Inhibition of Herpes Simplex Virus Type 1 Replication by Adeno-Associated Virus Rep Proteins Depends on Their Combined DNA-Binding and ATPase/Helicase Activities

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Adeno-associated virus (AAV) is a widespread, nonpathogenic human parvovirus with a unique biphasic life cycle. In the absence of a helper virus, AAV establishes a latent infection in the host cell mediated either by site-specific integration of the viral genome into human chromosome 19 or by episomal persistence of circularized virus genomes (reviewed in reference 53). In the presence of helper viruses such as a herpesvirus, adenovirus (Ad), or papillomavirus, AAV is rescued from latency and undergoes lytic replication. The AAV genome is a single-stranded DNA (ssDNA) of 4,680 nucleotides, which is packaged into an icosahedral capsid with a diameter of 20 nm. The AAV genome harbors two open reading frames (ORFs), rep and cap, which are flanked by two inverted terminal repeats (ITRs) containing viral origins of DNA replication. The cap ORF is transcribed from the p40 promoter and encodes the capsid proteins VP1, VP2, and VP3, which differ in their N termini due to alternative start codons. The rep ORF encodes the Rep proteins, which are expressed in four different forms due to transcription from two different promoters, p5 and p19, and alternative splicing at an intron at the C-terminal end. The different Rep proteins are termed Rep40, Rep52, Rep68, and Rep78 according to their apparent molecular weight. The Rep proteins are involved in diverse processes in the viral life cycle, such as DNA replication, regulation of gene expression, genome packaging, and site-specific integration (reviewed in reference 56). The biochemical activities of Rep required for AAV DNA metabolism include site-specific DNA-binding and endonuclease activities, as well as non-site-specific ATPase/helicase activities. While the ATPase/helicase activity is in all four Rep proteins, the site-specific DNA-binding and endonuclease activities are present only in the large Rep proteins Rep68 and Rep78 (Fig. 1A) (18, 74, 88, 91). It has recently become clear that the Rep proteins also have a variety of effects on the host cell, the overall purpose of which likely is the creation of a cellular environment favorable for AAV replication. These effects of the Rep proteins on the cell include DNA damage, cell cycle arrest, apoptosis, and inhibition of signal transduction by the protein kinases PKA and PRKX. Rep78 can induce a complete S-phase arrest, which is mediated by the protein’s ability to induce cellular DNA damage combined with its ability to bind to Cdc25A (5). The induction of DNA damage was postulated to require Rep endonuclease activity, while the interaction with Cdc25A was shown to be dependent on the zinc finger motifs in the C-terminal domain present in Rep52 and Rep78 (5) (Fig. 1A). The binding of Rep to Cdc25A prevents the latter from activating its substrates Cdk1 and Cdk2, resulting in the accumulation of hypophosphorylated (i.e., active) pRb, which in turn limits the cell’s progression through S phase (5, 65). In addition and probably related to its ability to arrest the cell cycle, Rep has been shown...
to induce p53-independent apoptosis via its DNA-binding and ATPase/helicase activities (66). Finally, the ability of Rep to inhibit the protein kinases PKA and PRKX, both members of the cyclic AMP (cAMP) signal transduction pathway, results in decreased expression of cAMP-responsive genes and contributes to Rep-mediated inhibition of Ad replication (16, 22, 23). Inhibition of PKA and PrKX was shown to depend on a PKI-like motif in the C-terminal domain present in Rep52 and Rep78 (Fig. 1A) (67).

Herpes simplex virus type 1 (HSV-1) is a complete helper virus for productive AAV replication (9). HSV-1 is a widespread human pathogen whose biphasic life cycle is characterized by lytic infection in epithelial cells of the mucosa and latent infection in the innervating sensory neurons. The HSV-1 genome is a 152-kb double-stranded DNA (dsDNA) and encodes approximately 80 gene products, which are expressed in a temporally regulated cascade comprising immediate-early (IE), early, and late phases (reviewed in reference 76). The HSV-1 helper factors for AAV replication act at two stages of the AAV life cycle, i.e., rep gene expression and DNA replication. The HSV-1 IE proteins ICP0, ICP4, and ICP22 synergistically transactivate rep expression, an effect which was shown to be particularly important for rescue of latent AAV (3, 28). The HSV-1 IE protein ICP27, in contrast, is inhibitory for AAV replication but essential for HSV-1 replication (3, 63). The HSV-1 helicase-primase complex (UL5/UL8/UL52), the ssDNA-binding protein ICP8 (UL29), and the DNA polymerase complex (UL30/UL42) are thought to become part of the AAV DNA replication complex, possibly through a direct interaction between ICP8 and Rep (3, 33, 71, 75, 80, 82). Although the helicase-primase complex and ICP8 are sufficient to support minimal levels of AAV DNA replication (82), the entire set of helper factors is required for the full helper activity (3).

The requirement of AAV for coinfection with a helper virus for productive replication inevitably leads to competition for cellular resources as well as for the helper factors themselves, which often are essential for both AAV and the helper virus. The fact that AAV inhibits the replication of its helper viruses Ad (10–12) and HSV (4, 31) suggests that it has developed strategies to influence this competition for its own advantage. In order to be successful, such strategies must limit the replication of the helper virus without affecting the synthesis of the helper factors required for AAV replication. In the case of HSV-1 as the helper virus, the helper factors are all expressed with IE or early kinetics preceding viral DNA replication (3, 81, 82). As expected for a successful strategy of helper virus inhibition, AAV limits mainly HSV-1 DNA replication and late gene expression, while IE and early gene expression is only marginally reduced (31). Similar observations were also made with Ad as the helper virus (77). Previous research from our laboratory has shown that AAV and HSV-1 replication proceeds in spatially separate replication compartments (RCs),
which recruit distinct sets of viral and cellular proteins. ICP8 was found in both HSV-1 and AAV RCs, although with differential staining patterns (31). It is therefore conceivable that the competition between HSV-1 and AAV RCs for ICP8 and possibly other HSV-1 replication factors contributes to inhibition of HSV-1 replication in coinfected cells. However, we and others have shown that the mere presence of AAV Rep protein in the absence of replicating AAV DNA is sufficient for inhibition of HSV-1 RC formation and HSV-1 oriS plasmid replication (31, 32). Similar observations were made for Ad in that Rep was shown to inhibit the maturation of Ad RCs (83).

Rep-mediated inhibition of HSV-1 has also been observed during the production of chimeric AAV/HSV gene delivery vectors. Such vectors are based on helper virus-free HSV-1 ampiclon vectors that incorporate the AAV ITRs flanking the transgene, as well as the AAV rep gene. HSV/AAV hybrid vectors combine the large transgene capacity of HSV-1 with the site-specific integration machinery of AAV and therefore support stable, long-term transgene expression (reviewed in reference 29). The presence of the rep gene on the hybrid vector genome, however, negatively affects vector replication, resulting in significantly reduced titers of vector stocks, and thus hampers efficient hybrid vector production (35).

To date, the molecular mechanisms underlying the inhibition of HSV-1 replication by AAV Rep protein remain poorly understood. We therefore further characterized Rep-mediated inhibition of HSV-1 replication in the present study. To this end, we investigated the effects of Rep on HSV-1 gene expression and DNA replication and assessed the Rep activities required for inhibition of HSV-1 replication using a panel of wild-type and mutant Rep proteins lacking defined domains and activities. Our data show that Rep mainly affects HSV-1 DNA replication and that the inhibitory effect of Rep depends on its DNA-binding and ATPase/helicase activities but not on its endonuclease activity. The Rep activities required for inhibition of HSV-1 replication precisely coincide with those required for the induction of cellular DNA damage and apoptosis, suggesting that these three processes are closely linked. Notably, the presence of Rep induced the hyperphosphorylation of a DNA damage marker, replication protein A (RPA), which has been reported not to be normally hyperphosphorylated during HSV-1 infection and to be sequestered away from HSV-1 replication compartments during infection. (84–86).

