Adeno-Associated Virus Type 2 Rep68 Can Bind to Consensus Rep-Binding Sites on the Herpes Simplex Virus 1 Genome

Michael Seyffert,a Daniel L. Glauser,a** Kurt Tobler,a Oleg Georgiev,b Rebecca Vogel,a Bernd Vogt,a Leticia Agúndez,c Michael Linden,c Hildegard Büning,d,e Mathias Ackermann,a,b Cornel Fraefelb

Institute of Virology, University of Zurich, Zurich, Switzerland; Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland; Department of Infectious Diseases, King’s College London, London, United Kingdom; Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany; Institute for Experimental Hematology, Hannover Medical School, Hannover, Germany

Adeno-associated virus type 2 is known to inhibit replication of herpes simplex virus 1 (HSV-1). This activity has been linked to the helicase- and DNA-binding domains of the Rep68/Rep78 proteins. Here, we show that Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome and that the Rep helicase activity can inhibit replication of any DNA if binding is facilitated. Therefore, we hypothesize that inhibition of HSV-1 replication involves direct binding of Rep68/Rep78 to the HSV-1 genome.

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deno-associated virus type 2 (AAV2) is a nonpathogenic human parvovirus with a unique biphasic life cycle. In the absence of a helper virus, AAV2 establishes a latent infection, while in the presence of a helper virus, such as adenovirus type 2 (AdV2), herpes simplex virus 1 (HSV-1), or human papillomavirus 16 (HPV-16), it undergoes lytic replication (1–4). The AAV2 genome is a single-stranded DNA (ssDNA) molecule of 4,680 nucleotides, which is packaged into an icosahedral capsid with a diameter of approximately 20 nm (5). The genome harbors two clusters of genes, rep and cap, which are flanked by inverted terminal repeats (ITRs). The ITRs form hairpin structures and contain a Rep-binding site (RBS) and a terminal resolution site (trs), which together act as the viral origin of DNA replication (6, 7). The cap gene 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 (8, 9). In addition, a nested open reading frame (ORF) within the cap gene encodes a protein designated assembly-activating protein (AAP), which is believed to be required for AAV2 capsid assembly in the nucleolus (10, 11). The rep gene encodes the Rep proteins, which are synthesized in four different forms due to transcription from two different promoters, p5 and p19, and alternative splicing of an intron near the C-terminal end (12). The different Rep proteins are termed Rep40, Rep52, Rep68, and Rep78 according to their apparent molecular weights. The Rep proteins are involved in diverse processes during the viral life cycle, such as DNA replication, regulation of gene expression, genome packaging, and site-specific genomic integration (13–18).

HSV-1 belongs to the subfamily of the *Alphaherpesvirinae* and is the reagent causing mucosal eruptions at the site of infection, which can recur at the same site upon reactivation from latency (19, 20). The HSV-1 virion is built up by three structural components, the capsid, the tegument, and the surrounding envelope. The viral genome is a linear double-stranded DNA (dsDNA) molecule 152 kb in size and has a unique structure. It is divided into two covalently joined segments, which contain unique segments (unique long [UL] and unique short [US]) and inverted repeat regions (TRs, IRs, IRq, and TRq). The IR sequences link the L and S segments (Fig. 1A). HSV-1 gene expression and replication occur in a temporally regulated cascade: immediate early (IE), early (E), and late. IE proteins exhibit mainly regulatory functions and initiate expression of the E genes. The E proteins comprise enzymes necessary for viral DNA replication and are therefore required for the expression of some of the late genes, as expression of these genes relies on DNA replication. All viral replication events take place in the nucleus within distinct areas termed replication compartments (RCs) (21). In the course of viral DNA replication, these RCs grow continuously and four different stages (I to IV) can be distinguished according to RC staining patterns (22–24). The minimal set of HSV-1 proteins required for initiating AAV2 replication consists of the E proteins UL5, UL8, and UL52, which together form the HSV-1 helicase-primase complex, as well as the ssDNA-binding protein ICP8 (UL29) (25–27). In addition, the HSV-1 IE proteins ICP4 and ICP0, the E protein complex forming the HSV-1 polymerase (UL30 and UL42), and the US1 gene product strongly enhance AAV2 replication (26). AAV2 has developed strategies to inhibit helper virus replication, likely to reduce competition (24, 28–33). For example, expression of the AAV2 nonstructural proteins Rep68 and Rep78 alone leads to significant inhibition of HSV-1 DNA replication (24, 28). Specifically, we demonstrated previously that the AAV2 Rep protein domains responsible for the inhibition of HSV-1 DNA replication include the DNA-binding and the ATPase/helicase activities, while the endonuclease activity is not required (28). We also showed that Rep-mediated inhibition of HSV-1 occurs even in the absence of AAV DNA and is not due to alterations of HSV-1 IE and E gene expression or to the Rep-mediated induction of toxic stress in the cell but rather occurs at the stage of HSV-1 DNA replication itself (28). We hypothesized that a possible mechanism of Rep68/Rep78-me-
mediated inhibition of HSV-1 DNA replication involves binding of Rep proteins to consensus RBSs on the HSV-1 genome and modification of the bound DNA substrate via the Rep helicase activity. To investigate this possibility, we now addressed the following questions: (i) do consensus RBSs exist on the HSV-1 genome, and if so, can AAV2 Rep proteins bind to these sites, and (ii) can the AAV2 Rep helicase activity inhibit replication of any DNA substrate when binding is facilitated?

