Definition of herpes simplex virus helper functions for the replication of adeno-associated virus type 5

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Adeno-associated virus (AAV) type 5 represents the genetically most distant AAV serotype and the only one isolated directly from human tissue. Seroepidemiological evidence suggests herpes simplex virus (HSV) as a helper virus for human AAV5 infections, underlining the in vivo relevance of the AAV–herpesvirus relationship. In this study we analysed, for the first time, HSV helper functions for productive AAV5 replication, and compared these to AAV2. Using a combination of HSV strains and plasmids for individual genes, the previously defined HSV helper functions for AAV2 replication were shown to induce AAV5 gene expression, DNA replication and production of infectious progeny. The helper functions comprise the replication genes for ICP8 (UL29), helicase–primase (UL5/8/52), and DNA polymerase (UL30/42). HSV immediate-early genes for ICP0 and ICP4 further enhanced AAV5 replication, mainly by induction of rep gene expression. In the presence of HSV helper functions, AAV5 Rep co-localized with ICP8 in nuclear replication compartments, and HSV alkaline exonuclease (UL12) enhanced AAV5 replication, similarly to AAV2. UL12, in combination with ICP8, was shown to induce DNA strand exchange on partially double-stranded templates to resolve and repair concatemeric HSV replication intermediates. Similarly, concatemeric AAV replication intermediates appeared to be processed to yield AAV unit-length molecules, ready for AAV packaging. Taken together, our findings show that productive AAV5 replication is promoted by the same combination of HSV helper functions as AAV2.

INTRODUCTION

Adeno-associated virus type 5 (AAV5) represents the genetically most distant member of the AAV family, with AAV2 being considered as the prototype strain. In contrast to AAV serotypes 1 to 4, which were isolated as contaminants of human or simian adenovirus stocks (Atchison et al., 1965; Hoggan et al., 1966; Parks et al., 1967), AAV5 was directly isolated from human tissue (Bantel-Schaal & zur Hausen, 1984). Although it was obvious from the beginning that AAV5 infection was prevalent in the human population (Georg-Fries et al., 1984; Samulski et al., 1982), research focused on AAV2, the first AAV strain to be cloned and sequenced (Samulski et al., 1982; Srivastava et al., 1983) as prerequisites for further molecular analysis and the development of AAV2-based gene therapy vectors.

The AAVs are small, replication-deficient, apathogenic viruses that require co-infection with an unrelated helper virus for productive infection. Besides adenovirus (Ad), all herpesvirus genera tested so far, including herpes simplex virus (HSV), cytomegalovirus, and human herpesvirus 6 have been shown to support productive AAV2 replication (Blacklow et al., 1967; Buller et al., 1981; Georg-Fries et al., 1984; McPherson et al., 1985; Thomson et al., 1994). In the absence of a helper virus, AAV2 was shown to establish latency. Both chromosomal integration and episomal modes of persistence have been described (Hüser et al., 2010, 2014; Schnepf et al., 2005).

AAV5 has a 4.7 kb ssDNA genome that comprises two open reading frames, one coding for the capsid proteins (cap) and another (rep) coding for a family of non-structural regulatory proteins (Chiorini et al., 1999b). Whereas AAV2 expresses Rep78, Rep52 and C-terminally spliced versions thereof, called Rep68 and Rep40, AAV5 only transcribes unspliced mRNAs leading to Rep78 and Rep52 (Qiu et al., 2002). In addition, a Rep40-like protein generated by alternative translation initiation was recently identified (Farris & Pintel, 2010, 2013). Analogously to AAV2, AAV5 Rep78 is assumed to regulate AAV gene expression and DNA replication. AAV5 Rep was shown to bind to the AAV5 inverted terminal repeats (ITR) that flank the genome and serve as origins of AAV5 DNA replication. Whereas the ITRs of most AAV serotypes are relatively conserved and in part interchangeable, the AAV5 ITR is more divergent and genetically unstable (Chiorini et al., 1999a, b). This has long hampered the cloning of a fully infectious WT AAV5 genome as a prerequisite for
further analysis (Chiorini et al., 1999b). On the other hand, Rep78 derived from AAV5 was the first Rep protein to be crystallized together with its cognate ITR, visualizing Rep-binding to the conserved Rep-binding site (RBS) within the AAV5 ITR (Hickman et al., 2002, 2004). A conformational change of the ITR upon binding of Rep leads to nicking of the adjacent terminal resolution site (trs) required for processive AAV DNA replication (Chiorini et al., 1999a).

