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RESEARCH ARTICLE

Transfection-free and scalable recombinant AAV vector production using HSV/AAV hybrids

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Adeno-associated virus (AAV) vectors are highly efficient tools for use in gene therapy. Current production methods rely on plasmid transfection and are not generally considered amenable to scale-up. To improve recombinant AAV (rAAV) vector production in terms of both final titre and simplicity, we constructed recombinant herpes simplex virus (HSV) vectors, either disabled (ICP27 deleted) or nondisabled, encoding the AAV rep and cap genes. We also integrated an rAAVGFP construct into the nondisabled vector and also into a second pair of HSV vectors (disabled and nondisabled) not expressing rep and cap. Transgene incorporation and expression was confirmed by Southern and Western blot, respectively. Optimal double-infection ratios were established for disabled and nondisabled pairs of rep/cap-expressing and rAAVGFP-containing vectors, resulting in up to 1.55×10^{12} rAAV capsids and 4×10^{9} expression units from approximately 1×10^{7} BHK producer cells. Functionality of the prepared vector was confirmed by the detection of abundant green fluorescent protein (GFP) expression following injections of rAAV preparations into the rat brain. This paper therefore describes a simple, efficient, and transfection-free rAAV production process based on the use of HSV and not relying on a proviral cell line that, with appropriate scale-up, could yield quantities of rAAV sufficient for routine clinical use.
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Introduction

Recombinant adeno-associated virus (rAAV) is an attractive vector for use in gene therapy as wild-type AAV is not associated with human disease, but is naturally defective requiring helper adenovirus or herpes simplex virus (HSV) coinfection for replication. rAAV vectors are deleted for all viral proteins, thereby reducing the risk of toxicity and immune responses. rAAV has been proven to transduce effectively both dividing and nondividing cells such as those of the eye,[1] heart,[2] brain,[3] liver,[4], lung,[5] and muscle,[6] in addition to many mammalian cell lines *in vitro*. Clinical trials in patients suffering from cystic fibrosis have progressed to phase II using rAAV to supply cystic fibrosis transmembrane conductance regulator to the lungs,[5] and phase I trials have been performed utilizing an rAAV vector to transduce muscle cells of haemophilia B patients leading to long-term expression of Factor IX.[7]

However, high-level rAAV production has so far proven problematic primarily due to the fact that rAAV production has relied on transfection techniques. These are hard to scale up. Traditionally, rAAV vectors for use in clinical trials were prepared using a plasmid containing the therapeutic gene flanked by AAV-inverted terminal repeats (ITRs), which is cotransfected with a second plasmid encoding the AAV *rep* and *cap* genes and the necessary adenovirus helper functions (either contained within the *rep*/*cap*-expressing plasmid[8] or a third plasmid[9]). Alternatively, and for much preclinical work, adenovirus helper functions have been supplied by coinfection with adenovirus. These production methods have been optimized to allow proof-of-principle experiments, including in man, but are not currently amenable to scale up for use in later stage clinical trials or in routine clinical practice should the need arise.

Using adenovirus as a helper raises additional problems due to the presence of infectious adenovirus particles within the rAAV vector stocks. Cesium chloride gradient centrifugation is often employed for the removal of adenovirus, although this purification method is not suitable for large volumes of cell lysates and tends not to eradicate all immunogenic adenovirus proteins.

However, recent modifications in rAAV purification have allowed for greater yields of rAAV to be achieved by using affinity chromatography techniques. Since AAV has been shown to bind to the heparin sulphate proteoglycan (HSPG) receptor during infection,[29] heparin sulphate affinity columns are now increasingly employed for purification of AAV in larger volumes of cell lysate.[10,31] Additionally, Brument *et al*[12] exploit a dual ion-exchange purification chromatography process for purification of different AAV serotypes.

