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Vaccinia virus as a subhelper for AAV replication and packaging

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Adeno-associated virus (AAV) has been widely used as a gene therapy vector to treat a variety of disorders. While these vectors are increasingly popular and successful in the clinic, there is still much to learn about the viruses. Understanding the biology of these viruses is essential in engineering better vectors and generating vectors more efficiently for large-scale use. AAV requires a helper for production and replication making this aspect of the viral life cycle crucial. Vaccinia virus (VV) has been widely cited as a helper virus for AAV. However, to date, there are no detailed analyses of its helper function. Here, the helper role of VV was studied in detail. In contrast to common belief, we demonstrated that VV was not a sufficient helper virus for AAV replication. Vaccinia failed to produce rAAV and activate AAV promoters. While this virus could not support rAAV production, Vaccinia could initiate AAV replication and packaging when AAV promoter activation is not necessary. This activity is due to the ability of Vaccinia-driven Rep78 to transcribe in the cytoplasm and subsequently translate in the nucleus and undergo typical functions in the AAV life cycle. As such, VV is subhelper for AAV compared to complete helper functions of adenovirus.

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INTRODUCTION

Adeno-associated virus (AAV) is a replication-deficient parvovirus that requires a helper virus to complete its lytic infection. It contains a single genome of approximately 4.7 kb flanked by two inverted terminal repeats (ITRs). The AAV genome contains two open reading frames, which encodes four Rep and three Cap proteins.[1] Helper viruses, such as adenovirus, are responsible for various functions in AAV replication. Adenovirus aids in many stages of the AAV life cycle including transcriptional activation, RNA transport, stability and translation,[2–4] DNA replication and viral assembly, and packaging.[3,5,6]

Along with adenovirus, various other viruses have been proposed as helper viruses for AAV replication. Herpes virus,[7] Human Papilloma Virus,[8] and vaccinia virus (VV),[9] have all been reported as being helper viruses for AAV. While the roles of Herpes virus, HPV, and adenovirus have been investigated extensively to date, there is only one single research article that describes VV functioning as an AAV helper virus.[9]

Here, we elaborate on previous findings that VV acts as a helper for AAV replication. Our studies found that vaccinia is a subprime helper for AAV. Unlike typical helper viruses, such as Adenovirus, vaccinia is unable to activate AAV promoters. Although the important initial helper functions required for AAV replication are not supplied by vaccinia, the virus is capable of replicating the AAV genome in the presence of Rep78 and also functions to assist in packaging of AAV. Understanding the functions of vaccinia and its role as a helper for AAV has aided in its usage in production of AAV vectors. This has allowed for the development of a scaled-up method for vector production that can assist in the necessity for large-scale vector manufacturing required for clinical use of AAV as a gene therapy vector.

RESULTS

VV failed to support rAAV production

In order to evaluate the previous findings that VV is a helper for AAV replication, we utilized the virus VV-T7 in a vector production system to create an AAV2-EGFP vector. We utilized the plasmid pAd/AAV,[10] which contains the entire AAV2 genome with its native promoters (p5, p19, and P40) but lacking AAV ITR along with the vector plasmid pscAAV-CB-EGFP.[11] The AAV2 vectors were produced by transfecting HeLa cells with the two plasmids and coinfection with VV-T7, adenovirus as a positive control or a mock transfection. The resulting cell lysate was evaluated by subsequent transduction of 16,095 cells and observation of vector production by the presence of enhanced green fluorescent protein (EGFP). Figure 1a shows rAAV2 particles generated using EGFP expression as an indicator. When using adenovirus as a helper virus for AAV replication, infectious rAAV2-EGFP could be observed. In contrast, VV super-infection failed to produce detectable amounts of rAAV vector. Since there are various factors that could cause the failure of rAAV production, the protein expression profile and the replication of vector DNA both were evaluated in vaccinia-infected, adenovirus-infected, and mock-infected cultures. We analyzed the Rep and Cap proteins expression profile using western blot analysis. As expected, Rep and Cap proteins (Figure 1b) were observed in samples that utilized adenovirus as a helper. However, Rep and Cap could not be detected in samples that used VV as a helper or mock infected samples. The replication of vector DNA was observed by Southern blot analysis of HeLa cultures. Figure 1c shows that AAV genome replication is observed only in the samples that were infected with adenovirus were multiple replication forms were