In cell culture, AAV5 can be propagated by co-infection with either adenovirus or HSV. In contrast to what was assumed from AAV1–4, seroepidemiology for AAV5 showed a parallel increase of HSV and AAV5 antibody titres from early childhood until adulthood, suggestive of HSV as a preferred in vivo helper virus (Georg-Fries et al., 1984). HSV functions involved in AAV5 replication have not yet been analysed. For productive AAV2 replication, the responsible HSV functions consist of a subset of six HSV replication genes comprising those for the HSV DNA-binding protein, also called ICP6 (UL29); the three-component helicase–primase complex (HP) encoded by UL5, UL8 and UL52; and the two-component HSV DNA polymerase (UL30/UL42). The minimal HSV four protein complex consisting of ICP8 and HP (UL5/8/52/29) leads to low-level, productive AAV2 replication (Weindler & Heilbronn, 1991). In vivo, AAV2 Rep co-localizes to ICP8 in a ssDNA-dependent manner, for which complex formation with UL5/8/52 is required (Alex et al., 2012; Heilbronn et al., 2003; Slanina et al., 2006). The HSV immediate-early (IE) gene for ICP0 was shown to transactivate rep gene expression from an integrated AAV2 genome (Geoffroy et al., 2004) and together with the HSV IE functions ICP4 and ICP22, further enhances AAV replication in the presence of the six identified HSV replication genes (Alazard-Dany et al., 2009; Nicolas et al., 2010). More recently, HSV alkaline exonuclease, encoded by UL12, was shown to add to AAV replication, likely by helping to resolve concatemeric AAV replication intermediates (Nicolas et al., 2010). Specific HSV mutants defective in these genes (Alazard-Dany et al., 2009; Nicolas et al., 2010; Slanina et al., 2006; Weindler & Heilbronn, 1991) corroborated the transfection data. The HSV ori-binding protein (UL9) is dispensable (Weindler & Heilbronn, 1991). This is not surprising, since AAV Rep serves as an ori-binding protein attracting HSV-ICP8 to the AAV ITR, comparable to the action of UL9 on the HSV ori (Alex et al., 2012; Heilbronn et al., 2003). Upon infection with HSVUL9, HSV DNA replication was blocked, whereas AAVwt replication was undiminished (Alazard-Dany et al., 2009; Weindler & Heilbronn, 1991). These findings clearly document that all HSV helper functions required for AAV2 replication are expressed before HSV DNA replication.

In this study, we tested the HSV helper functions previously identified for AAV2 for their role in AAV5 replication. By a combination of infection and transfection experiments, we analysed AAV5 gene expression, DNA replication and production of infectious AAV5 particles. The dependence of AAV5 replication on various combinations of transfected HSV functions was analysed, and compared side by side to the results of HSV-induced AAV2 replication.

RESULTS

Comparison of AAV5 and -2 replication with Ad-2 or HSV-1 as helper virus

Initial experiments were performed to compare AAV5 replication in HeLa cells upon co-infection with Ad-5, Ad-2, or HSV-1. All helper virus strains led to very comparable levels of rep and cap gene expression, AAV DNA replication and AAV particle production (data not shown). In time-course experiments, AAV2 and AAV5 DNA replication with either helper virus reached a plateau at 24 h that remained stable until 48 h post-infection (p.i.). To evaluate AAV5 replication upon plasmid transfection, pAAV5, containing the AAV5 WT genome, was transfected into HeLa cells, which were subsequently infected with HSV-1 (m.o.i. of 5) or Ad-2 (m.o.i. of 5). At 48 h p.i. cells were harvested for parallel analysis of AAV rep and cap gene expression, AAV DNA replication and AAV virus production. As shown in Fig. 1(a), AAV5 Rep78, Rep52 and a Rep40-like protein are induced with either HSV-1 or Ad-2 infection. The Rep expression profile is indistinguishable from the one described for AAV5 induced by Ad-5 helper genes (Nayak & Pintel, 2007), whereas expression of VP proteins is higher in the presence of HSV-1 (Fig. 1a). A parallel experiment was conducted transcfecting an AAV2 WT plasmid (pAAV2), leading to the typical AAV2 expression pattern for Rep and VP proteins (Fig. 1b). AAV DNA replication leads to higher order concatemers that are resolved by Rep during packaging of AAV ssDNA genomes. On Southern blots, AAV replication forms (RFs) are indicative of AAV DNA replication. RF1 represents 4.7 kb double-stranded AAV monomers, and RF2 represents 9.4 kb double-stranded AAV dimers (Fig. 1c). Production of AAV5 virus particles was quantified by quantitative PCR (qPCR) as genomic particles (gp ml⁻¹) determined in cell extracts. High titres of AAV5 were produced with either helper virus (Fig. 1d).

