Role of the Herpes Simplex Virus Helicase-Primase Complex during Adeno-Associated Virus DNA Replication

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A subset of DNA replication proteins of herpes simplex virus (HSV) comprising the single-strand DNA-binding protein, ICP8 (UL29), and the helicase-primase complex (UL5, UL8, and UL52 proteins) has previously been shown to be sufficient for the replication of adeno-associated virus (AAV). We recently demonstrated complex formation between ICP8, AAV Rep78, and the single-stranded DNA AAV genome, both in vitro and in the nuclear HSV replication domains of coinfected cells. In this study the functional role(s) of HSV helicase and primase during AAV DNA replication were analyzed. To differentiate between their necessity as structural components of the HSV replication complex or as active enzymes, point mutations within the helicase and primase catalytic domains were analyzed. In two complementary approaches the remaining HSV helper functions were either provided by infection with HSV mutants or by plasmid transfection. We show here that upon cotransfection of the minimal four HSV proteins (i.e., the four proteins constituting the minimal requirements for basal AAV replication), UL52 primase catalytic activity was not required for AAV DNA replication. In contrast, UL5 helicase activity was necessary for fully efficient replication. Confocal microscopy confirmed that all mutants retained the ability to support formation of ICP8-positive nuclear replication foci, to which AAV Rep78 colocalized in a manner strictly dependent on the presence of AAV single-stranded DNA (ssDNA). The data indicate that recruitment of AAV Rep78 and ssDNA to nuclear replication sites by the four HSV helper proteins is maintained in the absence of catalytic primase or helicase activities and suggest an involvement of the HSV UL5 helicase activity during AAV DNA replication.

Adeno-associated virus (AAV)-based gene therapy vectors have shown increasing promise over recent years, and the development of herpes simplex virus (HSV)-based packaging systems for recombinant AAV vectors has increased interest in the molecular interactions of AAV with its helper virus (6, 12, 38). AAV displays a characteristic bipartite life cycle (27). In the absence of a helper virus, AAV integrates into the host cell genome with a high preference for a specific site on human chromosome 19 (15, 19, 32). Productive AAV replication ensues upon infection with an unrelated helper virus like adenovirus or HSV (27).

AAV contains a 4.7-kb linear single-stranded DNA (ssDNA) genome with two open reading frames, rep and cap, which are flanked by 145-bp inverted terminal repeats (ITRs). These repeats contain both the origins of replication and the packaging signal and also serve as cis elements for chromosomal integration. AAV cap codes for three capsid proteins, and rep codes for four overlapping, nonstructural proteins. The Rep proteins comprise Rep78; a C-terminally spliced variant, Rep68; and N-terminally truncated versions of these proteins, Rep52 and Rep40, respectively. The Rep proteins are required for most steps of the AAV life cycle including regulation of gene expression, chromosomal integration, and DNA replication (27). Rep78/68 has been shown to initiate AAV DNA replication at the hairpin-structured ITRs. It binds to the Rep-binding site, unwinds the ITR due to its ATP-dependent helicase activity, and introduces a single-strand nick at the adjacent terminal resolution site (trs) (5, 16, 34). Rep78/68 can therefore be regarded as an ori-binding protein that initiates AAV DNA replication.

We previously identified a subset of six out of seven HSV type 1 (HSV-1) DNA replication proteins that provide helper functions for productive AAV replication (38). Of these, the HSV-1 single-strand DNA-binding protein (ICP8, infected cell protein 8) encoded by the UL29 gene, and a heterotrimeric helicase-primase complex encoded by the genes UL5, UL8, and UL52 were found to constitute the minimal requirements for basal AAV replication. The two-subunit HSV-1 DNA polymerase (UL30/UL42) is not necessary but enhances AAV replication. The HSV-1 origin-binding protein (UL9) is dispensable (38). This finding is consistent with the lack of sequence similarity between the HSV-1 and AAV origins.

In a recent study we provided evidence that on ITR-containing AAV templates, Rep78 functionally replaced HSV UL9 as an origin-binding protein by recruiting ICP8, a key component of the HSV replication complex. By three-dimensional immunofluorescence and quantitative colocalization analysis, we showed that upon coinfection of HSV and AAV, Rep colocalized with ICP8 in HSV replication compartments in a manner strictly dependent on the presence of ITR-flanked single-stranded AAV genomes. The data were confirmed in vitro by the demonstration of ternary complex formation between Rep78, ICP8, and AAV ssDNA (12). This unusual AAV ssDNA-dependent mechanism for targeting to components of the HSV replication complex appears to be needed to direct the incoming AAV genome to subnuclear HSV replication compartments for DNA replication.
Based on these findings we decided to further investigate the functional role of the heterotrimeric helicase-primase complex as a component of the minimal system of four HSV replication proteins required for AAV DNA replication. The major question was whether UL5 and UL52 were needed by virtue of their enzymatic activities or, rather, as structural components of the helicase-primase complex to recruit AAV ssDNA and Rep to nuclear HSV replication sites. Since AAV DNA replication is initiated on the AAV-ITR, a self-primed DNA template, a need for catalytic HSV primase activity was not expected. Similarly, the role of the UL5-encoded ATPase and helicase was not obvious in the light of similar activities documented for Rep78/68 (16). Stracker et al. recently reported that UL5 or UL52 mutant proteins retained the ability to recruit Rep to ICP8 in HSV replication foci and to initiate AAV DNA replication. The data were interpreted to indicate that neither primase nor helicase was required enzymatically but, rather, as structural components of the nuclear replication complex (36). However, the specific amino acid substitutions within UL5 lay outside conserved helicase motifs, and their effects had not been tested biochemically (36). In addition, the UL52 mutants were modified in the C-terminal zinc finger motif, which affects not only the primase activity but also the UL5 helicase function of the trimeric complex (1, 4).