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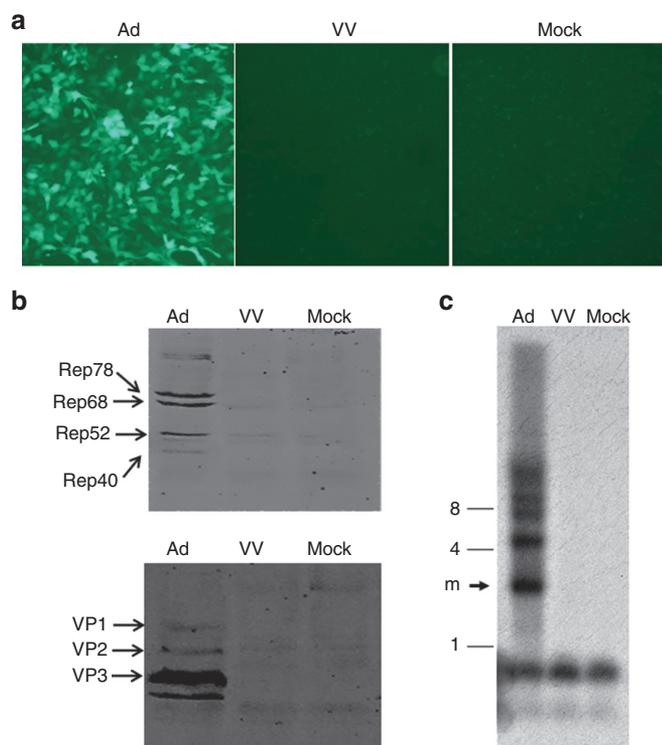
Figure 1 Vaccinia virus alone does not support recombinant adeno-associated virus (rAAV) production. HeLa cells were transfected with pAd/AAV and pscAAV-CB-EGFP. Upon transfection, cells were infected with adenovirus (Ad) or vaccinia virus (VV), or mock infection (mock) and were harvested at 48 hours post-infection. (**a**) Samples were subjected to three rounds of freezing and thawing and heat inactivated before being used to transduce 16,095 cells. rAAV production was visualized by observation of EGFP expression. (**b**) Cell lysate was prepared from collected cells and protein was extracted according to standard procedure and probed for Rep and Cap protein. The Rep and Cap expression profile of samples that were infected with adenovirus or vaccinia virus was observed. (**c**) For Southern blotting, 10 μg of DNA was applied to each lane. Southern blotting was performed according to standard procedures for DNA transfer and hybridized with a 32P-labeled GFP probe. The number on the left indicates the marker positions for 1, 4, and 8 kb and the (m) indicates the position of the monomer.

observed. Altogether, the positive control (adenovirus)-infected samples showed production of rAAV2-EGFP marked by EGFP expression from superinfection of HeLa cell lysate as well Rep and Cap protein expression and vector DNA replication. Vaccinia-infected samples failed to replicate vector DNA or produce Rep and Cap protein, which resulted in the lack of recombinant AAV produced from this method. This observation and discrepancy from previous findings prompted a further investigation into the role of vaccinia in the AAV life cycle.

VV cannot activate AAV promoters
Since Rep and Cap proteins were not expressed in the above experiment, we suspected that the main defect of VV could be due to its inability to activate AAV promoters. In order to investigate this hypothesis, three plasmids were constructed utilizing firefly luciferase as a reporter of promoter activation. The constructs were designed by cloning the AAV promoters into a backbone plasmid where the promoter would drive the transcription of firefly luciferase (Figure 2a). Each construct was transfected into HeLa cells and then infected with adenovirus, VV, or mock infection. The cells were collected at 48 hours post-transfection and lysed in order to detect firefly luciferase

activity. Figure 2b shows that the AAV p5 promoter, p19 promoter, and the p40 promoter were activated in Hela cells in the presence of adenovirus as noted by the significant increase in firefly luciferase activity compared to the mock infected control. However, there is no significant increase in all three AAV promoters when VV is added as compared to the mock infection control. Similar phenomena were observed in CV-1 cells (Figure 2c). The results suggested that unlike adenovirus, VV alone cannot activate the AAV promoters.