HSV helper functions for AAV5 replication are expressed before HSV DNA replication

To identify the HSV functions responsible for AAV5 replication, pAAV5-transfected cells were infected with HSVUL9, a replication-deficient host range mutant for the HSV ori-binding protein (Malik et al., 1992) previously shown to support AAV2 replication to a similar degree as WT HSV-1 (Weindler & Heilbronn, 1991). In the time-course experiments displayed in Fig. 2, the AAV5 Rep and VP protein profiles are identical in cells infected with HSV-1, or HSVUL9, respectively. HSV UL5 helicase, expressed as an ‘early’ protein, shows stable expression levels for either HSV strain, whereas UL9 is only expressed in HSV-1 infected cells. As expected, UL9 is not expressed in cells infected with HSVUL9 (Fig. 2b, d). Similar results were achieved in a parallel experiment with pAAV2 (Fig. 2c, d). AAV virus production was analysed by qPCR in benzene-treated freeze-thaw cell extracts. AAV5 titres of up to $5 \times 10^{10}$ gp ml⁻¹ were reached at 48 h p.i. with HSV-1, or
HSV1 UL9 as helper virus. AAV2 reached higher titres of up to $8 \times 10^{11}$ gp ml$^{-1}$ (Fig. 2e). In summary, the HSV functions required for AAV5 replication are fully expressed before and in the absence of HSV DNA replication, as shown previously for AAV2 (Weindler & Heilbronn, 1991).

**HSV IE genes induce AAV5 rep gene expression**

The results shown so far suggested that the previously identified set of HSV helper genes described for AAV2 replication are similarly involved in productive AAV5 replication. The set of HSV helper genes consists of the IE genes for ICP0 and ICP4, which transactivate the six identified ‘early’ HSV replication genes (UL5, $-8, -29, -30, -42, -52$). Together, these are required for productive AAV2 replication (Weindler & Heilbronn, 1991). Subsequently, ICP0 was shown to induce rep gene expression from chromosomally integrated AAV2 genomes (Alazard-Dany et al., 2009; Geoffroy et al., 2004). Here, the impact of HSV IE genes on AAV5 gene expression was analysed, as displayed in Fig. 2(f). The combination of ICP0 and ICP4 led to the highest Rep protein levels in the absence of VP proteins. Marginal VP3 levels were detected upon over-exposure of the blot (data not shown). ICP22 did not further enhance AAV5 expression (Fig. 2f). Therefore, the combination of ICP0 with ICP4 was selected for the subsequent analysis of HSV helper functions for AAV5 replication.

**Identification of HSV replication genes as helper functions for AAV5 replication**

The finding of undiminished productive AAV5 replication in the presence of HSV1UL9 was reminiscent of our previous study identifying the HSV helicase–primase complex...
Fig. 2. AAV5 and AAV2 replication with HSV-1 or HSVΔUL9 or induced by HSV IE genes. A time-course of pAAV5, or pAAV2 transfected cells after infection with WT HSV-1 or the replication-deficient strain HSVΔUL9 was performed as described in Fig. 1. (a, b) Western blots of pAAV5 transfected cells infected with HSV-1 or HSVΔUL9 as indicated. AAV Rep was detected with mAb 303.9, AAV VP with mAb B1, HSV-1 early proteins UL5 and UL9 with the mAb 152 (anti-UL5) or mAb 13924 (anti-UL9), respectively. Actin detected as outlined in Fig. 1 served as a control. (c, d) Western blots with pAAV2 transfected cells performed in parallel to (a) and (b). (e) AAV5 and AAV2 genomic particle yields with HSV-1wt or HSVΔUL9 were analysed as outlined in Fig. 1(d). (f) HeLa cells were co-transfected with pAAV5 and equal amounts of plasmids for the HSV IE genes, ICP0, ICP4, and ICP22 in the combinations depicted. Co-transfection with pAAV2 and ICP0 (lane 7) served as control. Cells were harvested 48 h post-transfection and analysed for Rep expression on Western blots with mAb 303.9 as described in Fig. 1.