We addressed the first question by screening the wild type (wt) HSV-1 (strain F) genome for the minimal AAV2 RBS motif GAGYGAGC as a prerequisite for the ability of Rep to specifically bind to dsDNA templates via its DNA-binding domain (34). We found that such sites are indeed present, as shown in Fig. 1. All consensus RBSs are located within coding sequences of genes found in the U_L segment of the HSV-1 genome (Fig. 1A). Sequence alignment revealed that all HSV-1 putative RBSs (pRBSs) consist of two complete GAGC repeats, with the exception of pRBS 7, which contains a T in place of a C nucleotide position 74604 but still complies with the GAGYGAGC consensus sequence (Fig. 1B). Importantly, no consensus trs (CCAACT) (6, 18) was found within 8 to 13 nucleotides (nt) after any HSV-1 pRBS, excluding the existence of an AAV2 integration site at these positions on the HSV-1 genome (35). We next tested the capability of AAV2 Rep proteins to bind via the DNA-binding domain to HSV-1 pRBSs by electrophoretic mobility shift assays (EMSAs) using purified His-tagged Rep68 proteins (His-Rep68). We designed 37-mer duplexed oligonucleotides harboring selected pRBSs (numbered 1 to 5) (Fig. 1B, light gray box), which were radioactively labeled with \[^{32}\text{P}]\text{ATP}. An oligonucleotide containing the native RBS from the AAV2 ITR was used as the positive control. An oligonucleotide containing a random sequence from within the UL44 gene harboring no pRBS was used as the negative control. Approximately 5 fmol of each duplexed oligonucleotide was incubated with 0, 120, or 240 ng of His-Rep68 protein for 30 min at room temperature and then subjected to 4% polyacrylamide gel electrophoresis. After 3 h, the gel was dried and exposed to Fujifilm imaging plates, which were developed with a Fujifilm FLA-7000 image plate reader. For all oligonucleotides harboring pRBSs 1 to 5 examined, we observed a dose-dependent shift compared to the unbound DNA template (Fig. 2A). The percent shift values for oligonucleotides 1 to 5 were slightly lower (63 to 76%) than that for the positive control (the ITR oligonucleotide; 87%). The negative-control oligonucleotide was shifted 5% only. Multiple shifted bands for oligonucleotides 1 to 5 as well as for the ITR oligonucleotide were observed, possibly due to the different oligomerization states Rep68 can form on dsDNA templates (36–38). To confirm that binding of His-Rep68 to the pRBS is indeed specific, we performed EMSAs as described above, except that 5