VV can replicate the genome in the presence of Rep78
While our findings showed that VV in incapable of supporting AAV replication in a wild-type setting, we wanted to further investigate the findings that Vaccinia is an AAV helper. While the previous data shows that Vaccinia does not activate the AAV p5, p19, and p40 promoters, it may be important in other aspects of the AAV life cycle. To investigate if VV may support AAV replication when AAV promoter activation is not necessary, we made a construct (pCI-Rep/Cap) that expressed rep78 directly from a CMV promoter instead of the p5 promoter. As shown in Figure 3a, the pCI-Rep/Cap plasmid contains the AAV Rep and Cap sequences without the ITRs along with a CMV promoter regulating *rep78,* the AAV p19 promoter regulating *rep52*, and the AAV p40 promoter regulating the *cap* gene. The two plasmids pCI-Rep/Cap and pscAAV-CB-EGFP were transfected into Hela cells and subsequently infected with adenovirus, VV, or mock infected with media only. Hirt DNA was extracted from the each culture condition and genome replication was observed by Southern blot. Figure 3b shows bands observed corresponding to a 1kb DNA ladder. In the presence of adenovirus, vector DNA can be observed in multiple replicative forms. With the addition of VV, replicated vector genome was observed as a monomer and a dimer. This data indicates that in the presence of Rep78, VV was capable of replicating the vector sequence. We also analyzed the Rep and Cap proteins expression profile using western blot analysis. As expected, Rep protein (Figure 3c) was observed in all samples but Cap protein was only detectable in samples infected with adenovirus. This result also indicates that Rep78 alone is not sufficient for genome replication since when this protein was present in mock-infected samples, genome replication was not observed. Rep78 along with VV or adenovirus is essential for vector genome replication.

VV-Rep/Cap can replicate the vector sequence and produce rAAV
Although vaccinia was able to replicate the vector genome in the experiment with a CMV driven expression of P5 rep protein, rAAV production was not detected (data not shown). We suspected this was due to the lack of capsid proteins, which were still under the control of the endogenous AAV P40 promoter. We hypothesized that we would be able to produce AAV vectors when a VV promoter was used to drive the expression of all AAV proteins.