In this report we focused on the role of the catalytic activities of primase and helicase for AAV DNA replication. We used UL52 mutants containing single amino acid exchange substitutions within the primase catalytic site, which had previously been shown to be deficient in RNA primer synthesis, both in vivo and in vitro (18). The selected UL5 mutants contained single amino acid exchange substitutions in two conserved helicase motifs. These mutants had been shown previously to be deficient for HSV DNA replication in vivo (42). In vitro, the mutants displayed an ATPase- and helicase-deficient phenotype, whereas the DNA-binding and primase activities of a purified UL5-UL52 subcomplex were retained (11). Here we show that either protein is needed as a structural component of the minimal nuclear HSV subcomplex that recruits Rep and AAV ssDNA for AAV DNA replication. We additionally show that the HSV primase catalytic activity is not required for AAV DNA replication, whereas the presence of a functional UL5 helicase contributes to replication efficiency.

MATERIALS AND METHODS

Cell lines. HeLa cells were maintained as monolayers in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO2. BL-1 cells, which complement the HSV-1 null mutant hr114 (with a deletion of UL52 [AUL52]) (10), and L-2.5 cells, which complement mutant hr99 (AUL5) (43), were maintained in the presence of 250 μg/ml G418, as described previously (38).

Plasmids and DNA transfections. Human cytomegalovirus promoter-driven expression constructs for HSV-1 replication genes have been described previously (14). Single amino acid point mutants of UL5 or UL52 were generated by site-directed mutagenesis using a QuickChange II site-directed mutagenesis kit (Stratagene) and verified by DNA sequence analysis. In the mutants, UL5(G102V) and UL5(K103A), the conserved helicase motif I was affected, whereas motif IV was mutated in UL5(R345K). Two UL52 mutants, UL52(D628Q) and UL52(D630A), were generated in the DXD motif of the respective primase catalytic site.

HSV strains and infection. The HSV-1 strain KOS-derived UL5 null mutant, hr99 (AUL5), and the UL52 null mutant, hr114 (AUL52), have been described before (10, 43). The viruses were plaque purified, propagated, and titrated on the respective complementing cell lines, as previously described (38). HSV infection was performed on the cell lines described above, according to methods described previously (13).

DNA transfection. Plasmid transfections were performed by the calcium phosphate coprecipitation protocol, essentially as described before (17). For the immunofluorescence experiments, DNAs were transfected with Lipofectamine (Invitrogen) as suggested by the supplier. Transfection efficiencies were monitored by transfection of a parallel plate with a green fluorescent protein expression construct and quantification of fluorescent cells.

AAV DNA replication. A total of 2 × 10⁶ HeLa cells were plated on 10-cm diameter dishes. Four to six hours later the cells were cotransfected with pTAV2-2, containing the wild-type (wt) AAV-2 genome, and combinations of plasmids encoding the four HSV-1 helper proteins UL29 (ICP8), UL5, UL52, and UL8, as indicated in the figure legends. In the experiments in which helper function was provided by virus infection, the cells were infected after 16 h. The cells were harvested 40 h posttransfection, and low-molecular-weight DNA was extracted by a modification of the Hirt extraction protocol as previously described (37). Equal proportions of DNA were digested with DpnI and separated on a 0.7% agarose gel for 20 h at 40 V. Fractionated DNA was transferred to a nylon membrane (Amersham) and UV cross-linked. The blots were hybridized to a [32P]-labeled AAV HincII fragment (nucleotide position 1386 to 2976).

To determine the extent of AAV replication, AAV-reactive bands were quantified by Phosphorimager analysis. The counts in the bands representing the AAV replication intermediates, RF1 and RF2, were added. An equivalent region of control lane on the same blot was used as a baseline value.