Finally, we demonstrate that the execution of apoptosis is not required for inhibition of HSV-1 replication and that the hyperphosphorylation of RPA per se is not inhibitory for HSV-1 replication, suggesting that these two processes are not directly responsible for the inhibition of HSV-1 replication by Rep.

**MATERIALS AND METHODS**

**Cell culture and viruses.** Vero and Vero 2-2 cells (72) were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. For culturing Vero 2-2 cells, 500 μg/ml G418 was included in addition.

HSV-1 strain F was grown and titers were determined in Vero cells. Briefly, confluent monolayers of Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.1 PFU and incubated until the cytopathic effect (CPE) reached 100%. The cells were then lysed by three cycles of freeze and thawing and cellular debris removed by centrifugation for 10 min at 1,900 × g. The cleared lysate was titrated on Vero cells using the Spearman-Karber method. AAV type 2 (AAV2) was grown and titrated as described previously (46). UV inactivation of AAV2 with 254-nm UV light at a dose of 960 ml/cm² was carried out in a UVC 500 UV cross-linker (Hoefer Pharma Biotech). The virus suspension was placed onto the lid of the 96-well tissue culture plate in order to form a layer of about 1 to 2 mm and kept on ice during the inactivation procedure. HSV-1 C12 is a recombinant HSV-1 strain SC16 containing a human cytomegalovirus (HCMV) IE1 enhancer/promoter-driven enhanced green fluorescent protein (EGFP) expression cassette in the US (g) locus and was kindly provided by S. Elatahitou (University of Cambridge, Cambridge, United Kingdom).

**Plasmids.** pdClDNA3.1+ was purchased from Invitrogen and pEGFP-N3 from Clontech. pHSVVGFP, an HSV-1 amplicon vector containing the EGFP-coding sequence under the control of the HSV-1 IE 4/5 promoter, was described previously (1). pAAV2GFP, a recombinant AAV (rAAV) plasmid containing the EGFP-coding sequence under the control of the HCMV IE1 enhancer/promoter flanked by the AAV2 ITRs, was described previously (35). HSVAdpα2C274kn and pEBHC/PC27 together represent a replication-competent, packaging-defective HSV-1 genome and were described previously (64). Plasmids pCM-UL5, -UL8, -UL9, -UL29, -UL30, and -UL42, expressing the corresponding HSV-1 replication factors from the HCMV IE1 enhancer/promoter (34), were kindly provided by R. Heilbronn (Free University of Berlin, Berlin, Germany). Plasmid pd-UL52, expressing HSV-1 UL52 from the short eukaryotic initiation factor 1 α subunit (seIF1a) promoter, was described previously (5). pAAVlacO, an rAAV plasmid containing 40 lac operator (lacO) repeats flanked by the AAV2 ITRs, was described previously (25). Plasmid pRep, containing the AAV2 rep ORF under control of its native p5 and p19 promoters, was described previously (35). Plasmid p52-EYFP/lacI, expressing enhanced yellow fluorescent protein (EYFP) linked to lac repressor protein (LacI) (79), was kindly provided by D. L. Spector (Cold Spring Harbor Laboratory, NY). For construction of plasmids pdClDNARep52, pdClDNARep68, pdClDNARep78, and pdClDNARep78(K340H), the corresponding AAV2 rep ORFs were PCR amplified from plasmids pBP52, pBP68, pBP78, and pBP78K340H (5, 65) (kindly provided by C. Berthet and P. Beard, ISREC, Lausanne, Switzerland) with primers containing BglIII and XbaI restriction sites and inserted between the BamHI and XbaI restriction sites of pdClDNA3.1+.

For construction of pdClDNARep68(Y156F), the 359-bp SacII-BamHI fragment from plasmid pCl-HisRep68Y156F (27) containing the Y156F mutation was transferred into the rep ORF in plasmid pdClDNARep78. For construction of pdClDNAEGFP, the EcoRI-NotI fragment from pEGFP-N3 containing the ORF encoding enhanced green fluorescent protein (EGFP) was introduced between the EcoRI and NotI sites of pdClDNA3.1+.

**Antibodies.** (i) Primary antibodies. The mouse anti-HSV-1 ICp8 monoclonal antibody (MAb) 7281 and the mouse anti-HSV-1 UL42 MAb Z1F11 were kindly provided by R. D. Everett (MRC Virology Unit, Glasgow, United Kingdom), the rabbit anti-HSV-1 ICp8 polyclonal antibody (Pab) 43 (41) by D. M. Knappe (Harvard Medical School, Boston, MA), the mouse anti-HSV-1 VP16 MAb LP1 (54) by A. Minson and H. Browne (University of Cambridge, Cambridge, United Kingdom), the rabbit anti-HSV-1 gC PAb R47 (17) by G. H. Cohen and R. J. Howard (The University of Pennsylvania, Philadelphia, PA), the mouse anti-HSV-1 UL8 MAb P77 (27) by L. J. Chesebro and P. Beard, ISREC, Lausanne, Switzerland, the mouse anti-HSV-1 VP2 PAb AGV103 by G. Elliott (Imperial College London, London, United Kingdom), and the rabbit anti-AAV Rep PAb 78 (78) by J. P. Trempe (Medical University of Ohio, Toledo, OH). The mouse anti-HSV-1 UL5 MAb 376, the mouse anti-HSV-1 UL8 MAb 817 (49, 55), the mouse anti-HSV-1 UL9 MAb 13924 (55), the mouse anti-HSV-1 UL30 MAb 13429 (48), and the mouse anti-HSV-1 UL52 MAb 14462 (48) were kindly provided by N. D. Stow (MRC Virology Unit, Glasgow, United Kingdom). The mouse anti-HSV-1 ICp4 MAb was purchased from Advanced Biotechnologies, the mouse anti-HSV-1 ICp8 MAb 10A3 from Abcam, the mouse anti-AAV Rep MAb clone 303.9 from Fitzgerald Industries International, the mouse anti-GFP MAb JL-8 from Clontech, the mouse antiactin MAb clone AC-47 from Sigma, the mouse anti-phonophos-phem-ATM S1961 MAb clone 10H11.E12 from Rockland Immunocchemicals, the rabbit anti-phonophos-PAP32 S4/S8 PAb BL647 from Bethyl Laboratories, and the mouse anti-phonophos-H2AX S139 PAb B40 from Bethyl Laboratories.

(ii) Secondary antibodies. Goat anti-mouse IgG(H+L)-Alexa Fluor 488 (AF488), goat anti-mouse IgG(H+L)-AF568, goat anti-mouse IgG(H+L)-AF647, goat anti-rabbit IgG(H+L)-AF594, goat anti-rabbit IgG(H+L)-AF488, goat anti-rabbit IgG(H+L)-AF658, and F(ab)2 fragment of goat anti-rabbit IgG(H+L)-AF594 were purchased from Molecular Probes (Invitrogen), rabbit anti-mouse IgGwhelone molecule)-peroxi-dase from Sigma, and goat anti-rabbit IgG(H+L)-horseradish peroxidase (HRP) from Southern Biotechnologies.

**Transfection.** Cells seeded on the previous day were transfected with Lipofectamine Plus reagents (Invitrogen) according to the manufacturer’s protocol, except for the data presented in Fig. 5, for which Lipofectamine2000 (Invitrogen) was used. Transfections were carried out in three different plate
forms: 6-cm plates (Southern blot analysis), 12-well plates (Western blot analysis), and 24-well plates (pHSVGF packaging, HSV-1 C12 replication, and annexin V and immunofluorescence staining). The relative amounts of DNA used for transfection of different plate formats were as follows: 6-cm plate, 100%; 12-well plate, 20%; and 24-well plate, 10%. For easier comparability of DNA amounts between the different assays, the DNA amounts indicated throughout correspond to values normalized to the 6-cm format.