As shown in Figure 4a, AAV rep and cap (VV-W5 and VV-W8) are under control of the vaccinia p7.5 promoter.[12] The plasmid pscAAV-CB-EGFP was transfected into HeLa cells and subsequently infected with the two VVes containing AAV Rep and Cap (VV-W5 and VV-W8). Serving as controls, cultures were either infected with adenovirus or transfected with the mini adenovirus plasmid pFdelta6. In the cells that were coinfected with adenovirus and VV-W5 and VV-W8, we observed a similar level of AAV production as the cells cotransfected with the mini adenovirus plasmid pFdelta6 and VV-W5+VV-W8 infection, at 1×10^4 AAV particles/cell and 9×10^3 AAV particles/cell respectively. In contrast, rAAV vectors are still produced in the presence of VV-W5+VV-W8 at 3×10^3 AAV particles/cell, albeit less than that of adenovirus infection
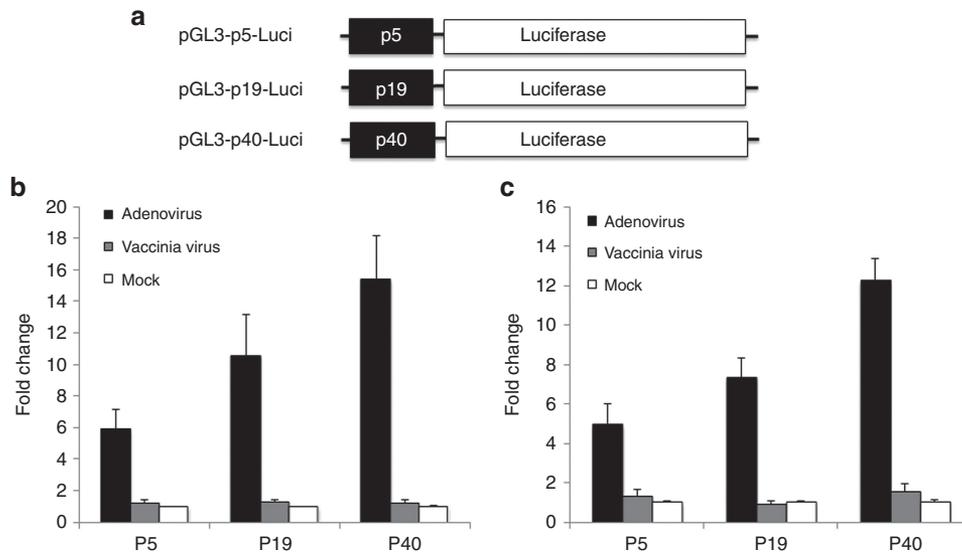
Figure 2 Vaccinia virus cannot activate adeno-associated virus (AAV) promoters. (**a**) Hela cells were transfected with firefly luciferase reporter plasmids pGL3-P5-luci, pGL3-p19-luci, and pGL3-p40-luci, which are under the control of the AAV P5, P19, or P40 promoters respectively. These cells were then infected with adenovirus or vaccinia virus or mock infection. Cells were harvested at 48 hours and then assayed for firefly luciferase activity. Promoter activation of the P5, P19, and P40 promoters was detected by luminescence output and plotted as fold change compared to uninfected control in hela (**b**) and CV1 (**c**) cells.
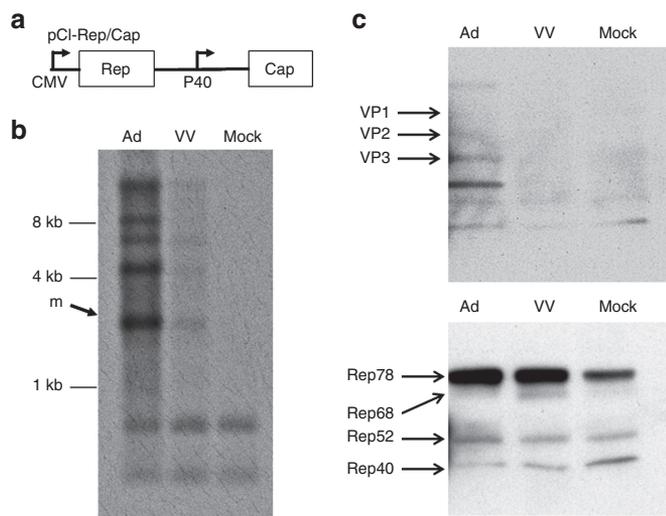
Figure 3 Vaccinia virus can replicate the genome in the presence of Rep78. (**a**) HeLa cells were transfected with pCI-Rep/Cap, pictured, and pscAAV-CB-EGFP. Upon transfection, cells were infected with adenovirus (Ad) or vaccinia virus (VV) or mock infection (mock) and were harvested at 48 hours post-infection. (**b**) Southern blotting was performed according to standard procedures for DNA transfer and hybridized with a 32P-labeled GFP probe. The numbers on the left indicate the marker positions for 1, 4, and 8 kb and the (m) indicates the position of the monomer. (**c**) Cell lysate was prepared from collected cells and protein was extracted according to standard procedure and probed for Rep and Cap protein. The Rep and Cap expression profile was observed.

(Figure 4b). Therefore, by bypassing the need for AAV promoter activation, delivering the necessary rep and cap proteins along with the VV was sufficient for vector production.

