expressing the N-terminal internal deletions of UL12 as indicated. Cells were fixed at 20 h posttransfection and tested for UL12 expression and localization by immunofluorescence. (C) Vero cells were transfected with plasmids expressing the indicated constructs described for B (lanes 1 to 4) or full-length UL12 (lane 5). Cells were collected at 20 h posttransfection and immunoprecipitated as described in the legend for Fig. 5.
resulting in aberrant nuclear egress and encapsidation (39). Thus, the role of viral exonucleases in DNA replication may be evolutionarily conserved among different dsDNA viruses. In addition, UL12 has been reported to play a role in resolving adenovirus replication intermediates generated when HSV-1 is used as a helper virus (38).

**Virusexoculeosis factors may collaborate to mediate viral recombination-dependent replication.** The overall mechanism of HSV-1 DNA replication and the formation of larger-than-unit-length concatemers are poorly understood. Although it was previously proposed that linear virion DNA circularizes in infected cells and that concatemers are generated by rolling circle replication (3, 18), several lines of evidence suggest that the fate of the viral genome in the infected cell and the mechanism of DNA replication is more complex than predicted by this model. It has been recognized for some time that the packaged HSV-1 genome contains nicks and gaps (69). If these nicks and gaps are left unrepairred, the passing of a replication fork would be expected to produce a DSB. Since DSBs are known to be highly recombigenic in all other systems studied (21), it is possible that DSBs generated during HSV-1 replication would stimulate recombination.

The two major kinases that sense DSBs are ATM and the DNA-protein kinase catalytic subunit (DNA-PKcs), stimulating the HRR and nonhomologous end-joining (NHEJ) pathways, respectively. DSBs are sensed by the MRN complex to signal ATM activation or by Ku70/Ku80 to activate DNA-PKcs. As mentioned above, ATM targets are phosphorylated in infected cells (30, 55, 56, 68), and virus yields are decreased in cells deficient in ATM, WRN, Chk2, Mre11, and Rad51 (29, 30, 37, 60), suggesting that these proteins may play a positive role in HSV infection. On the other hand, HSV-1 infection results in the degradation of DNA-PKcs in many cell types (28, 40), and viral yields increase severalfold in cells deficient for DNA-PKcs and Ku70 (40, 60), indicating that at least some components of NHEJ may be inhibitory for viral growth. Interestingly, cellular ubiquitin ligases RNF8 and RNF168 (components of the ATM pathway) are degraded during infection (31). Thus, HSV has apparently evolved a very complex interaction with the host DNA damage response pathways, activating some components and inactivating others.

The MRN complex, acts as a sensor of DSBs by binding to the sites of DNA damage and acting to recruit and activate the ATM kinase and its downstream targets (25, 26). Rad50 unwinds the DNA ends (36), enabling the recruitment of cellular nucleases such as Exo1 and Sae2, which play a role in resecting the broken ends (35). The 3'-single-stranded tail arising after resection serves as a substrate for the sequential recruitment RPA and Brca2, ultimately resulting in the formation of Rad51 nucleofilaments on the 3'-single-stranded tails. Rad51 nucleofilaments are capable of detecting homology in duplex DNA and participating in strand invasion to initiate HRR. It is interesting that, in addition to its role in the ATM-mediated HRR pathways, MRN has also been shown to participate in end resection in the classical NHEJ pathway (16) and the alternative (A-NHEJ) pathway (8). MRN also associates with telomeres and participates in the maintenance of normal telomere length (22, 72).

In summary, this paper provides evidence that the viral exonuclease UL12 can interact directly with the MRN components, important players in the DNA damage response of the cell. Taken together with our previous observation that UL12 and ICP8 can function as a two-subunit recombinase in vitro (48), these results may indicate that UL12 and ICP8 along with host recombination factors act to promote concatemer formation by stimulating homologous recombination. According to this scenario, UL12 in collaboration with ICP8 may steer the MRN complex toward the viral genome, resulting in end resection and the generation of 3' tails that can participate in strand invasion or strand annealing. Alternatively, since the MRN complex is known to play roles in multiple repair pathways, it is possible that the UL12-MRN interaction acts to regulate MRN, perhaps by influencing the repair pathway choice most beneficial for HSV. The pathway activated by HSV may influence the production of viral DNA concatemers that can be accurately processed and packaged to produce infectious virus. Current efforts are under way to prove the biological consequences of the UL12-MRN interaction.

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**REFERENCES**