HSV replication assays. HSV DNA replication was quantified as DpnI-resistant replication of a transfected HSV-1 ori₅-carrying plasmid, pH10, as outlined before (14). Briefly, expression plasmids for each of the seven HSV-1 DNA replication proteins were cotransfected together with the ori₅-carrying plasmid as described above. Forty hours later cells were harvested, and total genomic DNA was extracted. Equal aliquots of DNA samples (6 μg) were digested with DpnI and XbaI, separated in 0.7% agarose gels, and blotted onto nylon membranes as described above. HSV DNA replication was detected with a [32P]-labeled subfragment of ori₅, as described previously (14).

Monoclonal antibodies against UL5. The HSV helicase-primase complex (UL5-UL8-UL52 complex) was purified from Spodoptera frugiperda cells infected with a recombinant baculovirus that expresses all three proteins (AcUL5-UL8-UL52), essentially as described previously (7). The purified protein was used to immunize female BALB/c mice, and hybridoma lines secreting monoclonal antibodies (MAbs) were generated as described previously (26). MAbs reactive with the complex were initially identified by an enzyme-linked immunoorbent assay and subsequently assessed for their ability to interact with the individual components by Western blotting using extracts of S. frugiperda cells infected with baculoviruses expressing UL5, UL8, or UL52 (35). One antibody reactive to UL5 (MAB 376) was selected. This MAb specifically detected UL5 expression in cells transfected with the set of expression plasmids for HSV replication genes.

Western blot analysis. HSV DNA replication levels were examined in cell extracts prepared in parallel to the cells used for the determination of AAV and/or HSV replication. A total of 1 × 10⁶ HeLa cells were plated on 6-cm-diameter dishes and transfected as described above. At 40 h posttransfection protein extracts were prepared and analyzed by gel electrophoresis and Western blotting and stained with UL5 monoclonal antibody (MAB 376) essentially as described previously (17).

Immunofluorescence. HeLa cells were grown on coverslips and transfected with a combination of AAV and HSV plasmids. At 19 h posttransfection cells were fixed, permeabilized, and stained by double-label immunofluorescence for AAV Rep and HSV ICP8. Fixation was performed with 3.7% formaldehyde-phosphate buffered saline (PBS) for 30 min, followed by permeabilization in 1% Triton X-100–PBS for 10 min after a brief wash in PBS. For immunofluorescence staining, cells were incubated with a mixture of a rabbit polyclonal anti-Rep antibody (gift of J. Trempe) at a final dilution of 1:80 and a monoclonal anti-ICP8 antibody (HB-8180 from ATCC, Rockville, Md.) at a final dilution of 1:370. Antibodies were diluted in PBS-2% fetal calf serum for 60 min. The coverslips were washed three times in PBS for 5 min and then reacted for 30 min with a mixture of the secondary antibodies, fluorescein-labeled goat anti-rabbit immunoglobulin G, each diluted 1:800 in PBS-2% fetal calf serum. All incubations were performed at room temperature. Coverslips were mounted in polyvinyl alcohol (Elvanol) containing 1% 1,4-diazabicyclo-2,2,2-octane. Confocal fluorescence microscopy was performed using a Zeiss confocal laser scanning microscope with a 63× oil differential interference contrast objective (numerical aperture of 1.4). Single-image slices represent cellular cross sections of 0.6 μm.
RESULTS

Colocalization of Rep and ICP8 in the presence of the HSV helicase-primase complex is dependent on the presence of single-stranded AAV DNA. In a previous study using three-dimensional immunofluorescence and quantitative colocalization analysis, we demonstrated that upon coinfection of AAV and HSV, Rep78 colocalizes with ICP8 (UL29) in nuclear DNA replication compartments in a manner strictly dependent on the presence of ITR-flanked single-stranded AAV genomes (12).

To define the minimal components for ssDNA-dependent colocalization of Rep and ICP8, HeLa cells were cotransfected with plasmids specifying wt AAV-2 with and without the ITRs and cotransfected with expression plasmids for the minimal four HSV helper genes, UL29, UL5, UL8, and UL52. Nineteen hours later the cells were fixed and stained with an anti-Rep polyclonal antiserum and an anti-ICP8 MAb, as outlined in Materials and Methods. The nuclear distribution pattern of Rep and ICP8 was analyzed by immunofluorescence and confocal microscopy as shown in Fig. 1. ICP8 was detected in nuclear replication foci upon coexpression of the heterotrimeric helicase-primase complex with both AAV constructs (Fig. 1, panels A and B). The pattern of ICP8 staining was essentially similar to that seen previously when just the HSV proteins were expressed (24). Rep colocalized with ICP8 in the presence of the wt AAV construct (Fig. 1, panels A.I and A.II). In contrast, upon cotransfection of an ITR-deleted AAV genome, Rep expression remained diffuse, and the ability to localize to ICP8 foci was lost (Fig. 1, panel B). ITR-deleted AAV genomes lack 145-bp origin sequences at either genome end and, although AAV Rep is properly expressed, cannot, therefore, initiate AAV ssDNA synthesis. In summary, the previously described AAV ssDNA dependence of the colocalization of ICP8 and Rep upon HSV infection (12) is fully retained in the presence of the minimal four HSV helper genes. In control transfections where either UL52 or UL5 was omitted, preventing formation of the heterotrimeric helicase-primase complex, ICP8 remained diffuse, as previously described (24). As a consequence, ICP8-positive nuclear replication foci cannot be assembled. This leads to a diffuse nuclear staining pattern for ICP8, similar to the pattern seen for Rep (Fig. 1, panels C and D).