In order to evaluate the ability of VV-Rep/Cap to replicate vector DNA, we performed a Southern blot analysis of the above samples. Figure 4c shows that AAV genome replication is observed in all samples that were infected with VV-Rep/Cap. This result is consistent with previous observations as well. We also analyzed the Rep and Cap proteins expression profile using western blot analysis as previously described. As expected, Rep and Cap protein (Figure 4d) were observed in all samples. This data indicates that VV is able to facilitate replication of the vector genome as well as aiding in virion packaging. Similar results were obtained when a single stranded vector was generated instead of the self complementary vector (data not shown). Previous experiments using an AAV8 capsid yielded similar results.[12] Experiments using different ITR and Rep serotypes were not performed as most recombinant AAV vectors are AAV2 with a pseudotyped capsid.

Vaccinia replication aids in rAAV production

These results indicated that although VV is not a typical helper, it is still sufficient in order to replicate and package rAAV. In order to further evaluate these helper functions, we employed a mutant VV, Modified Vaccinia Ankara (MVA). MVA was derived by serial passages of CVA dermovaccinia in chicken embryo fibroblasts and is not permissive for replication in most cell lines. During this process, MVA lost approximately 15% of its genome and a number of virulent genes resulting in a change in plaque morphology.[13] In nonpermissive cell lines, MVA only produces immature viral particles.[14,15] HeLa cells were infected with rAd-AAV-eGFP to deliver the AAV vector sequence as previously shown.[12] These cells were subsequently infected with VV-W5 and VV-W8, each at a multiplicity of infection (MOI) = 1, or MVA-W5 and MVA-W8, each at MOI = 20. Previous unpublished experiments determined that a lower MOI of MVA did not result in infective AAV vectors. Figure 5a shows the rAAV obtained from infection with vaccinia (VV-W5 and VV-W8) or MVA (MVA-W5 and MVA-W8). Cells infected with MVA produced significantly fewer vectors as compared to those infected with VV with 7×10^3 AAV particles/cell and 3.5×10^4 AAV particles/cell respectively. In contrast to the vector yield, there was sufficient Rep protein produced (Figure 5b) and AAV genome replication (Figure 5c) as compared to cells infected with VV. This data indicates that MVA may be sufficient for AAV protein expression and replication, but additional vaccinia factors missing in MVA may be essential for rAAV production.