(UL5/UL8/UL52) and the single-strand DNA-binding protein ICP8 (UL29) as minimal helper functions for AAV2, and the two-component HSV DNA polymerase (UL30/UL42) further enhancing AAV2 replication (Weindler & Heilbronn, 1991). Recently, the HSV-1 alkaline exonuclease (UL12) was identified as an additional enhancing
factor, when combined with the identified set of HSV IE and DNA replication genes (Nicolas et al., 2010). To test the assumption that these genes are also involved in AAV5 replication induced by HSV, various combinations thereof were co-transfected with pAAV5, and analysed for expression of Rep and cap, AAV5 DNA replication and the production of infectious AAV5 particles (Fig. 3). Rep expression levels are highest with the combination of ICP0, ICP4 and the six HSV replication genes for ICP8 (UL29), helicase–primase (UL5/8/52) and DNA polymerase (UL30/UL42) (Fig. 3a, upper panel). This set of genes also led to the highest levels of VP proteins (Fig. 3a, lower panel). Expression of HSV

![Fig. 3. AAV5 and AAV2 replication induced by combinations of HSV-1 helper genes. Plasmids pAAV5 or pAAV2 were co-transfected in HeLa cells with combinations of HSV-1 genes ICP0, ICP4, UL5, UL8, UL52, UL29 (ICP8), UL30, UL42, UL12 or UL12exo, as indicated. AAV plasmid transfected cells infected with HSV-1 (m.o.i. of 5) served as references. (a, b) Cells were analysed on Western blots for AAV Rep, AAV VP and actin expression as outlined in Fig. 1. (c, d) AAV DNA replication was analysed on Southern blots with 32P-labelled rep gene-derived probes specific for either AAV5 or AAV2, as outlined in Fig. 1. Asterisks depict low intensity bands of the 4.7 kb AAV5 replication form (RF1). (e, f) Production of infectious AAV particles was analysed after second round infection of HeLa cells in triplicate cultures, infected with cleared, transfected cell extracts. AAV titres were analysed by qPCR as gp ml⁻¹, as described in Fig. 1.]

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exonuclease (UL12) did not further enhance AAV5 expression levels. The identical experimental set-up was performed several times with both AAV5 and AAV2, leading to comparable results (Fig. 3b). The data suggest that VP expression is most pronounced when HSV IE genes are combined with HSV replication genes that lead to AAV template amplification, as outlined below (Fig. 3c, d).

DNA replication of AAV5 or AAV2 was analysed on Southern blots of size-separated, genomic DNAs from Hirt extracts, which were digested with DpnI to degrade input plasmid DNA. Upon infection with HSV, the typical RF1 and RF2 bands are visible for AAV2 and for AAV5 (Fig. 1c; Fig. 3c, d: lane 10). Upon co-transfection of HSV helper genes, AAV2 replication is apparent as described before (Alazard-Dany et al., 2009; Weindler & Heilbronn, 1991) whereas AAV5 DNA replication is barely detectable (Fig. 3c). Upon co-transfection of UL12, RF1 and RF2 become clearly visible (Fig. 3c: lane 7). The specificity of the UL12 effect is underlined by the disappearance of RF bands upon addition of an exonuclease-deficient version of UL12 (Fig. 3c: lane 8), as initially described for AAV2 (Nicolas et al., 2010). In summary, AAV5 DNA replication increases with increasing numbers of HSV helper genes, which is consistent with a parallel increase of VP protein levels.