Functional requirement for UL52 primase during AAV DNA replication. Single amino acid exchange mutations of UL52 altering the primase catalytic site at amino acid positions 628 or 630, respectively, were shown before to lead to a selective and complete loss of primase function, both in vitro and in vivo (18). The ability of the mutated UL52 proteins to form a heterotrimeric complex with UL5 and UL8, which exhibited the associated helicase and ATPase activities, nevertheless remained intact (8, 18). These mutants therefore meet the prerequisites for investigating the requirement for primase catalytic activity on an AAV template, separate from other UL52-associated functions. To study the functional role of UL52 primase during AAV DNA replication, expression plasmids for wt UL52 or the UL52 catalytic site mutants were cotransfected with a plasmid for wt AAV-2. In addition, either the cells were infected with an HSV-UL52 null mutant, hr114 (38), or, in a second approach, plasmids for the wt forms of the remaining three of the minimal four HSV helper genes, namely UL5, UL8, and UL29 (ICP8), were included in the transfections.

In the first approach Hirt-DNAs were prepared 24 h postinfection with hr114, and Southern blots of DpnI-digested DNAs were hybridized to an AAV probe as shown in Fig. 2A. The appearance of the typical AAV replication intermediates, RF1 (4.7 kb) and RF2 (9.4 kb), is indicative of processive AAV DNA replication. The null mutant, hr114, replicates AAV efficiently when complemented with a wt UL52 construct (Fig. 2A, lane 2). Although controls documented that hr114 was

FIG. 1. AAV ITR-dependent colocalization of Rep and ICP8 at nuclear HSV replication sites. HeLa cells were grown on glass coverslips and transfected with plasmids for the HSV helicase-primase complex (UL5, UL8, and UL52) and ICP8 (UL29). (A.I and A.II) Cells were cotransfected with an AAV wt plasmid. (B) Cells were transfected with a plasmid for an ITR-deleted AAV mutant lacking the terminal 145-bp ori sequences, required for the generation of AAV ssDNA. (C and D) Cells were cotransfected with plasmids for wt AAV (+ITR), ICP8, and the helicase-primase complex proteins omitting either UL5 (D) or UL52 (C) as indicated. Cells were fixed and stained with a polyclonal antibody reactive to Rep and a MAb reactive to ICP8 as outlined in Materials and Methods. Confocal microscopy for the detection of colocalization of ICP8 and Rep was conducted as outlined in results. The expression of Rep and ICP8 was detected by secondary antibodies conjugated to rhodamine (represented in red) or fluorescein (represented in green), respectively. Merged images of 0.6 μm cell slices reveal colocalization in yellow. Panel A.I shows images taken at a low cutoff value also applied in panels B to D. Images of the same cell taken at a higher cutoff value are presented in panel A.II to better reveal the complete colocalization of ICP8- and Rep-positive foci. Virtually no Rep fluorescence could be detected for the cells shown in panels B to D with the cutoff value chosen for panel A.II.
entirely negative for HSV DNA replication (data not shown) as previously reported by others (10, 13), hr114 alone retained some activity for AAV DNA replication (Fig. 2A, lane 5). However, neither of the UL52 catalytic site mutants could complement AAV replication to the level observed with wt UL52 (Fig. 2A, lanes 6 and 7). To exclude variability of HSV expression levels as a possible reason for the differences in AAV DNA replication, cell extracts from the same experiment were analyzed by Western blotting for the expression of UL5 as an internal HSV marker protein. As displayed in Fig. 2B the HSV-infected samples showed comparable levels of UL5 expression, with the exception of lane 5, where UL52 is lacking.

The consistently reduced level of UL5 expression probably reflects protein instability in the absence of complex formation with UL52.

In further experiments, the requirement for UL52 primase function to support AAV DNA replication was analyzed in the simpler system of the four HSV helper protein assay. In the absence of any UL52 protein, AAV DNA replication was undetectable (Fig. 2C, lane 3). In contrast, in all transfections that included either wt UL52 or the catalytic-site mutants, similar levels of AAV DNA replication were observed (Fig. 2C, lanes 2, 4, and 5). To confirm their phenotypes, the UL52 mutants were tested for complementation of HSV oriS replication initia-
prepared and hybridized to a 32P-labeled HSV-ori S fragment. Replication and DpnI, as outlined in the legend of Fig. 2. A Southern blot was used for the analysis of AAV DNA replication. Similar UL5 protein levels were compared on Western blots of cell extracts prepared, in parallel, from the cells transfected with a plasmid containing HSV-ori S (plasmid pH 10) and expression constructs for the seven HSV DNA replication proteins. In lanes 6 and 7 the wt UL5 plasmid was replaced by UL52 catalytic site mutants, as indicated. Total genomic DNA was extracted 40 h later and digested with XbaI and DpnI, as outlined in the legend of Fig. 2. A Southern blot was prepared and hybridized to a 32P-labeled HSV-ori S fragment. Replicated ori S is detected as a DpnI-resistant band of 4.3 kb, as indicated by the arrow. Plasmid pH 10 digested with either XbaI alone or with a combination of XbaI and DpnI served as markers run in parallel.