Western blot analysis. For the results shown in Fig. 1B, Vero cells were transfected with 0.5 μg of the individual pcDNA Rep plasmids or empty pcDNA vector together with 0.05 μg pEGFP-N3. For the results presented in Fig. 2D, Vero 2-2 cells were transfected with 0.5 μg pHSVGFV, 2 μg HSVVpaca272kam, and 0.2 μg pEBHICP27 together with the indicated amounts of pcDNA.Rep plasmids or empty pcDNA vector. For the results presented in Fig. 3A, Vero cells were transfected with 0.5 μg pHSVGFV, 0.2 μg each of pCM-UL5/8/9/30/42 plasmid and 0.4 μg of plDNA-L52 together with the indicated amounts of pcDNA.Rep plasmids or empty pcDNA vector. At 48 h after transfection, the cells were lysed and processed for Western blot analysis as described previously (31). Primary antibodies were used at the following dilutions: mouse anti-AAV Rep Mab clone 303/9, 1:200; mouse anti-GFP Mab JB-8, 1:8,000; mouse anti-HSV-1 ICP4 Mab, 1:10,000; rabbit anti-HSV-1 ICP8 PAB 4-83, 1:5,000; mouse anti-HSV-1 ICP8 Mab 10A3, 1:1,000; mouse anti-HSV-1 UL5 Mab 376, 1:1,000; mouse anti-HSV-1 UL5 Mab 917, 1:500; mouse anti-HSV-1 UL9 Mab 13924, 1:500; mouse anti-HSV-1 UL30 Mab 13429, 1:500; mouse anti-HSV-1 UL52 Mab 14462, 1:200; mouse anti-HSV-1 UL42 Mab Z1F11, 1:1,000; mouse anti-HSV-1 VP16 Mab LPI, 1:5,000; rabbit anti-HSV-1 VP22 AGV031, 1:10,000; rabbit anti-HSV-1 gC PAB R47, 1:10,000; and mouse antiaction MAb clone AC-47, 1:10,000. Secondary antibodies were used at the following dilutions: rabbit-anti-mouse IgG(whole molecule)-peroxidase, 1:10,000; goat anti-rabbit IgG(H + L)-HRP, 1:10,000.

Live visualization assay for AAV DNA replication. The live visualization assay for AAV DNA replication has been described previously (25). Briefly, Vero cells grown on coverslips were transfected with 0.5 μg of the pAAVlacO replicon, 0.1 μg of the EYFP-LacI-expressing plasmid pSV2-EYFP/lacI, and 0.1 μg of the individual pcDNA.Rep plasmids or empty pcDNA vector. For the following day, the cells were transfected with 0.5 μg of pEBHICP27 together with the indicated amounts of pcDNA.Rep plasmid with empty pcDNA vector. For the results presented in Fig. 6E, Vero cells were transfected with 0.5 μg of pHSVGFV, 0.1 μg of pEBHICP27, and 0.4 μg each of pCM-UL5/8/9/30/42 and p-DUL52 plasmids together with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector. For the results presented in Fig. 3C, Vero 2-2 cells were transfected with 0.5 μg of pHSVGFV, 0.1 μg of pEBHICP27, and 0.4 μg each of pCM-UL5/8/9/30/42 and p-DUL52 plasmids together with the indicated amounts of pcDNA.Rep78 or empty pcDNA vector. Three days later, the cells were harvested and extrachromosomal DNA extracted by the procedure described by Hirt (36). The DNA was digested as indicated in the text, separated on 1% agarose gels, and transferred to positively charged nylon membranes (Hybond N+; Amersham). Hybridization with a digoxigenin (DIG)-labeled probe specific for the EFGP-coding sequence and immunological detection using an alkaline phosphate-conjugated anti-DIG antibody and chemiluminescence substrate (CDP Star) were performed as described by the supplier (Roche). The DIG-labeled probe was produced by PCR amplification of the ORF encoding EFGP from plasmid pEGFP-N3 using the PCR DIG probe synthesis kit (Roche). The PCR product was purified with the QIAquick PCR purification kit (Qiagen) before being used for hybridization.

Results

Expression of wild-type and mutant AAV Rep proteins. For high-level, constitutive expression of Rep proteins, the corresponding ORFs were placed under control of the HCMV IE1 enhancer/promoter in the pcDNA3.1+ expression vector. A map of the Rep proteins used in this study is shown in Fig. 1A, and their activities are summarized in Table 1. Briefly, we compared the full-length Rep78 protein with (i) Rep52, which lacks the N-terminal site-specific DNA-binding and endonuclease activities (60); (ii) Rep68, which lacks the C-terminal PKI-like and zinc finger motifs (22, 37); (iii) Rep78(Y156F),

Table 1. Summary of Rep constructs and their activities

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<th>Rep protein</th>
<th>DNA binding</th>
<th>Endonuclease on:</th>
<th>ATPase/helicase</th>
<th>C-terminal domain</th>
<th>Replication of AAV</th>
<th>Inhibition of HSV-1</th>
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which contains a mutation in rolling-circle replication motif 3 (RCR3), thereby abolishing site-specific endonuclease activity (20, 73); and (iv) Rep78(K340H), which contains a mutation in the consensus nucleoside triphosphate (NTP)-binding site, thereby abolishing ATPase/helicase activity (13, 43). The loss of ATPase/helicase activity in the K340H mutant results in a loss of endonuclease activity on dsDNA substrates, because endonuclease activity on dsDNA requires prior unwinding via the helicase activity (50, 60). Of note also is that the K340H mutant still retains endonuclease activity on ssDNA substrates, while the Y156F mutant lacks endonuclease activity on both ssDNA and dsDNA substrates (20, 73) (Table 1).

To assess the expression levels from the different constructs, Vero cells were transfected with the Rep-encoding plasmids together with a plasmid expressing EGFP, which served as transfection and loading control. Western blotting with Rep- and EGFP-specific antibodies showed that all Rep proteins had the expected sizes and were expressed at comparable levels (Fig. 1B). To ascertain the functionality of the expressed Rep proteins, we tested their ability to support AAV DNA replication. To this end, we employed a previously described live cell visualization assay for AAV replication, in which AAV RCs are visualized by the binding of an EYFP-LacI fusion protein to lacO repeats present in a recombiant AAV (rAAV) genome (25). The rAAV genome and the plasmid encoding the EYFP-LacI fusion protein were transfected together with the individual Rep-encoding plasmids, while the helper factors for AAV replication were provided by infection with HSV-1. Transfected cells were identified by EYFP-LacI expression, while HSV-1-infected cells were identified by staining for HSV-1 ICP8, which localizes to HSV-1 and AAV RCs. AAV DNA replication is known to require Rep DNA-binding, endonuclease, and ATPase/helicase activities (7, 8, 38, 39, 50).

Figure 2E shows that transfection of the Rep78 plasmid led to a pronounced, dose-dependent inhibition of HSV-1 DNA replication (the nonessential \( \gamma_1,34.5 \) gene is also deleted due to deletion of pac). In the presence of trans-expressed ICP27, the HSV-1 helper BAC represents a replication-competent, packaging-defective HSV-1 genome providing helper functions for replication and packaging of HSV-1 ampiclon plasmids (59, 64). Although Vero 2-2 cells express ICP27, the cotransfection of an ICP27-expressing plasmid is required for high titers of HSV-1 ampiclon vector stocks (64). Packaged HSV-1 ampiclon stocks were harvested at 72 h after transfection and the titers determined by enumerating EGFP+ cells upon infection of Vero cells. By providing the helper functions from a packaging-defective HSV-1 genome, we ensured that replication of the HSV-1 ampiclon could occur only in transfected cells and that HSV-1 infection could not spread to neighboring, untransfected cells. The effects of Rep on HSV-1 replication could therefore readily be assessed by cotransfection of Rep encoding plasmids together with the HSV-1 ampiclon and HSV-1 helper DNA. As shown in Fig. 2A, the cotransfection of increasing amounts of the construct encoding the full-length Rep protein, Rep78, led to a pronounced, dose-dependent inhibition of HSV-1 replication, demonstrating that the HSV-1 ampiclon packaging assay provides a sensitive means to assess the effects of Rep on HSV-1 replication efficiency.