Production of infectious AAV particles was analysed in second round infection with cleared supernatants of freeze-thaw extracts. Since AAV titres were initially higher with AAV2 (Fig. 2a), the AAV2 extracts were pre-diluted. AAV titres were quantified in triplicate cultures of Ad-2-infected HeLa cells, as gp ml⁻¹ by qPCR. All combinations of HSV helper functions led to production of infectious AAV and titres increased with increasing numbers of HSV helper genes (Fig. 3e, f). In summary, HSV helper functions for AAV5 replication are identical to the ones described before for AAV2 (Alazard-Dany et al., 2009; Nicolas et al., 2010; Weindler & Heilbronn, 1991). The highest AAV5 replication rates are achieved by the combined expression of the HSV IE genes, ICP0 and ICP4; six HSV replication genes, UL5/8/29/30/42/52; and HSV alkaline exonuclease, UL12.

**Nuclear co-localization of AAV5 Rep and HSV ICP8**

We have shown before that Rep of AAV2 co-localizes to HSV ICP8 in nuclear replication compartments in an AAV ssDNA-dependent manner (Alex et al., 2012; Heilbronn et al., 2003; Slanina et al., 2006). ICP8 and the helicase–primase complex (UL5/8/52) are required to initiate the formation of the replication compartments, which further mature when the two-component HSV polymerase (UL30/42) is co-expressed (Wilkinson & Weller, 2004). Here we studied co-localization of AAV5 Rep (tagged with HA at the carboxy-terminus) with HSV ICP8, in the presence of different combinations of HSV helper functions. Although co-localization of AAV5 Rep with ICP8 was not as pronounced as previously described for AAV2 (Alex et al., 2012; Heilbronn et al., 2003; Slanina et al., 2006), it was clearly apparent when UL30/42 and ICP0/4 were added (Fig. 4a). Thus, AAV5 Rep can be recruited by HSV to nuclear replication compartments, similarly to Rep of AAV2 (Alex et al., 2012; Heilbronn et al., 2003; Slanina et al., 2006). The relatively reduced frequency of co-localization may reflect less efficient AAV5 plasmid rescue due to the more complex AAV5 ITR (Chiorini et al., 1999b), leading to reduced AAV5 replication as described above (Fig. 2e).

**DISCUSSION**

In this study, the HSV helper functions for productive AAV5 replication were identified to consist of the HSV IE genes for ICP0 and ICP4; the HSV replication functions for ssDNA-binding protein ICP8 (UL29); the helicase–primase complex (UL5/8/52); and the two-subunit DNA polymerase (UL30/42). In addition, alkaline exonuclease (UL12) adds to AAV5 replication. The results are corroborated by side-by-side analysis with AAV2, confirming the findings described previously (Alazard-Dany et al., 2009; Nicolas et al., 2010; Weindler & Heilbronn, 1991).

**Comparison of HSV and Ad helper functions for AAV5**

The major viral helpers for productive AAV2 replication, adeno virus and HSV, were also the most intensely studied in the past. For AAV2 replication five Ad-5 functions, namely E1A, E1B, E2A, E4Orf6 and VA RNA were identified to support DNA replication and production of progeny virus (Muzyczka & Berns, 2001). These induce AAV2 replication by a combination of effects on AAV transcription, translation, induction of DNA second-strand synthesis and diverse modulatory effects on host cell proteins. AAV5 displays a divergent transcription profile, wherein only unspliced mRNAs are transcribed for expression of Rep78 and Rep52 (Qiu et al., 2002). An additional Rep40-like protein is initiated downstream of Rep52 from the same mRNA (Farris & Pintel, 2010; Fasina & Pintel, 2013). In spite of these variations, the same combinations of Ad-5 genes that replicate AAV2 were identified to replicate AAV5 (Nayak & Pintel, 2007). In view of the similarity of the HSV-induced with the Ad-induced AAV5 expression profile described here, it was not surprising to find that the same HSV helper functions identified for AAV2 were active with AAV5 as well. Other than adeno virus, HSV exerts its helper effects by a combination of transcriptional activation, DNA replication and genome maturation for both AAV2 and AAV5 (Fig. 4b).