Fig. 3. HSV UL52 mutants are deficient in ori S-dependent HSV DNA replication. HeLa cells were cotransfected with a plasmid containing HSV-ori S (plasmid pH 10) and expression constructs for the seven HSV DNA replication proteins. In lanes 6 and 7 the wt UL5 plasmid was replaced by UL52 catalytic site mutants, as indicated. Total genomic DNA was extracted 40 h later and digested with XbaI and DpnI, as outlined in the legend of Fig. 2. A Southern blot was prepared and hybridized to a 32P-labeled HSV-ori S fragment. Replicated ori S is detected as a DpnI-resistant band of 4.3 kb, as indicated by the arrow. Plasmid pH 10 digested with either XbaI alone or with a combination of XbaI and DpnI served as markers run in parallel.

As expected, the UL52 mutants were entirely deficient for HSV ori S replication (Fig. 3).

These results demonstrate that the primase catalytic activity is not required for AAV DNA replication when supported by the minimal four HSV genes, which is consistent with our initial assumption that the AAV genome can function as a self-primed ssDNA template. The presence of a UL52 polypeptide is nevertheless required for AAV DNA replication in this assay, presumably as a structural component of the helicase-primase complex.

To demonstrate that the UL52 mutants retain the ability to form a trimeric complex in vivo, colocalization experiments similar to those described in the legend of Fig. 1 were performed. As shown in Fig. 4, the primase catalytic site mutants are indistinguishable from wt UL52 in their ability to support formation of ICP8-positive nuclear foci and to recruit colocalizing Rep78 in the presence of the wt AAV genome (Fig. 4, panels A, B, and C). Although similar ICP8 foci were formed in the presence of the ITR-deleted AAV genome, Rep78 remained diffuse and did not colocalize with ICP8 (Fig. 4, panels G, H, and I). In summary, AAV ssDNA-dependent colocalization of ICP8 and Rep is dependent on the presence of UL52 protein but does not require its primase activity.

In contrast to the above finding with the minimal set of HSV helper functions, UL52 primase activity was required for fully efficient AAV replication upon HSV infection. A possible explanation for this finding is that primase mutants with catalytic defects are unable to recruit the two-component HSV DNA polymerase to HSV replication foci and, consequently, cannot proceed to form fully mature HSV replication compartments (3). These compartments are assumed to support AAV replication better than the so-called “prereplicative” sites formed by the minimal set of helper functions. Support for this assumption was provided when we compared AAV DNA replication upon transfection of the minimal four HSV genes to that after infection with HSV mutants. As shown in Fig. 2 (panel D), AAV DNA replication in the presence of the four HSV helper proteins was less than 1% of that achieved in cells transfected with a wt UL52 plasmid and superinfected with hr114. Significantly, AAV DNA replication initiated by HSV infection in the absence of functional UL52 protein remained detectable at approximately 17% of the level observed in the presence of wt UL52. These differences cannot readily be explained by differences in transfection efficiencies, which were assayed in parallel in all experiments and were found to be in the range of 30 to 40% positive cells.

Functional requirements for the UL5 helicase during AAV DNA replication. The UL5 encoded protein carries ATPase and helicase active site. However, in vitro helicase activity can only be measured when UL5 forms a complex with UL52. In cotransfection experiments UL5 was absolutely required for AAV DNA replication initiated by the set of HSV replication functions (38). In view of the fact that AAV Rep displays both ATPase and helicase activities, it was not obvious why AAV would need the enzymatic activity of an additional ATPase and helicase for its replication.

To evaluate this point we first tested the need for UL5 expression in the context of HSV infection. We show here, for the first time, that an HSV strain with a deletion of UL5, hr99 (43), is significantly reduced for AAV DNA replication as demonstrated by the strong complementation achieved by transfection of a wt UL5-expressing plasmid (Fig. 5A, compare lanes 5 and 2). Quantification of the AAV RF1 and RF2 intermediates shows that the level of replication in the presence of hr99 infection alone achieved only 12% of the AAV replication achieved when the complementing plasmid expressing wt UL5 was included (Fig. 5E).