We have previously demonstrated that AAV-mediated inhibition of HSV-1 replication in the context of AAV/HSV-1 coinfection occurs mainly at the stage of DNA replication (31). In order to find out if this also holds true for inhibition of HSV-1 by expression of Rep78, we assessed the amounts of replicated HSV-1 ampiclon DNA by Southern blotting. Cells transfected with increasing amounts of the Rep78 construct together with HSV-1 ampiclon DNA (pHSVGFp) and HSV-1 helper DNA were harvested 72 h later for extraction of extra-chromosomal DNA. To distinguish between transfected and newly synthesized HSV-1 ampiclon DNA, the DNA was digested with DpnI, which selectively cleaves the transfected DNA of bacterial origin (i.e., Dam-methylated DNA). The DNA was also cleaved with HindIII, which cuts once on pHSVGFp and therefore reduces the concatemeric replication products into linear monomers. HSV-1 ampiclon DNA was detected with a probe specific for the EGFP-coding sequence. Figure 2E shows that transfection of the Rep78 plasmid led to a dose-dependent reduction of the DpnI-resistant HSV-1 ampiclon replication products, demonstrating that Rep inhibits HSV-1 DNA replication.

Inhibited HSV-1 DNA replication can theoretically result either from blocks at the stages of IE or early gene expression or from a block of DNA replication itself. To determine which of the above possibilities holds true for our experimental system, we analyzed HSV-1 gene expression in the presence of Rep78. Cells transfected with increasing amounts of the Rep78 construct together with HSV-1 ampiclon DNA (pHSVGFp) and HSV-1 helper DNA were harvested 48 h later and subjected to Western analysis with antibodies specific for HSV-1 proteins of all kinetic classes. As shown in Fig. 2D, expression of Rep78 resulted in a dose-dependent inhibition of the levels of the IE protein ICP4; the early proteins UL9, ICP8, UL5, and UL42; and the late proteins VP16, VP22, and glycoprotein C (gC). Of note, the inhibitory effect on the late proteins was more pronounced than that on the IE and early proteins.

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FIG. 2. (A) Effects of Rep78 on HSV-1 productivity. Vero 2-2 cells were transfected with pHSVGFp, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. The pHSVGFp amplicon vectors were harvested 72 h later and titrated on Vero cells. (B) Screening of Rep proteins for inhibitory activity on HSV-1 replication. Vero 2-2 cells were transfected with pHSVGFp, fHSVΔpacΔ27Δkn, pEBHICP27, and 0.5 μg each of the pcDNA.Rep constructs or empty pcDNA vector. The pHSVGFp amplicon vectors were harvested 72 h later and titrated on Vero cells. (C) trans-complementation of Rep DNA-binding and ATPase/helicase activities. Vero 2-2 cells were transfected with pHSVGFp, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of the pcDNA.Rep constructs or empty pcDNA vector. The pHSVGFp amplicon vectors were harvested 72 h later and titrated on Vero cells. The data in panels A to C are shown as means ± standard deviations (SD) from triplicate experiments. (D) Effects of Rep on HSV-1 protein levels. Vero 2-2 cells were transfected with pHSVGFp, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pcDNA.Rep78 or empty pcDNA vector. At 48 h after transfection, the cells were lysed and analyzed by Western blotting with antibodies specific for AAV Rep and HSV-1 ICP4, UL9, ICP8, UL5, UL42, VP16, VP22, and gC. Detection of actin served as a loading control. The asterisk indicates an unspecific band. (E) Effects of Rep78 on HSV-1 DNA replication. Vero 2-2 cells were transfected as described for panel D. At 72 h after transfection, extrachromosomal DNA was extracted, digested with DpnI and HindIII, and analyzed by Southern blotting with a DIG-labeled probe specific for the EGFP-coding sequence on pHSVGFp. The linearized pHSVGFp amplicon products (DpnI resistant) and the DpnI fragments of the transfected pHSVGFp plasmid (DpnI sensitive) are indicated. One or 10 ng of linearized pHSVGFp plasmid was loaded as a positive control and size marker (M). (F) Effects of Rep on AAV DNA replication. Vero 2-2 cells were transfected with pAV2GFP, fHSVΔpacΔ27Δkn, pEBHICP27, and the indicated amounts (in μg) of pRep, pcDNA.Rep78, or empty pcDNA vector. At 72 h after transfection, extrachromosomal DNA was extracted, digested with DpnI, and analyzed by Southern blotting with a DIG-labeled probe specific for the EGFP-coding sequence on rAV2GFP. Rescued monomeric (ITRm) and dimeric (ITRd) rAV2GFP replication intermediates (DpnI resistant) and the DpnI fragments of the transfected pAV2GFP plasmid (DpnI sensitive) are indicated. One nanogram of the BglII-excised ITR cassette from pAV2GFP was loaded as a positive control and size marker (M).
also attempted to detect UL8, UL52, and UL30, but the levels expressed in HSV-1 helper BAC-transfected cells were too low for detection by Western blotting.)

To assess if the observed inhibition of HSV-1 gene expression and DNA replication is specific for HSV-1 and not due to general cytotoxicity of Rep, we assessed whether the amounts of Rep78 plasmid used were compatible with AAV DNA replication. General cytotoxic effects of Rep would be expected to inhibit both HSV-1 and AAV DNA replication. To this end, we performed an replication assay equivalent to that shown in Fig. 2E, except that an rAAV plasmid (pAV2GFP) was used instead of the HSV-1 amplicon. AAV DNA replication from pAV2GFP results in the rescue of DpnI-resistant mono- and multimeric ITR cassettes (i.e., rAAV replication intermediates). rAAV DNA was detected with a probe specific for the EGFP-coding sequence. Transfection of a plasmid encoding all four Rep proteins under control of the native p5 and p19 promoters, pRep, served as a control for physiological Rep levels. The blot in Fig. 2F shows that all the amounts of Rep78 plasmid used supported AAV DNA replication, while no AAV DNA replication was observed in the absence of Rep. Specifically, the levels of the AAV replication intermediates were comparable between pRep and 0.1 to 0.5 μg of pcDNA.Rep78, while the levels were slightly reduced with 1 μg of pcDNA.Rep78, indicating that very high Rep levels are inhibitory to AAV replication (Fig. 2F).

The seven essential HSV-1 replication factors, UL9, ICP8, UL5/8/52, and UL30/42, are all expressed with early kinetics (81). We therefore aimed to find out if the observed inhibition of the HSV-1 early gene expression (Fig. 2D), in particular the expression of the replication factors, was responsible for the observed inhibition of HSV-1 DNA replication or if, alternatively, Rep rather had a direct inhibitory effect on HSV-1 DNA replication. If the former was the case, Rep would not be expected to inhibit HSV-1 DNA replication if abundant levels of HSV-1 replication factors were provided by overexpression from constitutive promoters. If, however, Rep acted directly on HSV-1 DNA replication, inhibition of HSV-1 DNA replication would be expected even in the presence of overexpressed replication factors. We first tested the effect of Rep78 on the levels of the seven HSV-1 replication factors when expressed from the HCMV IE1 enhancer/promoter (UL9, ICP8, UL5, UL8, UL30, and UL42) or the short eukaryotic initiation factor 1 subunit (sEIF1α) promoter (UL52). As shown in Fig. 3A, Rep78 had no significant inhibitory effect on the levels of the HSV-1 replication factors expressed from constitutive promoters. We next assessed the effect of Rep78 on pHSVGFP amplicon replication mediated by HSV-1 helper BAC and overexpressed HSV-1 replication factors (Fig. 3B) or by overexpressed HSV-1 replication factors alone (Fig. 3C). In either situation, Rep78 significantly inhibited the replication of the HSV-1 amplicon, demonstrating that Rep inhibits HSV-1 DNA replication even in the presence of abundant HSV-1 replication factors.