**Mechanisms of AAV induction by HSV IE genes**

The major HSV IE proteins ICP0 and ICP4 serve as transcriptional activators and are constituents of HSV transcriptional activator complexes. Their role as transactivators of silent HSV ‘early’ replication genes is...
with antibodies against HSV ICP8 and the HA-tag of AAV5 Rep, and were analysed by confocal microscopy. Displayed images represent cross sections of 0.8 μm. Anti-ICP8 reactivity is displayed in green, while Rep is represented in red. Merged foci appearing in yellow indicate co-localization. To determine the extent of co-localization, the correlation coefficient R of green (ICP8) and red (Rep) fluorescence above a fixed background value was determined for a total of 4 to 10 cells for each construct, and is displayed as mean ± SEM. NA, not applicable.

(b) A model of HSV-induced AAV DNA replication is depicted. The AAV5 genome with essential genetic elements is depicted on top. In the middle, the initiation of Rep78-dependent AAV DNA replication is depicted, proposing a stepwise recruitment of HSV replication proteins, ssDNA-binding protein (ICP8), helicase–primase complex (UL5/8/52) and two-component HSV DNA polymerase (UL30/42).

**AAV ITR-dependent initiation of DNA replication by HSV replication functions**

For the initial identification of HSV helper functions for AAV replication, HSV ‘early’ replication genes were expressed from constitutive heterologous promoters to make them independent of transactivation by HSV IE genes. This led to the identification of the minimal set of four helper genes for AAV replication, encoding ICP8 and the helicase–primase complex. These were sufficient for low-level AAV2 production including rep gene expression (Alex et al., 2012; Slanina et al., 2006; Weindler & Heilbronn, 1991). In the absence of a helper virus, AAV2 gene expression is repressed by Rep78/68, which binds to AAV2 promoters (Beaton et al., 1989; Kyo¨stio¨ et al., 1994). ICP0 was shown to induce Rep in a cell line harbouring latent AAV2 (Geoffroy et al., 2004). It is long established that Rep78/68 is required, but not sufficient, for AAV replication. Ad E1A, a potent transcriptional activator, relieves the repression and strongly stimulates rep gene expression (Chang et al., 1989). HSV ICP0 appears to exert a comparable effect when inducing rep from an integrated silent AAV2 genome (Geoffroy et al., 2004) or from transfected plasmids for WT AAV2 or AAV5, as shown here. Similar to E1A, ICP0 does not bind to DNA directly, but exerts its transcriptional effects indirectly. HSV mutants with specific amino acid exchanges in ICP0 showed that ICP0 exploits the ubiquitin-proteasome pathway, presumably to degrade factors that repress the AAV2 p5 promoter. Neither the localization of ICP0 to nuclear ND10 domains, nor their destruction, was required for rep gene expression (Geoffroy et al., 2004). HSV ICP4, the major HSV IE transcriptional activator had hardly an effect on Rep induction, but enhanced the stimulatory effect of ICP0 (Alazard-Dany et al., 2009). The additive effect of ICP0 and ICP4 on Rep of AAV5 shown here, is consistent with previous findings for AAV2 (Alazard-Dany et al., 2009).

**Fig. 4.** Co-localization of AAV5 Rep and HSV-1 ICP8 and model of HSV-dependent AAV replication. (a) HeLa cells grown on coverslips were co-transfected with pAAV5-Rep-HA and different combinations of HSV-1 genes for ICP0, ICP4, UL5, UL8, UL52, ICP8 (UL29), UL30 and UL42, as indicated. Cells were stained

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1991). Here we show that ICP8 and helicase–primase also constitute the minimal helper genes for AAV5 replication, although the efficiency is very low. Addition of ICP0/4 stimulates Rep and VP levels and boosts AAV production. In the absence of HSV DNA polymerase, a cellular polymerase is assumed to induce AAV5 DNA replication. Leading-strand DNA polI was shown to replicate AAV2 in vitro (Nash et al., 2008) and to co-localize in vivo with Rep in nuclear HSV/AAV replication compartments (Nicolas et al., 2010). The full replication complex is assembled when the two-component HSV DNA polymerase joins in (Fig. 4b). AAV5 DNA replication and particle production are enhanced by more than tenfold, as previously shown for AAV2 replication (Alazard-Dany et al., 2009; Weindler & Heilbronn, 1991).