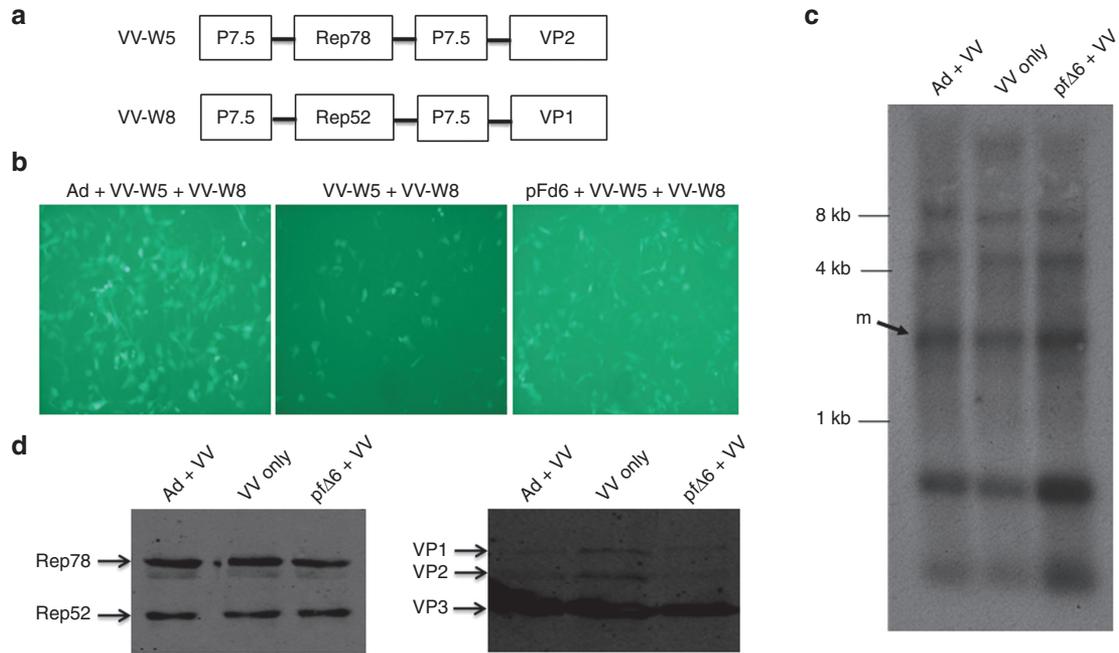
Figure 4 VV-Rep/Cap can replicate the vector sequence and produce recombinant adeno-associated virus (rAAV). (**a**) HeLa cells were transfected with pscAAV-CB-EGFP as a reporter and the helper plasmid pfdelta6 or pCI as a control. Upon transfection, cells were infected with vaccinia viruses containing AAV *Rep* and *Cap* sequences under the control of the vaccinia p7.5 promoter (VV-W5 and VV-W8 as pictured). Cells were additionally infected with adenovirus as a positive control. (**b**) Samples were harvested and subjected to three rounds of freezing and thawing and heat inactivated before being used to transduce 16,095 cells. rAAV production was visualized by observation of EGFP expression and positive cells were counted. Left panel—pscAAV-cB-EGFP + pCI + VV-W5 + VV-W8 + Adenovirus; Middle panel—pscAAV-cB-EGFP + pCI + VV-W5 + VV-W8; Right panel—pscAAV-cB-EGFP + pfdelta6 + VV-W5 + VV-W8. (**c**) Southern blotting was performed according to standard procedures for DNA transfer and hybridized with a 32P-labeled GFP probe. The number on the left indicates the marker positions for 1, 4, and 8 kb and the (m) indicates the position of the monomer. (**d**) Cell lysate was prepared from collected cells and protein was extracted according to standard procedure and probed for Rep and Cap protein. The Rep and Cap expression profile was observed.
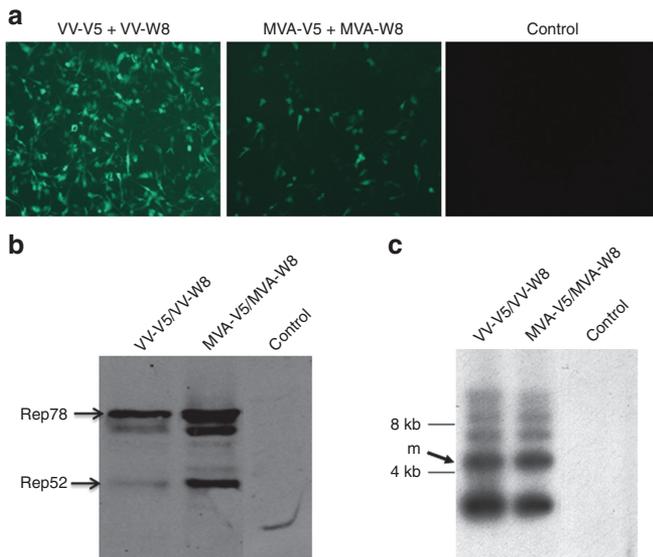
Figure 5 Vaccinia replication aids in recombinant adeno-associated virus (rAAV) packaging. HeLa cells were transduced with Ad-AAV-eGFP as a reporter and either VV-W5 and VV-W8 or MVA-W5 and MVA-W8 or no vaccinia virus as a mock. (**a**) Samples were harvested and subjected to three rounds of freezing and thawing and heat inactivated before being used to transduce 16095 cells. rAAV production was visualized by observation of EGFP expression and positive cells were counted. (**b**) Southern blotting was performed according to standard procedures for DNA transfer and hybridized with a 32P-labeled GFP probe. The number on the left indicates the marker positions for 4 and 8 kb and the (m) indicates the position of the monomer. (**c**) Cell lysate was prepared from collected cells and protein was extracted according to standard procedure and probed for Rep protein. The Rep expression profile was observed.