Taken together, these findings show that the expression of Rep78 from the HCMV IE1 enhancer/promoter strongly inhibits HSV-1 productivity (Fig. 2A), HSV-1 gene expression (Fig. 2D), and HSV-1 DNA replication (Fig. 2E). The inhibitory effects of Rep78 on HSV-1 appeared to be specific and not due to general cytotoxicity, since the Rep levels employed were compatible with AAV DNA replication (Fig. 2F). Furthermore, the data show that the inhibition of HSV-1 DNA replication by Rep was not due to the reduction of the expression of the HSV-1 replication factors but presumably was caused by a direct inhibitory effect of Rep on HSV-1 DNA replication (Fig. 3). The finding that Rep levels compatible with efficient AAV DNA replication are strongly inhibitory for HSV-1 DNA replication (Fig. 2E and F) further corroborates this notion, since both AAV and HSV-1 DNA replication depend on the HSV-1 replication factors (3).

Rep DNA-binding and ATPase/helicase activities are required for inhibition of HSV-1. We next aimed to identify which activities of Rep78 were required for the observed inhibition of HSV-1 replication. For this, all Rep constructs presented in Fig. 1 were screened for their ability to inhibit HSV-1 replication. This time we transfected 0.5 μg of the Rep plasmids, since this amount was sufficient for considerable inhibition of HSV-1 replication by Rep78 (Fig. 2A and E). As shown in Fig. 2B, Rep68, Rep78, and Rep78(Y156F) all inhibited HSV-1 replication at comparable levels, while almost no inhibition was observed in the presence of Rep52 and Rep78 (K340H). The lack of inhibitory activity of Rep52 and Rep78(K340H) demonstrates that DNA-binding and ATPase/helicase activities are essential for inhibition of HSV-1 replication. The finding that Rep78(Y156F) and Rep68 inhibit HSV-1 replication allows to conclude that endonuclease activity and the C-terminal domain containing the PKI-like and the zinc finger motifs are not required for inhibition of HSV-1.

We then asked if Rep DNA-binding and ATPase/helicase activities need to be present on the same Rep molecule or if trans-complementation of DNA-binding and ATPase/helicase activities would restore the inhibitory effect. Although Rep78 (K340) has a dominant-negative effect on the helicase activity of wild-type Rep78, it does not have any dominant-negative effect on Rep52 helicase activity (74). Transfection of cells with Rep78(K340H), which has site-specific DNA-binding activity but no ATPase/helicase activity, together with Rep52, which has ATPase/helicase activity but no site-specific DNA-binding activity, therefore allows us to assess if Rep DNA-binding and ATPase/helicase activities act separately or if the ATPase/helicase activity of Rep needs to be targeted to a dsDNA substrate via the site-specific DNA-binding domain. As shown in Fig. 2C, cotransfection of Rep78(K340H) with Rep52 did not restore the inhibitory effect, suggesting that the two activities need to be present in cis.

Taken together, these results demonstrate that Rep-mediated inhibition of HSV-1 replication does not depend on the endonuclease activity or the C-terminal domain but requires DNA-binding and ATPase/helicase activities on the same Rep molecule (summarized in Table 1).

The activities of Rep required for inhibition of HSV-1 replication coincide with those required for induction of apoptosis. Rep proteins have previously been described to induce apoptosis in HL-60 and NT2 cells, which are a p53-null promyelocoid cell line and a p53-containing embryonal carcinoma cell line, respectively (66). The induction of apoptosis has been shown to be mediated by the DNA-binding and ATPase/helicase activities of Rep (66), notably the same activities which are required for inhibition of HSV-1 replication.

We therefore first wanted to determine if Rep expression is also able to induce apoptosis in Vero cells. In the following
experiments, unless otherwise stated, cells were transfected with 0.5 μg of the Rep constructs. In a first experiment, we assessed the time course of Rep-mediated apoptosis. For this, Vero cells were transfected with the Rep78 construct or the empty vector, together with a plasmid expressing EGFP (pEGFP-N3), and analyzed 24, 48, and 72 h later by staining with annexin V followed by flow cytometry. Transfected cells were identified by EGFP fluorescence. The results shown in Fig. 4A show that Rep78 is able to induce apoptosis in Vero cells starting from 48 h after transfection. We next assessed if the observed induction of apoptosis in Vero cells also depends on the DNA-binding and ATPase/helicase activities of Rep, as previously reported for HL-60 and NT2 cells (66). This was indeed the case, as apoptosis was readily induced by Rep68,
Rep78, and Rep78(Y156F), while Rep52, Rep78(K340H), and the empty vector induced only low levels of apoptosis (Fig. 4B). Analogous to Rep-mediated inhibition of HSV-1 replication, Rep-mediated apoptosis required DNA-binding and ATPase/helicase activities to be on the same Rep molecule, since transcomplementation of DNA-binding and ATPase/helicase activities did not restore efficient induction of apoptosis (Fig. 4C). The Rep activities required for induction of apoptosis are summarized in Table 1.

The finding that the requirements for Rep-mediated apo-
tosis perfectly coincide with those for Rep-mediated inhibition of HSV-1 replication may suggest that the induction of apoptosis is part of the mechanism by which Rep inhibits HSV-1 replication. We therefore determined if apoptosis is indeed induced in Rep-expressing and HSV-1 infected cells and if pharmacological inhibition of apoptosis could prevent Rep-mediated inhibition of HSV-1 replication. To first find out if Rep induces apoptosis during HSV-1 infection, cells were transfected with either the Rep78 plasmid or the empty vector and infected immediately after transfection with HSV-1 at a high MOI (10 PFU). Cells were then harvested every 12 h until the CPE reached 100% (48 h p.i.) and analyzed by annexin V staining. As shown in Fig. 4D, annexin V staining remained low throughout the HSV-1 infection both in Rep78-expressing cells and in cells transfected with the empty vector, showing that the presence of Rep78 does not lead to apoptosis in the course of HSV-1 infection. This result is not unexpected, since HSV-1 has previously been shown to encode antipapoptotic factors (reviewed in reference 58). To assess the effect of pharmacological inhibition of caspases on Rep-mediated inhibition of HSV-1 replication, cells transfected with Rep78 or the empty vector were treated with two different broad-range caspase inhibitors, Z-VAD-FMK and Boc-D-FMK. As shown in Fig. 4E, both inhibitors prevented Rep-mediated apoptosis. However, the presence of caspase inhibitors did not prevent Rep-mediated inhibition of HSV-1 replication (Fig. 4F).

In summary, these data show that the Rep protein per se induces apoptosis in Vero cells and that the activities of Rep required for this effect precisely coincide with those required for inhibition of HSV-1 (i.e., DNA-binding and ATPase/helicase activities on the same Rep molecule). However, Rep does not induce apoptosis during HSV-1 infection, nor does treatment with caspase inhibitors prevent Rep-mediated inhibition of HSV-1 replication, suggesting that the execution of apoptosis is not part of the mechanism by which Rep inhibits HSV-1 replication.