AAV DNA replication is initiated by binding of Rep78/68 to the RBS of the ITR. Although the primary DNA sequences are divergent, both AAV2 and AAV5 display comparable hairpin-structured ITRs and the RBS sequences are conserved (Chiorini et al., 1999a, b; Hickman et al., 2004). AAV2- and AAV5-Rep78 display DNA-binding, ATPase, helicase and endonuclease activities (Chiorini et al., 1999a; Maggin et al., 2012) required for the initiation of AAV DNA replication. Co-crystallization of the endonuclease domain of AAV5 Rep with the AAV5-ITR identified conserved amino acids that contact the RBS and the adjacent trs on the opposite DNA strand (Hickman et al., 2004). Although the sequence of the trs cleavage site differs between AAV2 and AAV5, the mechanisms appear to be similar and nicking is required for ongoing AAV DNA replication (Chiorini et al., 1999a).

HSV-induced AAV2 DNA replication is initiated by AAV ssDNA-dependent ternary complex formation of Rep78 and ICP8 on the hairpin-structured AAV ITR as shown in vitro. In vivo, Rep78 was shown to co-localize to ICP8 in nuclear replication compartments in an AAV ssDNA-dependent manner (Alex et al., 2012; Heilbronn et al., 2003). Ternary complex formation of Rep and ICP8 on the AAV ITR is reminiscent of HSV ori-binding by the HSV ori-binding protein UL9, which first recruits ICP8 to cover and stabilize ssDNA regions of the unwinding HSV ori (Weller & Coen, 2012). The growing replication fork then recruits the helicase–primase complex, as shown in vitro (Biswas & Weller, 2001; Chen et al., 2011; Weller & Coen, 2012). In vivo, ICP8 and HP initiate the formation of nuclear HSV replication compartments (Livingston et al., 2008; Weller & Coen, 2012). With AAV ssDNA genomes as templates, Rep co-localizes to ICP8 in these nuclear foci, and the four helper proteins initiate AAV DNA replication (Slanina et al., 2006). We show here that this is the case also for AAV5. In contrast to the HSV ori, the AAV ITR represents a partially double-stranded genome that can adopt a fork-shaped DNA configuration. It contains a self-primed free 3’ end, ready for elongation by DNA polymerase even in the absence of UL52 primase activity, as shown by us before (Slanina et al., 2006). The three protein components of the helicase–primase complex were needed, apparently serving as a structural backbone of the replication complex. Here we show that the components of the HSV replication complex for AAV5 replication are the same, leading to the assumption that the details of their interactions can be extrapolated from earlier findings with AAV2 (Fig. 4b).

Role of UL12 in AAV genome maturation

The most recent addition to the list of HSV helper genes was UL12, which codes for alkaline exonuclease. UL12 was identified as a Rep-interacting protein, and was shown to enhance the accumulation of unit-length AAV RFs (Nicolas et al., 2010). UL12 is not directly involved in HSV DNA replication. In cooperation with ICP8, UL12 mediates DNA strand exchange of newly replicated HSV DNA concatemers, apparently for repair of DNA damage emerging during replication (Reuven et al., 2003). HSV infection was shown to induce DNA repair by homologous recombination, and UL12 was identified to interact with the components of the ATM pathway (Balasubramanian et al., 2010). Recently, isolated UL12 exonuclease proved to be sufficient to induce DNA repair by the single-strand annealing pathway that exploits small DNA sequence homologies to anneal double-strand breaks at the expense of small deletions of varying sizes. (Schumacher et al., 2012; Weller & Coen, 2012). Here we used catalytically active or inactive UL12 variants to demonstrate that the AAV5 replication pattern with predominant AAV intermediates of undefined lengths is relieved by UL12. Unit-length AAV RFs accumulate, leading to a moderate enhancement of AAV5 production, as shown before for AAV2 (Nicolas et al., 2010). It will be interesting to see whether UL12, by binding to both Rep and ICP8, induces AAV DNA strand exchange of concatemeric AAV replication intermediates. The AAV ITR contains multiple short DNA repeats which are known to invert and recombine during AAV DNA replication, resulting in variable alternative ITR conformations. It is conceivable that UL12 exploits these DNA repeats to mediate DNA strand exchange of AAV genomes in conjunction with ICP8. Thereby, unit-length AAV ssDNA strands will accumulate, ready for packaging. The titre of infectious AAVs directly depends on the proportion of particles with packaged full-length genomes, likely explaining the enhancement of infectivity by addition of UL12 seen here for AAV5, and reported before for AAV2 (Nicolas et al., 2010).