FIG 1  The HSV-1 genome contains nine pRBSs. (A) The minimal AAV2 RBS motif GAGYGAGC was used to identify consensus RBSs within the HSV-1 (strain F) genome, which can be divided into U₁ and U₅ segments that are both flanked by internal and terminal inverted repeats (TR₁, IR₁, IR₅, and TR₅). The arrowheads facing down represent pRBSs located on the plus strand, and the arrowheads facing up represent pRBSs located on the minus strand. The pRBSs were numbered randomly from 1 to 9 as indicated. The HSV-1 genes containing a consensus pRBS are highlighted. (B) Alignment of sequences from the AAV2 ITR and the nine HSV-1 pRBSs identified in panel A. The consensus RBS (motif GAGYGAGC) and the trs located on the AAV2 ITR are indicated (dark gray boxes). Sequences 1 to 5, analyzed in in vitro experiments, are squared in a light gray box. The illustration was generated using the prettypplot function of the online tool EMBoss (http://emboss.sourceforge.net). The numbers on the right indicate the nucleotide position within the AAV2 genome (ITR) or the HSV-1 genome (sequences 1 to 9). Conserved nucleotides are indicated below as a schematic representation created with WebLogo (63).
Rep-binding assays. (A) EMSAs were performed with purified His-tagged Rep68 proteins (His-Rep68) and radiolabeled duplex oligonucleotides (oligonuc.) 1 to 5 containing pRBSs, the negative control (nc), or the positive control (ITR); see the light gray box in Fig. 1B for oligonucleotide sequences. The immunoprecipitated DNA oligonucleotides were then analyzed by qPCR using primers specific for consensus RBSs 1 to 9 (Fig. 1 and Table 1). Primers for amplification of a sequence from the HSV-1 genome containing no proximal RBS (US1) served as a negative control (Table 1). The qPCR mix was the following: 0.25 μl of each primer (10 μM), 10 μl of SYBR green PCR master mix (Applied Biosystems), and 2.5 μl of DNA in a final volume of 20 μl. The reaction was carried out as follows: 95°C for 3 min, 39 cycles of 95°C for 15 s and 60°C for 1 min, and a final elongation step at 95°C for 10 min. The raw threshold cycle \((CT)\) values were analyzed using the percent input method as follows: % input \(= 100 \times 2^{-\Delta CT}\), where \(\Delta CT = CT_{(input)} − \log_2\text{(input dilution factor)} − CT_{(ChIP sample)}\). For each primer pair tested (those for no RBS and pRBSs 1 to 9), the percent input values were calculated from cells expressing either Rep52-GFP or Rep68-GFP (Fig. 2C). Using these data, we next addressed (i) whether Rep68 binding to the HSV-1 DNA is more efficient at a pRBS and (ii) whether the DNA-binding domain of Rep68 is required for binding.

To address the first point, we calculated the difference between each value obtained from binding of Rep68-GFP to a pRBS and the control value (no RBS), which was set as 1. The graph in Fig. 2D shows that Rep68-GFP can bind more efficiently to the HSV-1 DNA that harbors a pRBS than to the control with no RBS, with one exception; binding to pRBS 7 was not more efficient than binding to the control with no RBS, likely because the pRBS 7 consensus sequence is slightly aberrant (Fig. 1B). Nevertheless, we can conclude that binding of Rep68 to HSV-1 DNA is more efficient when the DNA harbors a pRBS. To address the second point, we calculated the difference (percent input ratio) between the Rep68-GFP and the Rep52-GFP values for each consensus binding site 1 to 9 and the negative control (no RBS). The ratios were normalized to the no-RBS (US1) control ratio and are shown in Fig. 2D. As expected, the ratio between Rep68-GFP and Rep52-GFP was the smallest in the absence of a consensus RBS (no RBS), and this value was set as 1. The ratios for all pRBSs 1 to 9 were clearly higher, and those for pRBSs 3 to 8 were statistically significant. We can therefore conclude that the DNA-binding domain

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**TABLE 1** Primer pairs used for ChIP-qPCR