DISCUSSION

Vaccinia is a virus that undergoes its entire life cycle in the cytoplasm of the host cell. A vast majority of its gene products can only function in the cytoplasm as well. In contrast, AAV is a nuclear virus where the replication and packaging all take place in the nucleus. Well-characterized AAV helper viruses such as adenovirus or herpes virus are all nuclear viruses. Genes of helper viruses identified to support AAV replication and packaging such as E1a, E4ORF6, etc. each have their functions in the nucleus. Nevertheless, cytoplasmic VV has been widely cited as a helper virus for AAV.[16–19] Therefore, this warranted further clarification of its role in AAV life cycle.

In this study, we clearly showed that VV could activate none of the AAV promoters. This is understandable since VV gene products are mainly located in the cytoplasm. It is unlikely that vaccinia could induce some cellular factors to activate AAV P5, P19, or P40 promoters.

Despite its inability to activate AAV promoters, VV has some characteristics of a helper virus. It has the capability to replicate the AAV genome in the presence of Rep78 and properly package the AAV virion when P19 and p40 products were expressed, which has also been reported in another study.[20] One of the hallmarks of a helper for AAV replication is the ability to aid in replication of the vector genome. Studies have shown that in addition to Rep proteins another factor is needed in order to facilitate replication. While research indicates that Rep78 or Rep68 is needed for AAV replication,[21] adenovirus infection[22] or HSV infection[23,24] are also essential for genome replication. Similarly, we found that VV is capable of replicating the AAV genome but unlike its adenovirus and Herpes virus counterparts, it cannot complete all functions of a helper making it a subhelper for the AAV life-cycle. The difference between wild-type vaccinia and MVA in AAV packaging further confirms this conclusion.

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While it has been previously reported that VV is a helper for AAV replication,[9] the SV40-transformed NB-E cells were used to come to this conclusion. SV40 has been found to provide a weak helper function to AAV in the absence of adenovirus.[25,26] SV40 T antigen has also been found to interact with AAV Rep78. This interaction may be at least partially responsible for SV40-mediated expression of AAV2 Rep proteins.[1] Overall, VV with some minimal assistance can act as a weak helper for AAV replication.

MATERIALS AND METHODS

Cell lines

HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg of penicillin/ml, and 100 U of streptomycin/ml (Invitrogen, Carlsbad, CA). CV-1 cells were cultured in minimum essential media with 10% fetal bovine serum, 100 µg of penicillin/ml, and 100 U of streptomycin/ml. 16,095 cells (a human fibroblast cell line from the Coriell Institute) were cultured in minimum essential media with 10% fetal bovine serum, 100 µg of penicillin/ml, and 100 U of streptomycin/ml. Chicken embryo fibroblast cells obtained from American Type Culture Collection (CRL-12203) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg of penicillin/ml, and 100 U of streptomycin/ml (Invitrogen). All cells were maintained in a humidified 37 °C incubator with 5% CO_2.

Plasmid constructs

The plasmid pGL3-basic (Promega, Madison, WI) was digested with MluI and NheI (NEB, Ipswich, MA). The digested plasmid was used as the backbone to construct all plasmids having the AAV promoters driving the firefly luciferase gene. AAV p5, p19, and p40 promoters were amplified by polymerase chain reaction by using plasmid pSub201 as the template. The plasmid pCI-neo was digested with NheI and XmaI (NEB). The digested plasmid was used as the backbone for the pCI-Rep/Cap plasmid. The plasmid pH22 was digested with BsaI (NEB) and used as the insert for the pCI-Rep/Cap plasmid. The MVA plasmid constructs were produced by digestion of pLW44 with BamHI (NEB). The AAV Rep and Cap sequences were removed from the pRB21-W5 and pRB21-W8 plasmids and cloned into the pLW44 backbone.