The UL5 helicase sequence comprises several amino acid motifs that are highly conserved among a large family of helicase proteins of various species (42). Point mutations of critical amino acids within the so-called Walker motifs were shown to abolish helicase activity, both in vitro and in vivo (11). In addition, HSV mutants carrying single amino acid substitutions in either helicase motif I or motif IV were shown to be negative for HSV DNA replication (42). UL5 expression constructs containing these mutations in helicase motif I and motif IV were therefore similarly tested for their ability to enhance AAV replication in hr99-infected cells. All three helicase mutant plasmids complemented hr99 for AAV DNA replication to a certain degree (Fig. 5A, lanes 6 to 8). However, in repeated experiments with various time courses and multiplicities of infection, the maximum level of replication was only about one-third of that achieved with UL5 (Fig. 5E). To exclude the possibility that the mutant proteins were less efficiently expressed, UL5 protein levels were compared on Western blots of cell extracts prepared, in parallel, from the cells used for the analysis of AAV DNA replication. Similar UL5...
protein levels were detected with all four plasmids (Fig. 5B, lanes 2, 6, 7, and 8), whereas the controls, including non-complemented hr99-infected cells, proved negative for UL5 expression (lanes 3 to 5).

The requirement for UL5 helicase activity for AAV DNA replication was next tested in the context of the minimal HSV helper functions. Cells were cotransfected with the wt AAV construct and expression constructs for ICP8, UL8, UL52, and wt or mutant UL5 proteins. Replacement of wt UL5 by mutants in either of the helicase motifs greatly decreased AAV DNA replication (Fig. 5C, compare lanes 4, 5, and 6 with lane 2). Nevertheless, in contrast to the cells that received no UL5 expression (lanes 3 to 5).

To exclude the possibility that the mutants were defective in the assembly of the HSV replication foci, they were also examined in an immunofluorescence colocalization assay. Figure 4 shows that in the presence of a cotransfected wt AAV plasmid, all three mutants behaved indistinguishably from wt UL5 in terms of the formation of discrete ICP8 foci to which Rep efficiently colocalized (Fig. 4, panels D, E, and F). In the absence of AAV ssDNA synthesis, colocalization was lost, and nuclear Rep expression remained diffuse. ICP8, however, re-

**FIG. 4.** Colocalization of AAV Rep and HSV ICP8 is retained in the presence of HSV helicase or primase catalytic site mutants. The experiment was performed as outlined in the legend of Fig. 1. HeLa cells were grown on glass coverslips and transfected with plasmids encoding the HSV helicase-primase complex (UL5/8/52) and ICP8 (UL29). Panels A and G show wt HSV proteins, and in panels B to F and H to L, the wt HSV UL5 or UL52 proteins were replaced as indicated by catalytic site mutants. Plasmids specifying wt AAV or the ITR-deleted AAV mutant were also present in panels A to F and G to L, respectively. The anti-rabbit antibody reactive to Rep conjugated to rhodamine is shown in red and the anti-mouse antibody reactive to ICP8 conjugated to fluorescein is represented in green. Merged foci appear in yellow. A lower cutoff value was chosen for panels G to L compared to panels A to F to display the diffuse nuclear Rep staining pattern of low intensity in the absence of the AAV-ITRs.
tained its ability to form nuclear replication foci, irrespective of the UL5 mutant expressed (Fig. 4, panels J, K, and L). In summary, AAV ssDNA-dependent colocalization of ICP8 and Rep in the presence of the minimal four HSV helper proteins is retained upon expression of helicase-deficient UL5 mutant proteins. These data taken together suggest that the consistently higher AAV replication rates in the presence of wt UL5 are due to a direct involvement of the UL5 helicase activity at some stage during AAV DNA replication.

DISCUSSION

In this report we studied the need for the catalytic activities of the HSV UL52 primase and UL5 helicase during AAV DNA replication. The following observations were made utilizing expression constructs containing single amino acid point mutations within the UL5 and UL52 catalytic sites in complementary HSV infection and DNA transfection approaches. (i) In extension of our previous finding that in HSV-infected cells
ICP8 colocalizes to AAV Rep in a manner strictly dependent on AAV ssDNA (12), we show here that ssDNA-dependent colocalization is fully retained upon coexpression of only ICP8 and the helicase-primase complex. (ii) Catalytic activity of HSV primase is dispensable for AAV DNA replication in the minimal four helper protein assay. (iii) In contrast, UL5 helicase activity is required for the full extent of AAV DNA replication. (iv) Irrespective of their catalytic functions, mutant primase activities of the holoenzyme were unaffected, as was the ability of the active site mutants to support leading-strand synthesis on a preformed, forked DNA substrate (8, 18). The retained ability of the active site mutants to support leading-strand DNA synthesis (18) is consistent with the current model for DNA replication of the self-primed single-stranded AAV genome by leading-strand DNA polymerase δ (29).