<table>
<thead>
<tr>
<th>pRBSs or control</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RBS/US1</td>
<td>GCTTCCCTTGGTTTGGAGACCA</td>
<td>GTCCAGCTCAAACCTCCCAAA</td>
</tr>
<tr>
<td>1</td>
<td>CACGTTGCGAGCATTCTGGTC</td>
<td>CGGACGACCCACACCAAT</td>
</tr>
<tr>
<td>2</td>
<td>ATCGTGTGTTATGCCTGCCAC</td>
<td>GAGGAGATGCGCCATGCTG</td>
</tr>
<tr>
<td>3</td>
<td>CACCCTAACTCCTCCCTCCACG</td>
<td>GGACTGCTGCTGCTCTGTTG</td>
</tr>
<tr>
<td>4</td>
<td>CAAAGGCTCTGAAACTACCACG</td>
<td>GGTTGTATAGACCCACAGG</td>
</tr>
<tr>
<td>5</td>
<td>GCTAAATGGCGACCTCCCTTCC</td>
<td>CGATGTGCTGCGTCTGAG</td>
</tr>
<tr>
<td>6</td>
<td>GCCTTTGTTGGTGCGATGATAG</td>
<td>ACATGATCCTGCTCTCACC</td>
</tr>
<tr>
<td>7</td>
<td>AACCCGCTATCCAGGGTGATAC</td>
<td>ATATAGGGCTGAGCCAAAGG</td>
</tr>
<tr>
<td>8</td>
<td>GACCCAGGTTGTTATGAAAGT</td>
<td>CGTTAGGGTGTCCTTACCC</td>
</tr>
<tr>
<td>ICP0 promoter</td>
<td>ATACAATGCGATCCCTGGCAGA</td>
<td>GCTGCGTCCGTCGTCG</td>
</tr>
</tbody>
</table>

a Primers were generated using the online tool Primer3 (64, 65). The primer pair for the ICP0 promoter was described elsewhere (40). For all primer pairs, the efficiency coefficients \([E]\) were determined with serial dilutions of purified HSV-1 (strain F) DNA under the same qPCR conditions described in the legend to Fig. 2C and used to standardize the percent input calculations.

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fmol of nonlabeled (cold) duplex ITR oligonucleotide was used as competitor. The cold ITR competitor DNA appeared to prevent binding of His-Rep68 to the pRBS oligonucleotides, as no shift was observed under these conditions for oligonucleotides 1 to 3 and a clearly reduced shift was observed for oligonucleotides 4 and 5 (Fig. 2B).

In order to investigate whether AAV2 Rep68 is able to bind to consensus RBSs on the HSV-1 genome also in HSV-1-infected cells, we performed chromatin immunoprecipitation (ChIP) assays followed by quantitative PCR (qPCR) analysis (ChIP-qPCR). For this analysis, Vero cells were transfected with plasmids expressing either Rep68 or Rep52 protein fused with enhanced green fluorescent protein (EGFP). The next day, the cells were infected with wt HSV-1 (strain F) at a multiplicity of infection (MOI) of 40. At 16 h after infection, the cells were fixed with 4% paraformaldehyde, sonicated for 10 min, and processed for ChIP using the GFP-Trap kit (ChromoTek). The immunoprecipitated DNA oligonucleotides were then analyzed by qPCR using primers specific for consensus RBSs 1 to 9 (Fig. 1 and Table 1). Primers for amplification of a sequence from the HSV-1 genome containing no proximal RBS (US1) served as a negative control (Table 1). The qPCR mix was the following: 0.25 μl of each primer (10 μM), 10 μl of SYBR green PCR master mix (Applied Biosystems), and 2.5 μl of DNA in a final volume of 20 μl. The reaction was carried out as follows: 95°C for 3 min, 39 cycles of 95°C for 15 s and 60°C for 1 min, and a final elongation step at 95°C for 10 min. The raw threshold cycle (\((CT)\)) values were analyzed using the percent input method as follows: % input \(= 100 \times 2^{-\Delta CT}\), where \(\Delta CT = CT_{(input)} − \log_2\text{(input dilution factor)} − CT_{(ChIP sample)}\). For each primer pair tested (those for no RBS and pRBSs 1 to 9), the percent input values were calculated from cells expressing either Rep52-GFP or Rep68-GFP (Fig. 2C). Using these data, we next addressed (i) whether Rep68 binding to the HSV-1 DNA is more efficient at a pRBS and (ii) whether the DNA-binding domain of Rep68 is required for binding.