The activities of Rep required for inhibition of HSV-1 replication coincide with those required for induction of DNA damage. Expression of Rep78 has previously been shown to induce DNA damage in U2OS and HeLa cells (5). The resulting DNA damage response has been shown to be mediated by ataxia telangiectasia-mutated (ATM) and to result in the activation of the checkpoint effector kinase Chk2 and histone H2AX. The authors postulated that Rep endonuclease activity induced nicks in the cellular DNA and that this was responsible for the DNA damage response. To reach that conclusion, Berthet and coworkers (5) used a mutant Rep protein lacking DNA-binding activity, Rep78A1–171, as well as the Rep78(K340H) mutant lacking ATPase/helicase activity (and consequently endonuclease activity on dsDNA). However, they did not use the Rep78(Y156F) mutant lacking endonuclease activity and retaining ATPase/helicase activity. Their data therefore do not elucidate if endonuclease or, rather, ATPase/helicase activity is required for the induction of cellular DNA damage.

We therefore assessed the requirements of Rep for the induction of a DNA damage response using our panel of Rep mutants. For this, Vero cells were transfected with Rep-encoding plasmids or a plasmid expressing EGFP as a negative control. Transfected cells were identified by staining with Rep-specific antibodies or by EGFP fluorescence. As markers of a DNA damage response, we used antibodies specific for hyperphosphorylated (i.e., activated) ATM (p-ATM S1981), RPA (p-RPA32 S4/S8), and H2AX (γH2AX S139). In Rep78-transfected cells, the activation of all three markers was observed as early as 24 h after transfection (data not shown). Such activation was also observed in cells treated with broad-spectrum caspase inhibitors, demonstrating that it was not a consequence of Rep-mediated apoptosis (data not shown). To identify the Rep activities required for activation of the DNA damage responses, cells were transfected with our panel of Rep plasmids and analyzed 48 h later by staining for the DNA damage markers. Examples of Rep78-transfected cells scored as positive or EGFP-transfected cells scored as negative are shown in Fig. 5A. Rep-positive cells showing an activated DNA damage response also showed a somewhat altered chromatin structure (Fig. 5A), corroborating the notion that Rep damages cellular chromatin (5). Activation of ATM, RPA, and H2AX was observed in approximately 30 to 50% of cells transfected with Rep68, Rep78, and Rep78(Y156F), while transfection with Rep52 and Rep78(K340), as well as a combination of the two, did not induce more DNA damage than the negative control (Fig. 5B).

Taken together, these data demonstrate that Rep induces a cellular DNA damage response in Vero cells characterized by hyperphosphorylation of ATM, RPA, and H2AX and that this requires DNA-binding and ATPase/helicase activities on the same Rep molecule. As such, they show that the activities of Rep required for induction of cellular DNA damage precisely coincide with those required for the inhibition of HSV-1 replication and induction of apoptosis (summarized in Table 1).

The hyperphosphorylation of RPA per se is not responsible for the inhibition of HSV-1 replication. It has recently become clear that differential DNA damage responses are induced during HSV-1 and AAV replication. Specifically, HSV-1 infection induces hyperphosphorylation of ATM (44, 70) and H2AX (84), but not RPA (85, 86). In contrast, productive AAV infection leads to hyperphosphorylation of ATM, RPA, and H2AX (68). We therefore hypothesized that components of the DNA damage response elicited by Rep, specifically the hyperphosphorylation of RPA, may be involved in the inhibition of HSV-1 replication. HSV-1 replication does not induce hyperphosphorylation of RPA unless the HSV-1 DNA polymerase is blocked by inhibitors such as PAA or acyclovir (85, 86). In addition, endogenous p-RPA is sequestered into virus-induced chaperone-enriched (VICE) domains during productive replication (84), suggesting that p-RPA may be inhibitory to HSV-1 replication and that HSV-1 may prevent such inhibition by excluding p-RPA from HSV-1 RCs. To assess if hyperphosphorylation of RPA is mediating the inhibition of HSV-1 replication, we induced hyperphosphorylation of RPA by pretreatment of cells with hydroxyurea (HU) (47) or camptothecin (CPT) (69) or infection with UV-inactivated AAV (UV-AAV) (26, 40) and assessed the effect of these treatments on HSV-1 replication. While HU and CPT treatments result in damaging of cellular DNA, infection of cells with UV-AAV induces a DNA damage response without actually damaging cellular DNA (26, 40). The DNA damage response to UV-AAV resembles stalled replication fork signaling and involves DNA polymerase δ, ATR, TopBP1, RPA, the Rad9/Rad1/
Hus1 complex, H2AX, and Chk1 but not ATM or NBS1 (26, 40). Although the cellular DNA itself is not damaged, such DNA damage signaling has profound effects on the cell cycle in that it results in G2 arrest (40, 61).

We first confirmed the induction of a DNA damage response in Vero cells upon treatment with HU (2.5 mM), CPT (1 μM), and UV-AAV (MOI of 10^4 and 5 × 10^5 gcps). All three treatments induced a strong hyperphosphorylation of RPA (p-RPA32 S4/S8) and H2AX (γH2AX S139) compared to that in control cells (Fig. 6A to C). ATM was strongly hyperphosphorylated (p-ATM S1981) upon HU and CPT treatment but only weakly hyperphosphorylated upon infection with UV-AAV (Fig. 6A to C). ATM was strongly hyperphosphorylated (p-ATM S1981) upon HU and CPT treatment but only weakly hyperphosphorylated upon infection with UV-AAV (Fig. 6A to C). When CPE reached 100%, the HSV-1 C12 produced was harvested and titrated. As shown in Fig. 6E, HU treatment significantly inhibited HSV-1 replication compared to untreated cells, while both UV-AAV and CPT had no significant effect on HSV-1 productivity.

Taken together, these results demonstrate that although all three pretreatments, HU, CPT, and UV-AAV, strongly induced the hyperphosphorylation of RPA, only HU led to significant inhibition of HSV-1 replication, while CPT and UV-AAV did not affect HSV-1 productivity. As such, they suggest that the hyperphosphorylation of RPA per se is not inhibitory for HSV-1 replication and that HU inhibits HSV-1 replication...
FIG. 6. (A to C) Immunofluorescence staining for DNA damage markers upon HU, CPT, and UV-AAV treatment. Vero cells were treated for the indicated times with HU (2.5 mM) or left untreated (nil) (A), with CPT (1 μM) or its solvent DMSO alone (B), or with UV-AAV (MOI of 10⁴ or 5 × 10⁴ gcp) or left untreated (nil) (C). The cells were then fixed and stained for p-ATM S1981, p-RPA32 S4/S8, or γH2AX S139 (red). Chromatin was stained with DAPI (blue; inset). Scale bars, 10 μm. Images were recorded by CLSM and show single z stacks of representative cells. (D) Western blot for Rep proteins upon live AAV or UV-AAV infection. Vero cells were left uninfected or infected with live AAV or UV-AAV (MOI of 5 × 10⁴ gcp). Twenty-four hours later, the cells were superinfected with HSV-1 (MOI of 5 PFU) or left uninfected. Twenty-four hours after HSV-1 infection, the cells were lysed and processed for Western blotting with a Rep-specific MAb. Detection of actin served as a loading control. (E) HSV-1 replication in cells pretreated with HU, CPT, and UV-AAV. Vero cells were left untreated (nil) or treated with HU (2.5 mM), UV-AAV (MOI of 10⁴ or 5 × 10⁴ gcp), and CPT (1 μM) or its solvent DMSO alone for the indicated times. After three washes in PBS, the cells were infected with HSV-1 C12 (expressing EGFP) at an MOI of 5 PFU. At 24 h after HSV-1 C12 infection, virus was harvested and titrated on Vero cells by enumerating EGFP⁺ cells. The data are shown as means ± SD from triplicate experiments. The asterisks indicate statistically significant reductions compared to untreated cells ($P < 0.05$).
by a different mechanism, presumably the depletion of deoxynucleoside triphosphate (dNTP) pools by inhibition of ribonucleotide reductase (2).