In a recent report by Stracker et al., UL52 zinc finger mutants as components in the four HSV helper protein assay, with some mutants displaying significant impairments (36). We independently demonstrated that another UL52 zinc finger mutant, CC3,4AA (1), exhibited reduced levels of AAV DNA replication but retained the ability to mediate nuclear colocalization of ICP8 and Rep (H. Slanina and R. Heilbronn, unpublished observations). In summary, these findings argue that it is the ability of UL52 to promote assembly of functional foci containing Rep, AAV DNA, and the other HSV helper proteins, rather than primase catalytic activity, that is important for productive AAV DNA replication.

In the context of HSV infection the situation is slightly more complex. AAV DNA replication occurs in the absence of UL52 protein at approximately 17% of the level seen when complementing wt UL52 is present. In addition, the UL52 catalytic site mutants did not enhance AAV DNA replication to the level reached with wt protein. The latter finding may be explained by the observation that primase mutants with catalytic defects are unable to couple to the two-component HSV DNA polymerase (3), the expression of which enhances AAV DNA replication, as shown previously (38). The lack of coupling to the HSV polymerase is linked to the defects in the maturation of the HSV replication foci from the so-called stage IIIA to the stage IIIB. Formation of stage IIIB foci involves incorporation of the two-component HSV DNA polymerase and cellular proteins including promyelocytic leukemia protein. The occurrence of HSV-induced AAV DNA replication in the absence of UL52 protein suggests that an alternative cellular pathway for AAV DNA replication may exist, which appears to predominate when the HSV replication complex cannot be properly assembled. It is intriguing to speculate that this pathway represents that by which genotoxic agents can induce AAV DNA replication (39, 40). The recent finding that ICP8 colocalizes with not only AAV Rep but also cellular replication protein A (36) is consistent with such an alternative replication pathway. This pathway, utilizing cellular DNA polymerase, is obviously less efficient than AAV replication in the presence of wt HSV infection (12, 38) and may explain our finding that the extent of AAV DNA replication in the presence of the minimal four HSV replication proteins represents only a few percent of that measured upon HSV infection.

Role for the UL52 primase in AAV replication. The HSV UL52 primase catalytic subunit of the heterotrimeric helicase-primase complex represents a well-studied enzyme, both in vivo and in vitro. Single amino acid exchanges in the critical UL52 DXD motif led to a loss of catalytic activity (8, 18), whereas the interactions with the UL5 and UL8 components of the complex were retained. DNA-binding, ATPase, and helicase activities of the holoenzyme were unaffected, as was the ability of the mutant primase to support displacement synthesis on a preformed, forked DNA substrate (8, 18). The retained activity of the active site mutants to support leading-strand DNA synthesis (18) is consistent with the current model for DNA replication of the self-primed single-stranded AAV genome by leading-strand DNA polymerase δ (29).

In a recent report by Stracker et al., UL52 zinc finger mutants were tested for AAV DNA replication (36). These mutants display a more complex phenotype, affecting not only the primase but also the ATPase and helicase activities of the heterotrimeric complex (1-4). In this report we show that the UL5 helicase activity is required for fully efficient AAV DNA replication. This may explain why variable AAV DNA replication levels were previously observed upon cotransfection of UL52 zinc finger mutants as components in the four HSV helper protein assay, with some mutants displaying significant impairments (36). We independently demonstrated that another UL52 zinc finger mutant, CC3,4AA (1), exhibited reduced levels of AAV DNA replication but retained the ability to mediate nuclear colocalization of ICP8 and Rep (H. Slanina and R. Heilbronn, unpublished observations). In summary, these findings argue that it is the ability of UL52 to promote assembly of functional foci containing Rep, AAV DNA, and the other HSV helper proteins, rather than primase catalytic activity, that is important for productive AAV DNA replication.

In the context of HSV infection the situation is slightly more complex. AAV DNA replication occurs in the absence of UL52 protein at approximately 17% of the level seen when complementing wt UL52 is present. In addition, the UL52 catalytic site mutants did not enhance AAV DNA replication to the level reached with wt protein. The latter finding may be explained by the observation that primase mutants with catalytic defects are unable to couple to the two-component HSV DNA polymerase (3), the expression of which enhances AAV DNA replication, as shown previously (38). The lack of coupling to the HSV polymerase is linked to the defects in the maturation of the HSV replication foci from the so-called stage IIIA to the stage IIIB. Formation of stage IIIB foci involves incorporation of the two-component HSV DNA polymerase and cellular proteins including promyelocytic leukemia protein. The occurrence of HSV-induced AAV DNA replication in the absence of UL52 protein suggests that an alternative cellular pathway for AAV DNA replication may exist, which appears to predominate when the HSV replication complex cannot be properly assembled. It is intriguing to speculate that this pathway represents that by which genotoxic agents can induce AAV DNA replication (39, 40). The recent finding that ICP8 colocalizes with not only AAV Rep but also cellular replication protein A (36) is consistent with such an alternative replication pathway. This pathway, utilizing cellular DNA polymerase, is obviously less efficient than AAV replication in the presence of wt HSV infection (12, 38) and may explain our finding that the extent of AAV DNA replication in the presence of the minimal four HSV replication proteins represents only a few percent of that measured upon HSV infection.