METHODS

Cells and viruses. HSV-1 strain KOS and the HSVΔUL9 mutant (hr94) (Malik et al., 1992) were grown in Vero cells, as described (Heilbronn et al., 1990). Adenovirus type 2 (Ad-2) was propagated in HeLa cells.

Plasmids. The AAV5 plasmids p5TRV-RepCap and p5TRV-Rep380HACap were kind gifts of D. Pintel. p5TRV-RepCap spans the AAV5 WT genome with two 4 nt insertions between the ITRs and...
the coding region, and is abbreviated as pAAV5. Plasmid p5TRV-Rep380HACap contains a haemagglutinin tag (HA) at AAV5 nt position 2158 (Farris & Pintel, 2010). The AAV2 plasmid (pTA2V-0) comprises the AAV2 WT genome (abbreviated as pAAV2). Expression plasmids for the HSV-1 replication genes UL5, UL8, UL52, UL29 (ICP8), UL30 (pol) and UL42, and plasmids for HSV-1 ICP0 and ICP4 under their cognate promoters, were described before (Heilbronn & zur Hausen, 1989). Plasmid pTF3pol for ICP4/0/22, and UL30/UL42 (Alazard-Dany et al., 2009), pSAKUL12 and pUL12exo+ for HSV-1 exonuclease (Nicolas et al., 2010; Reuven et al., 2003) were under heterologous promoter control.

**DNA transfection and cell infection.** DNA transfection of HeLa cells was performed as described (Winter et al., 2012), and infection with HSV-1 WT or HSV5UL9 was performed at an m.o.i. of 2–5, or Ad-2 at an m.o.i. of 5. For AAV infections, equal proportions (1/30) of AAV-containing freeze–thaw supernatants were mixed with Ad-2 (m.o.i. of 20) to co-infect HeLa cells in a total volume of 200 μl per well for 1.5 h. AAV2-containing cleared supernatants were serially diluted up to 10⁻⁴ before infection. The inoculum was sucked off, fresh medium was added and the cells were incubated for another 48 h.

**Southern blot analysis.** DNA samples were generated by Hirt extraction as described (Winter et al., 2012). For the detection of AAV5 and AAV2 DNA replication intermediates, DNA probes were labelled with a DecaLabel DNA Labelling kit (Termo Scientific) and α-³²P-dCTP for radioactive labelling, or biotin-11-dUTP labelling, according to the manufacturer’s protocol. The AAV5 probe spans a 1.1 kb EcoRl fragment within the rep ORF (AAV5 nt 532–1637). For AAV2 a 1.1 kb Ncol/EcoRl fragment located in the rep ORF (AAV2 nt 626–1768) was used. Membranes hybridized with ³²P-labelled DNA probes were detected by autoradiography on X-ray films. Biotinylated probes were detected with Streptavidin HRP (High-Sensitivity HRP, Thermo Scientific) by enhanced chemiluminescence (ECL-kit, Perkin Elmer).

**Western blot analysis.** Western blots were prepared as described (Winter et al., 2012). Protein extracts were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Protran BA85, GE Healthcare). The membrane was reacted with mAb 303.9 (anti-Rep) or mAb B1 (anti-VP) (Progen) at a 1:10 dilution, mAb 152 (anti-UL5) diluted 1:15 (Slanina et al., 2006), and mAb 13924 (anti-UL9) diluted 1:2500 (Stow et al., 1998). The anti-β-actin rabbit polyclonal antibody was diluted 1:5000. A HRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:2000 (antibodies were diluted in 2 % FCS/PBS). Secondary antibody mixture consisted of rhodamine-labelled goat anti-rabbit and fluorescein-labelled goat anti-mouse IgG, both diluted 1:1000. Coverslips were washed three times in PBS and mounted in polyvinyl alcohol (Elvanol) containing 1 % 1,4-Diazobicyclo-(2,2,2)-octane (DABCO) as an anti-fading agent. Image acquisition was performed with a Zeiss LSM 510 laser-scanning microscope.

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