**DISCUSSION**

In a first set of experiments, we characterized the inhibitory effect of Rep on HSV-1 replication. For this purpose, we assessed the levels of infectious HSV-1 particles, the levels of replicated HSV-1 DNA, and the levels of expressed HSV-1 gene products in the presence of Rep78 expressed from the HCMV IE1 enhancer/promoter. The presence of Rep78 resulted in a dose-dependent inhibition of HSV-1 productivity, DNA replication, and gene expression. The inhibitory effect on the latter was not uniform in that Rep78 had a stronger effect on the late gene products VP16, VP22, and gC than on the IE gene product ICP4 and the early gene products UL9, ICP8, UL5, and UL42 (Fig. 2). These findings are consistent with our previous observations that in the situation of AAV/HSV-1 coinfection, HSV-1 replication is inhibited mainly at the levels of DNA replication and late gene expression, while IE and early gene expression is only marginally reduced (31). It appeared, however, that the expression of Rep78 from the HCMV IE1 enhancer/promoter had a more pronounced effect on the levels of HSV-1 IE and early gene products than AAV coinfection, an observation which may be explained by the different expression kinetics of rep. Specifically, high-level expression of the AAV rep gene from its native promoters (p5 and p19) requires transactivation by the HSV-1 IE early gene products ICP0, ICP4, and ICP22 (3). Consequently, in the situation of AAV/HSV-1 coinfection, expression of the rep gene is expected to occur with kinetics similar to that of HSV-1 early genes. In the present study, in contrast, Rep78 was expressed with IE kinetics, which may explain why the inhibitory effect on HSV-1 IE and early gene products was more pronounced than in the situation of AAV/HSV-1 coinfection (31). However, the reduced levels of HSV-1 early gene products, in particular the HSV-1 replication factors, appeared not to be responsible for the inhibition of HSV-1 DNA replication, since Rep inhibited HSV-1 DNA replication even in the presence of overexpressed HSV-1 replication factors (Fig. 3). In addition, the Rep levels employed were compatible with AAV DNA replication, which also depends on the HSV-1 replication machinery (Fig. 2). These findings suggest that Rep may have a direct inhibitory effect on HSV-1 DNA replication.

In a second set of experiments, we identified the Rep activities required for inhibition of HSV-1 replication. The results demonstrate that the inhibition of HSV-1 replication requires Rep DNA-binding and ATPase/helicase activities on the same Rep molecule, while the C terminus of Rep78 and Rep endonuclease activity are not required (Fig. 2 and Table 1). The finding that Rep DNA-binding and ATPase/helicase activities are required and that they need to be present on the same Rep molecule is consistent with a model in which the inhibition of HSV-1 replication involves the interaction of Rep with DNA and in which the Rep helicase activity needs to be targeted to a dsDNA substrate via the DNA-binding domain. Theoretically, the inhibition of HSV-1 replication could result either from a direct effect of Rep on HSV-1 DNA or else from an effect on cellular DNA. Rep is known to have a variety of effects on the host cell, including the induction of DNA damage, cell cycle arrest, and apoptosis (5, 65, 66, 68). Apoptosis has been shown to require Rep DNA-binding and ATPase/helicase activities (66), notably the same activities required for inhibition of HSV-1 replication. We therefore hypothesized that Rep-mediated induction of apoptosis may be part of the mechanism by which Rep inhibits HSV-1 replication. Our data show that although the activities required for induction of apoptosis and for inhibition of HSV-1 replication coincide precisely (Fig. 4 and Table 1), execution of apoptosis is not part of the inhibitory mechanism. First, apoptosis did not occur in cells expressing Rep78 and infected with HSV-1, and second, treatment with caspase inhibitors did not abolish the inhibitory activity of Rep on HSV-1 replication (Fig. 4). We concluded from these data that the ability of Rep to induce apoptosis must be closely linked to its ability to inhibit HSV-1 replication, possibly by acting in the same pathway. Furthermore, the finding that the execution of apoptosis is not required for inhibition of HSV-1 replication suggests that Rep-mediated apoptosis may only be the consequence of an upstream Rep effect leading to inhibition of HSV-1 replication. A possible candidate for such an upstream effect is the ability of Rep to induce cellular DNA damage, an effect which results in DNA damage signaling, cell cycle arrest, and presumably apoptosis (5). We therefore assessed which activities of Rep were required for the induction of DNA damage. Intriguingly, the Rep activities required for induction of cellular DNA damage precisely corresponded to those required for induction of apoptosis and inhibition of HSV-1 replication, corroborating the notion that these three Rep effects are linked in a common pathway (Fig. 5 and Table 1). Consistent with previous findings, expression of Rep78 resulted in the hyperphosphorylation of ATM (p-ATM S1981) and H2AX (γH2AX S139) (5, 68). The phosphatidylinositol 3-kinase-like kinase (PIKK) ATM is a master regulator of the cellular response to double-strand breaks and activates a variety of substrates involved in cell cycle checkpoints and DNA repair. ATM protein kinase activity is activated upon DNA damage stimuli, and this activation has been attributed to intermolecular autophosphorylation on serine 1981 and dissociation of multimeric ATM to an active, monomeric form (reviewed in reference 42). ATM has been shown to be essential for Rep78-induced DNA damage signaling, in that expression of Rep78 did not result in cell cycle arrest and activation of H2AX in ATM-null cell lines (5). H2AX is a histone protein which can be hyperphosphorylated at serine 139 by ATM in response to double-strand breaks or, alternatively, by ATM- and Rad3-related (ATR) kinase in response to replication stalling and single-strand breaks and is involved in the assembly and retention of DNA repair factors at the DNA lesion (reviewed in reference 24). We also observed the hyperphosphorylation of RPA (p-RPA32 S4/S8), a heterotrimeric ssDNA-binding protein consisting of 70-, 32-, and 14-kDa subunits which is involved in diverse processes such as DNA replication, DNA repair, recombination, and DNA damage checkpoints (reviewed in reference 92). RPA hyperphosphorylation occurs upon DNA damage signaling and can be mediated by the PIKKs ATM, ATR, and DNA-dependent protein kinase (DNA-PK), depending on the nature of the DNA lesion (reviewed in references 6 and 19). Theoretically, there are two
different possible explanations for the observation that the ability of Rep to induce DNA damage correlates with its ability to inhibit HSV-1 replication. First, the Rep-induced DNA damage itself, presumably acting on both cellular and HSV-1 DNAs, could be responsible for inhibition of HSV-1 replication. Second, the cellular response to Rep-induced DNA damage could be responsible for inhibition of HSV-1 replication by creating a cellular environment unfavorable for HSV-1 replication. Indeed, AAV and HSV-1 have recently been demonstrated to induce differential DNA damage signaling and to be differentially affected by DNA damage and repair proteins. Specifically, productive AAV infection induces the hyperphosphorylation of ATM, RPA, and H2AX (68), while HSV-1 infection leads to hyperphosphorylation of ATM (44, 70) and H2AX (84) but not RPA (85, 86). To find out if the Rep-mediated activation of RPA may be responsible for the inhibition of HSV-1 replication, we assessed the replication efficiency of HSV-1 in cells in which RPA hyperphosphorylation had previously been induced by treatment with HU or CPT or by infection with UV-AAV. Although all three agents efficiently induced hyperphosphorylation of RPA, only HU pretreatment significantly inhibited HSV-1 replication (Fig. 6). This suggests that Rep-induced activation of RPA per se is not responsible for the inhibition of HSV-1 replication, and that the observation of inhibition of HSV-1 replication by HU involves mechanisms other than RPA hyperphosphorylation, presumably the depletion of dNTP pools by inhibition of ribonucleotide reductase (2). These findings may be interpreted in at least two different ways. First, they may mean that the hyperphosphorylation of RPA does not interfere with efficient HSV-1 replication. Second, they may mean that HSV-1 can efficiently circumvent a potential inhibitory effect by sequestering p-RPA away from its RCs into VICE domains (84).