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of Rep68 enhances binding to pRBSs in this assay. To appreciate the quality of the binding of Rep68 to pRBSs, we tested binding of an HSV-1 DNA-binding protein, ICp4, to the ICp4-binding site in the HSV-1 ICp0 promoter relative to an unspecific DNA that does not contain an ICp4-binding site (US1). For this test, we infected Vero cells with a recombinant HSV-1 (rHSV-EYFP-ICp4) which expresses the ICp4 protein fused to the enhanced yellow fluorescent protein (EYFP) (39). One day later, we processed the cells for ChIP and qPCR as described above. We found that the ICp4 protein binds specifically to the ICp0 promoter. Binding efficiency to the ICp0 promoter sequence was approximately 2.5-fold higher than to the US1 control sequence (Fig. 2F) and is comparable to previous findings (40). We can therefore conclude that binding of AAV2 Rep68 to the HSV-1 DNA at a pRBS is at least as efficient as binding of the native HSV-1 DNA-binding protein ICp4 to its binding site in the HSV-1 ICp0 promoter (Fig. 2F).

To investigate the question of whether the AAV2 Rep helicase domain can inhibit replication of any DNA substrate when binding is facilitated, we used a well-established assay to investigate the replication of HSV-1 and AAV2 DNA (21, 24, 41, 42). Briefly, cells are cotransfected with two plasmids; the first contains 40 binding sites for the lac repressor protein LacI and an HSV-1 origin of DNA replication (plasmid pH5V-lacO), and the second plasmid encodes the lac operon-binding motif of LacI fused with enhanced yellow fluorescent protein (EYFP-LacI). In the presence of HSV-1 helper functions, pH5V-lacO replicons (and therefore the binding sites for LacI) are amplified (because of the HSV-1 origin of DNA replication) and recruitment of autofluorescent LacI protein then allows visualization of pH5V-lacO DNA replication. We have now modified this assay by transfecting a third plasmid encoding the AAV2 Rep40/Rep52 proteins, which contain the helicase domain but not the DNA-binding domain, fused with the lac operon-binding motif of LacI (Rep40/Rep52-LacI) (Fig. 3A and B). The modified Rep40/Rep52 proteins are prone to bind to lac operon-binding sequences present on the pH5V-lacO replicon and therefore allowed us to study the effect of the Rep helicase activity independent of the interaction between the AAV2 Rep DNA-binding domain and the RBS. The results shown in Fig. 3C to E demonstrate that the Rep40/Rep52-LacI proteins were indeed able to inhibit the replication of pH5V-lacO, as cells containing mature stage IV RCs were not observed (Fig. 3C, panel n). In contrast, the Rep40/Rep52 proteins, which lack any DNA-binding domain, did not prevent the formation of mature stage IV HSV-1 RCs (Fig. 3C, panel i). Neither did Rep40/Rep52K340H-LacI proteins (where Rep52K340H is Rep52 with the K340H amino acid change), which can bind to the pH5V-lacO replicon but contain a point mutation that inactivates the helicase activity (43), although the number of stage IV RCs was reduced by approximately 50%, indicating that these fusion proteins can inhibit the replication of the HSV-1 replicon to some extent (Fig. 3C, panel s). Also, in the cultures transfected with the Rep40/Rep52-LacI-encoding plasmid, the frequency of stage III RCs was reduced by approximately 3-fold (Fig. 3C, panel m) while the frequency of cells showing diffuse EYFP fluorescence (stage I; no RCs) was much higher than that in the control cultures, those with no Rep (Fig. 3C, panel a), Rep40/Rep52 (Fig. 3C, panel f), and Rep40/Rep52K340H-LacI (Fig. 3C, panel p). We confirmed that Rep40/Rep52-LacI proteins indeed bind to LacI repressor-binding sites on the pH5V-lacO reporter DNA by determining the degree of colocalization of the LacI-EYFP and the Rep-Alexa Fluor 594 signals in the merged images of panels l to n and q to s in Fig. 3E. The colocalization values for the smaller RCs (stages II and III) were lower in trend than the values for the mature RCs (stage IV). This observation may be explained as follows: at early time points when the numbers of pH5V-lacO replicons are small, there is more competition for Lac repressor-binding sites between the Rep-LacI construct and the LacI-EYFP reporter protein. The distributions of stage I to IV RCs were comparable in cells transfected with the plasmids encoding Rep40/Rep52 (Fig. 3C, panels f to i) and Rep40/Rep52K340H-LacI (Fig. 3C, panels p to s), indicating that Rep40/Rep52-LacI competition with EYFP-LacI did not interfere with detection of RCs. Moreover, while Rep68/Rep78 can efficiently inhibit wt HSV-1 DNA replication (24, 28), Rep40/Rep52, Rep40/Rep52-LacI, and Rep40/Rep52K340H-LacI do not (28; data not shown). These results indicate that the AAV2 Rep helicase activity in the absence of the Rep DNA-binding and endonuclease activities can inhibit the replication of any DNA template as long as it can bind to it. Of note, we have observed 5.3% ± 9.2% and 17.3% ± 4.6% of cells (means ± standard deviations) showing numerous nuclear foci when transfected with Rep40/Rep52-LacI (Fig. 3C, panel o) and the helicase mutant Rep40/Rep52K340H-LacI (Fig. 3C, panel t), respectively. Such foci were not observed when cells were transfected with the plasmid encoding Rep40/Rep52 (Fig. 3C, panel j) or the empty backbone plasmid pcDNA3.1+ (Fig. 3C, panel e). Some of the foci represent background binding of EYFP-LacI to LacI repressor-binding sites on the pH5V-lacO input plasmid because foci were observed also in