Role for HSV UL5 helicase in AAV DNA replication. The experimental data presented in this report suggest that in contrast to UL52 primase, which is catalytically dispensable, a functional UL5 helicase enhances AAV replication, both in the context of HSV infection and upon transfection of the four HSV helper proteins. This conclusion is based upon the inefficient complementation of AAV DNA replication exhibited by three independent UL5 mutants with single amino acid exchanges in conserved helicase motifs affecting DNA-dependent ATPase and helicase activities in vitro (11). Helicase motif I is directly involved in ATP-binding and hydrolysis, whereas motif IV appears to be preferentially involved in the coupling of ATP hydrolysis with DNA-binding during the process of DNA unwinding by the helicase-primase complex (11). We show here that UL5 helicase motif I and motif IV mutant proteins support the formation of nuclear foci, similar to those described before for wt UL5 (22, 24). In addition, all three mutants retained the ability to complement ssDNA-dependent colocalization of ICP8 and Rep (12). These observations indi-
cate that the observed intermediate level of AAV DNA replication seen in both assays is likely a direct result of an enzymatic deficiency of the UL5 mutants.

**Model of HSV-mediated processive AAV DNA replication.**

The UL5 protein is characterized by the presence of specific conserved motifs, which identify it as a member of helicase superfamily SF1, and by the fact that it functions as part of a heterotrimeric complex. AAV Rep78/68, in contrast, belongs to the SF3 class of DNA helicases and acts as a hexameric complex (33). In addition, Rep86/78 acts as 3' to 5' helicase (16, 41), whereas UL5 unwinds in 5' to 3' direction (21). HSV encodes a second helicase unwinding in 3' to 5' orientation, the origin-binding protein UL9, which was shown previously to be dispensable for AAV DNA replication (38). UL5 is characterized by strong and sequence-specific binding to the HSV origins of replication, but it displays only limited unwinding activity (20). UL9 serves to recruit ICP8 to the HSV origins, which in turn recruits the heterotrimeric helicase-primase complex (9, 25). This subassembly of HSV proteins is necessary and sufficient to initiate the formation of nuclear HSV “pre-replication” foci in vivo (22, 23). Further recruitment of the two-component HSV-1 DNA polymerase by the primase then allows viral DNA synthesis to be initiated (3). This cascade of interactions, initiated by UL9 binding, enables UL9 to attract all components of the HSV replication complex to assemble at the HSV origins of replication.

AAV Rep likely functions as an analogous ori-binding protein on the AAV-ITR. Rep78/68 binds site specifically to the Rep-binding site on the AAV-ITR. As shown before, ICP8 forms a ternary complex with Rep78 and single-stranded AAV DNA (12). By interaction with ICP8 bound to AAV ssDNA, additional replication factors can be recruited. Incorporation of the heterotrimeric helicase-primase complex and possibly cellular DNA polymerase δ (28) may then be sufficient to allow initiation of DNA replication. It is possible that UL5, as the helicase component of the heterotrimeric complex, enhances AAV replication by acting in coordination with AAV Rep by mechanisms comparable to those thought to coordinate action of UL5 and UL9 helicases on an HSV template.

AAV DNA represents a self-primed ssDNA template that is replicated by continuous leading-strand DNA synthesis. Thus, replication of the complementary strand is not expected to require helicase activity at all. We therefore hypothesize that UL5 helicase probably plays a role at a later stage. The generation of higher-order, multimeric AAV DNA intermediates during continuous leading-strand DNA synthesis requires unwinding of the double-stranded AAV DNA template. UL5 helicase, as a component of the heterotrimeric complex, was shown to prefer ssDNA over double-stranded DNA with a preference for “forked” DNA substrates (11). Rep78/68 binds ssDNA without obvious preference for a replication fork (41). Additionally, or alternatively, UL5 helicase may be operative in DNA recombination on an AAV template. Two recent in vitro studies showed that ICP8 in conjunction with the helicase-primase complex promotes ssDNA-binding, ssDNA invasion, and ssDNA strand exchange, suggestive of a role for the minimal HSV four proteins in HSV DNA recombination (30, 31). In line with current models of processive AAV DNA replication, it can be speculated that DNA recombination plays a role in ongoing AAV DNA replication where large multimeric replication intermediates are resolved for packaging of the monomeric, ssDNA (27). A helicase with complementary orientation directionality to Rep78/68 could use this mechanism for the resolution of AAV DNA replication intermediates. The ability to study the individual enzymatic functions of the heterotrimeric helicase-primase complex in the context of the previously described ternary complex comprising ICP8, Rep, and AAV ssDNA (12) should help to further elucidate the mechanisms by which the HSV DNA replication proteins function on the heterologous AAV origin.

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**REFERENCES**


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