Although the finding that inhibition of HSV-1 replication requires Rep DNA-binding and ATPase/helicase activities on the same Rep molecule is consistent with the idea that the inhibition of HSV-1 replication involves the interaction of Rep with target DNA, it is also conceivable that Rep inhibits HSV-1 replication by its interaction with the HSV-1 DNA replication complex. It is thought that the direct interaction between Rep and ICP8 (33, 75) leads to the recruitment of the HSV-1 DNA replication complex to AAV replication origins. Consistent with this, essential roles of ICP8, the HSV-1 helicase-primase complex, and the HSV-1 DNA polymerase holoenzyme in AAV replication have been demonstrated (3, 82). However, it is also possible that the Rep-ICP8 interaction directs Rep to DNA replication complexes on HSV-1 replication origins, where it possibly interferes with efficient HSV-1 DNA replication. In analogy, the interaction of Rep with RPA (75) might direct Rep to cellular DNA replication complexes, leading to inhibition of cellular DNA replication. Direct interactions with the ssDNA-binding proteins ICP8, RPA, and Ad-DBP have been demonstrated for Rep68 and Rep78 (33, 75), and these interactions have been shown to enhance binding of Rep68 and Rep78 to the Rep-binding site (RBS) (75). The enhancement of Rep DNA binding by all three ssDNA-binding proteins was also observed for a mutant Rep protein lacking ATPase/helicase activity, Rep68(K340H/Y121H), implying that the mutant Rep protein can still interact with ICP8, RPA, and Ad-DBP (75). As such, these findings are consistent with the idea that the inability of the Rep78(K340H) mutant to inhibit the replication of HSV-1 is not due to a loss of the direct interaction with ICP8 but rather is due to the loss of the ATPase/helicase activity itself. We therefore consider it more likely that the inhibition of HSV-1 replication involves the interaction of Rep with target DNA rather than that with ICP8.

How could Rep DNA-binding and ATPase/helicase activities result in cellular DNA damage and apoptosis, as well as inhibition of HSV-1 replication? Rep displays sequence-specific binding to dsDNA, which targets the protein to the RBSs within the AAV replication origins, as well as to its preintegration site on human chromosome 19 (AAVS1). The sequence-specific DNA-binding activity requires the amino-terminal 224 amino acids, which are present in Rep68 and Rep78 but not in Rep40 and Rep52 (60). The sequence recognized by Rep consists of an array of GAGC tetranucleotide repeats, with the binding affinity being determined by the number of perfect GAGC repeats present as well as secondary structures neighboring the RBSs (14, 15, 30, 51, 52, 62, 87). The AAV genome, for instance, contains RBSs within the ITR replication origins (GAGC GAGC GAGC GCGC), as well as within the p5 promoter (GAGT GAGC AGGC AGGG), with the binding affinity of Rep being higher for the ITR than for the p5 RBS (30). In addition to the RBS present in the AAV preintegration site (CAGC GAGC GAGC GAGC), the human genome contains 105 potential RBSs if they are defined as GAGY GAGC motifs (90). By applying the same definition, nine minimal RBSs can be found in the HSV-1 genome. However, the sequence requirements for low-affinity Rep binding are less stringent, in that a single GAGC repeat followed by a run of G bases is sufficient (15), suggesting that the above numbers of potential RBSs within the cellular and HSV-1 genomes are underestimated. In contrast, appropriately spaced terminal resolution sites (TRSs), where Rep endonuclease activity can induce a site- and strand-specific nick, are present only in the AAV replication origins and in AAVS1, and therefore, only these sites function as Rep-dependent replication origins. The fact that the cellular RBSs outside AAVS1 lack functional TRSs is thought to determine the specificity of AAV integration into AAVS1 (45, 87, 90). In the absence of a helper virus, Rep concentrations are low and Rep is expected to be targeted to the rather strong RBSs within the AAV replication origins and the AAVS1 preintegration site, resulting in site-specific integration and establishment of latent infection. In the presence of a helper virus such as HSV-1, Rep concentrations are high and Rep is expected to additionally bind to more degenerate RBSs present on the cellular and HSV-1 genomes. Binding of Rep to degenerate RBSs lacking an appropriately spaced TRS is expected to be followed by unwinding via the helicase activity but not by nicking. The helicase activity of Rep has no specific sequence requirement for unwinding of dsDNA substrates. However, Rep helicase activity requires prior binding to the DNA substrate. If the substrate is blunt-ended dsDNA, Rep68/78 helicase activity requires the presence of an RBS, to which the protein binds via its N-terminal DNA-binding domain (88, 91). If the DNA substrate does not contain an RBS, Rep68/78 and Rep52 helicase activity require a single-stranded 3′ tail, to which Rep binds and along which it moves in a 3′-to-5′ direction while unwinding the stretch of...
dsDNA (74, 88, 91). In contrast, Rep40 not only can bind to 3‘-end single-stranded tails but in addition can initiate helicase activity on a blunt-ended dsDNA substrate (18). Our finding that Rep68 and Rep78, but not Rep52, inhibit HSV-1 replication suggests that the substrate for Rep helicase activity is dsDNA and that Rep binds to its substrate via its sequence-specific DNA-binding domain. It is conceivable that Rep DNA binding followed by unwinding of the DNA double strand interferes with transcription and replication, since it may block access of transcription and replication factors. However, the mechanism by which Rep DNA binding and unwinding induces a cellular DNA damage response is more difficult to understand. One straightforward explanation would be that the ssDNA exposed upon Rep helicase activity is sensed as DNA damage, resulting in the activation of the corresponding DNA damage signaling. Accumulation of ssDNA as it occurs upon replication stalling is not required for induction of cellular DNA damage (Fig. 5), however, suggests that the mechanism is more complex. It is possible that the process of Rep DNA binding and unwinding recruits other cellular factors which then induce the actual DNA damage. Consistent with such a hypothesis, Rep has been shown to interact with a variety of cellular proteins involved in DNA replication and repair (57).

Whatever the mechanism, our data show that Rep DNA-binding and ATPase/helicase activities on the same Rep molecule are required for induction of DNA damage and apoptosis, as well as for inhibition of HSV-1 replication, and as such they suggest that these three processes are closely linked. Since execution of apoptosis is not involved in inhibition of HSV-1 replication (Fig. 4), we consider it possible that the ability of Rep to induce DNA damage is actually responsible for inhibition of HSV-1. Such Rep-induced DNA damage might occur not only on cellular DNA but also directly on HSV-1 DNA, the latter of which would presumably interfere with HSV-1 transcription and DNA replication. On AAV DNA, in contrast, the process of Rep binding and unwinding is followed by nicking and is targeted to the AAV replication origins (ITR and p5) where it fulfills an essential step in the AAV DNA replication cycle, i.e., the creation of a free 3‘ end for repair synthesis of the ITRs. Finally, the inhibition of HSV-1 replication would confer a competitive advantage to AAV, since it would limit the competition of HSV-1 for replication factors. Although such an inhibitory mechanism would not be specific for HSV-1 DNA replication in that it is also expected to affect HSV-1 transcription, it would still allow the largely undisrupted expression of the HSV-1 helper factors for AAV replication, since rep expression occurs with early kinetics and therefore is expected to have only a limited inhibitory effect on HSV-1 IE and early gene transcription.

At present, such a model certainly remains speculative, since several aspects still need to be addressed experimentally, in particular the interaction of Rep with cellular and HSV-1 DNAs and the consequences of this interaction for the transcription and replication of these DNA substrates. Nonetheless, it represents an intriguing explanation for the observation that AAV Rep can inhibit the replication of such diverse DNA substrates as cellular (5, 65, 89), Ad (77), simian virus 40 (SV40) (89), and HSV-1 (32) DNAs, inasmuch as it would predict inhibited replication of every DNA substrate on which Rep binding and unwinding occur at random locations and for which it is not part of the replicative cycle.

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