FIG 3 Inhibition of DNA replication by the AAV2 Rep helicase activity. (A) Schematic representation of the Rep constructs analyzed in this experiment. The functional protein domains of interest for this study are indicated. The arrowhead indicates the splicing site of the Rep gene. (B) Western blot analysis of Rep40/Rep52, Rep40/Rep52-LacI, and Rep40/Rep52K340H-LacI. Vero cells (200,000 cells/well) were transfected with 0.1 μg of a plasmid encoding either Rep40/Rep52, Rep40/Rep52-LacI, or Rep40/Rep52K340H-LacI or the empty plasmid backbone pcDNA3.1+ (no Rep). After 24 h, the cells were harvested and processed for Western blotting using a Rep-specific antibody (mouse anti-Rep monoclonal antibody [MAb] clone 303.9 [Fitzgerald]; 1:100). Actin staining served as a loading control. (C) HSV-1 DNA replication assay. Vero cells (150,000 cells/well) were cotransfected with 0.01 μg of the reporter plasmid pSV2EYFP-LacI, and 0.05 μg of a plasmid encoding either Rep40/Rep52-LacI, the helicase-deficient mutant Rep40/Rep52K340H-LacI, or wt Rep40/Rep52 or the empty plasmid backbone pcDNA3.1+ (no Rep). One day after transfection, the cells were superinfected with wt HSV-1 (strain F) at an MOI of 5. After 16 h, the cells were fixed with 4% paraformaldehyde, immunostained for Rep with a primary mouse anti-Rep MAb (clone 303.9 [Fitzgerald]; 1:100) and secondary goat anti-mouse IgG (H+L)–Alexa Fluor 594 (Molecular Probes; 1:500) (red insets), and then subjected to confocal laser scanning microscopy with an SP2 confocal laser scanning microscope from Leica. The percentage of cells displaying pHSV-lacO RCs at stage I, II, III, or IV or the percentage of cells showing numerous foci is indicated. n/o, not observed. Scale bars, 5 μm. (D) Graph representing the data shown in panel C. Error bars show standard deviations from three independent experiments with 80 to 100 cells counted in each experiment. Asterisks indicate statistically significant differences based on a paired two-tailed Student t test. (E) Panels I to n and q to s from Fig. 3C are shown as merged images. The blue insets represent the DAPI (4′,6-diamidino-2-phenylindole) stain of the corresponding cells. The degree of colocalization (expressed as a percentage) was calculated using the colocal function of the software Imaris (Bitplane).
the presence of pHSV-lacO and EYFP-LacI and in the absence of helper virus (data not shown). This hypothesis is supported by the fact that Rep52-LacI proteins, which can also bind to the Lac repressor-binding sites on the pHSV-lacO replicons, colocalized with the numerous EYFP-LacI foci (Fig. 3C, insets in panels o and t). While we do not know why the number of foci increases markedly in the presence of Rep40/Rep52-LacI constructs or the absence of HSV-1 helper virus, similar patterns have been observed before to occur when HSV-1 replication is inhibited by treating the cells with viral polymerase inhibitors such as phosphonoacetic acid, transfecting the cells with Rep68/Rep78-encoding plasmids, or using polymerase-deficient HSV-1 (24, 44, 45).