The plasmids pFdelta6, pAd/AAV, and pscAAV-EGFP were previously described.[10,11]

Recombinant AAV vector production

rAAV vectors were produced by transfection and transduction methods previously described in particular vaccinia constructs VV-W5 and VV-W8 (ref. 12). Vectors were produced by transfection using Fugene 6 (Roche, Indianapolis, IN) at a concentration of 1 µg per plasmid per 1×10^6 cells. HeLa cells were infected with adenovirus (MOI = 10), VV (MOI = 1) at 8 hours post-transfection. Cells were then harvested at 48 hours post-transfection. Samples underwent three rounds of freezing and thawing and debris was removed by centrifugation. Viruses were inactivated by heating the samples at 56 °C for 45 minutes and the cell lysate was used to transduce 16,095 cells. GFP was visualized with a Nikon microscope using a fluorescein isothiocyanate filter.

Luciferase assay

The p5-, p19-, and p40-luciferase constructs were transfected into HeLa cells at a concentration of 1 µg per 1×10^6 cells. The cells were then infected with adenovirus, VV, or mock infection at 8 hours post-transfection. The cells were collected at 48 hours post-transfection and lysed by the addition of lysis buffer (100 mmol/l potassium phosphate, pH 7.8, and 1 mmol/l dithiothreitol (DTT)) to the cell pellet and undergoing three rounds of freezing and thawing. Cell lysate was diluted in assay buffer (25 mmol/l glycylglycine, pH 7.8, 15 mmol/l potassium phosphate, pH 7.8, 15 mmol/l $MgSO_4$, 4 mmol/l EGTA, 2 mmol/l ATP, and 1 mmol/l DTT). After the addition luciferin solution (1 mmol/l D-Luciferin (Goldbio, St. Louis, MO), 25 mmol/l glycylglycine, pH 7.8, and 10 mmol/l DTT), light output was read using a luminescence reader.

Western blot analysis

Total proteins were extracted with lysis buffer, which consisted of 50 mmol/l Tris at pH 8.0, 150 mmol/l NaCl, 1% Triton X-100, 10 mmol/l DTT, and 1× protein inhibitor (Roche). Cell lysates were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking the membrane with 5% nonfat dry milk in TBST buffer, which contains 25 mmol/l Tris-HCl at pH 8.0, 150 mmol/l NaCl, and 0.1% Tween20, the membrane was incubated with the primary antibody, anti-AAV capsid (B1, American Research Products, Belmont, MA) or anti-AAV Rep (303.9, American Research Products) at a dilution of 1:500 at 4 °C overnight. The membrane was washed and incubated with a horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Sigma, St. Louis, MO) at a dilution of 1:2,000. The membrane was developed using an enhanced chemiluminescent substrate (Amersham-Pharmacia Biotech, Piscataway, NJ).

Southern blotting hybridization

Alkaline agarose gel electrophoresis was used to analyze DNA replication of the AAV genome as described previously.[27] Briefly, HIRT DNA was extracted using standard methods and 10 µg of DNA was resolved on a 1% alkaline agarose gel. After gel blotting, the membrane was hybridized with 32P-labeled probe that was produced using a fragment specific to the EGFP portion of each vector. The purified fragment was labeled by a [α-32P] dCTP using Prime-IT II Random Primer Labeling Kit (Stratagene, La Jolla, CA). The membrane was analyzed by X-ray autoradiography.

Recombinant MVA production

MVA virus and plasmid pLW44 was kindly provided by Bernard Moss. To generate MVA, Chicken embryo fibroblast cells were seeded in a six-well plate and infected with MVA at an MOI of 0.05. The cells were subsequently transfected with the transfer plasmid 1.5 hours following infection with Fugene per manufacturer instructions. The cells were incubated overnight at 37 °C in 5% CO_2 and the media was replaced. At ~48 hours postinfection the cells were harvested in 1 ml of media and the sample underwent three rounds of freezing and thawing. The resulting lysate was used to reinfect Chicken embryo fibroblast cells and plaque purified for several rounds as previously described.[28]

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