In this report, we demonstrate that the AAV2 Rep68 protein is capable of binding to pRBs on the double-stranded HSV-1 genome in silico, in vitro, and in HSV-1-infected cells. EMSA and ChIP-qPCR data revealed that binding of Rep68 to consensus RBs is specific and requires the Rep DNA-binding domain. Moreover, Rep68 is not able to bind efficiently to random HSV-1 sequences (UL44 and US1). In addition, we showed that the Rep helicase activity can inhibit replication of a random DNA substrate if binding is facilitated.

Generally, replicative stress is rapidly sensitized in the cell by numerous stress response factors and pathways such as DNA damage response (DDR) or DNA damage tolerance (DDT) (reviewed in reference 46). One of the best understood stress responses resembles the DDR pathway initiated by the sensor kinase ataxia-telangiectasia mutated kinase (ATM) - and Rad3-related kinase (ATR), which is activated upon stalling of the replication fork (RF). At a stalled RF, the helicase activity of the mini-chromosome maintenance protein complex (e.g., MCM2) is uncoupled from the replication complex and continues unwinding, which generates a stretch of ssDNA recognized and covered by replication protein A (RPA) (47–50). This leads to the activation of ATR, which then induces a DDR resulting in cell cycle arrest, allowing the cell to resolve the stalled RF (51–55). However, persisting replication stress can result in the collapse of the RF, associated with double-strand breaks and the consecutive inhibition of DNA replication (56).

Hence, we hypothesize that binding of Rep to consensus RBs and the helicase activity may generate a situation resembling a stalled RF, in particular when the Rep helicase activity is uncoupled from the replication activity, either because of the absence of a functional trs or because of the absence of the endonuclease activity. Support for this theory comes from previous observations which showed that the AAV2 Rep68/Rep78 proteins induce a cellular DDR which is characterized by the activation of RPA and ATM, leading to an S-phase arrest (28, 57–59). It was initially hypothesized that the activation of ATM is a response to nicks induced by the endonuclease activity of Rep at multiple trs’s located on the cellular chromatin (59). However, we have shown in a later study that the activation of RPA and ATM also occurs with a Rep68/Rep78 mutant lacking endonuclease activity (the Y156F mutant) and that the DNA-binding and ATPase/helicase activities of Rep are necessary for activation of these DNA damage markers (28). We therefore hypothesize that the activation of RPA and ATM in fact due to Rep-induced dsDNA breaks caused by persistent stalling of RFs, although this possibility remains to be investigated.

It has been shown previously that the AAV2 Rep proteins are also capable of binding to the DNA of other helper viruses, such as AdV. In particular, Rep68 proteins can bind specifically to the AdV E2A promoter (60), as well as to a 55-bp DNA fragment within the AdV major late transcription promoter (MLP) via interaction with the cellular TATA box-binding protein (TBP) (61). These interactions of Rep68 with the AdV genome mediate inhibition of transcription and therefore may directly affect AdV replication. However, in contrast to binding of Rep to the HSV-1 genome, binding of Rep68 to the AdV genome occurs independently of consensus RBs, and inhibition of AdV replication does not imply the Rep helicase activity. Whether the AAV2 Rep proteins are capable of binding to the HSV-1 genome independently of a consensus RBs, e.g., via viral or cellular proteins that bind to the HSV-1 DNA, is a matter of current investigations. Of note, the Rep40/Rep52 proteins, which have no DNA-binding domain, are not capable of binding to dsDNA templates, either to consensus RBs or to random sequences. However, Rep40/Rep52 can very well bind to ssDNA templates, which is facilitated by two lysine residues (Lys-404 and Lys-406) located within the helicase domain (62). We indeed observed some unspecific binding of Rep52 to consensus RBs within the HSV-1 genome (Fig. 2C), which may occur when stretches of ssDNA are exposed naturally during replication of the HSV-1 genome. However, since Rep40/Rep52 alone is not capable of inhibiting HSV-1 DNA replication (28), we consider that unspecific binding of Rep40/Rep52 to consensus RBs on the HSV-1 genome may not exhibit sufficient binding capacity to inhibit HSV-1 DNA replication.

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