There have also been recent advances in methods of rAAV production that do not rely solely on transfection. These include using HSV amplicon plasmids to increase *rep*/*cap* gene expression and rAAV copy number with

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helper functions supplied by a replication-incompetent HSV virus,[13] and integrating rAAV as a provirus into a stable cell line followed by infection with a *rep*/*cap*-expressing HSV.[14]

Our aim was to build on this previous work to develop a completely transfection-free, scalable rAAV production system by integrating both the AAV *rep*/*cap* gene sequence and an AAVGFP vector genome into the backbone of replication competent and incompetent HSVs. This would allow rAAV production from a variety of cell lines by a single infection step.

Materials and methods

Plasmids

The plasmid pUL43/repcap contained the AAV *rep*/*cap* sequence flanked by the HSV-1 UL43 gene region. This was constructed by amplifying the AAV *rep*/*cap* sequence, by polymerase chain reaction (PCR), from the plasmid pΔBal using primers containing *Nsi*I restriction sites (sense primer 5′-ccaatgcatggaggggtggagtcgtgacgtgaatt-3′, antisense primer 5′-ccaatgcattgattaacccgccatgctacttatctac-3′). The *rep*/*cap* PCR product and plasmid pUL43, containing the HSV-1 UL43 gene sequence, were both digested using *Nsi*I and ligated. The expression of all rep and cap proteins was driven by the endogenous AAV p5, p19, and p40 promoters. The plasmid pAT15.3/AAVGFP contained an AAV proviral construct expressing eGFP under human CMV promoter control flanked by the HSV-1 US5 gene region. The US5 gene was initially inserted into the standard cloning vector pAT153 as a *Bam*H1 (136289)/*Eco*N1 (139328) fragment (pAT/5.1) followed by blunt-end ligation of the AAV-GFP construct (*Pac*I/*Sca*I fragment) into the *Xba*I site of US5. The distance between the AAV terminal repeats was filled downstream of eGFP with a stuffer fragment of 2.7 kb amplified from the *lacZ* region of plasmid pCH110 by PCR.

Cell lines

Replication competent and incompetent viruses were propagated on baby hamster kidney (BHK) and B130 cells, respectively. The B130 cell line is a neomycin-resistant BHK-derived cell line that constitutively expresses the HSV-1 ICP27 protein and has been previously described.[15] AAV preparations were titred using 293-T cells obtained from the European Collection of Animal Cell Cultures. Cells were cultured in full growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) complemented with 10% foetal calf serum, (with 800 μg/ ml of neomycin for culture of B130 cells). Tissue culture reagents were supplied by Invitrogen unless otherwise stated.

HSV-1 viruses

Plasmid pAT15.3/AAVGFP was inserted into virus strains 17+ (wild-type laboratory HSV-1 strain) and 17+27− (17+ HSV-1 rendered replication incompetent by removal of ICP27) by standard homologous recombination techniques into purified viral genomic DNA to generate 17+/AAVGFP and 17+27−/AAVGFP, respectively. Green fluorescent protein (GFP)-expressing viral plaques were purified from parental white plagues and positional insertion confirmed by Southern blotting. The *rep*/*cap* genes were inserted into 17+ and 17+27− genomic DNA firstly by the generation and purification of intermediate viruses 17+/GFP and 17+27−/GFP, as described above, using plasmid pUL43/GFP. GFP was then substituted by *rep*/*cap* in the UL43 gene region of the intermediate viruses by homologous recombination using pUL43/rc producing 17+/rc and 17+27−/rc. The virus encoding both *rep*/*cap* and AAVGFP within the same HSV-1 backbone (17+/rc/AAVGFP) was generated by homologous recombination of the rAAVGFP construct into the US5 gene region of the 17+/rc virus (generated as described above) using pUS5/AAVGFP. Positional insertion was confirmed by Southern blotting and transgene expression analysed by Western blotting. BHK cells were used throughout for the generation and growth of replication competent viruses and B130 cells similarly used for ICP27-deleted viruses. Once grown to high titre, the virus stocks were titrated by standard plaque assay on cell monolayers to determine pore-forming units/ml. Multiplicity of infection (MOI) is used throughout as an expression of pore-forming unit/host cell ratio.

Recombinant AAV production

Tissue culture flasks (175 cm^2) were seeded with 5×10^5 of either BHK, B130, or 293-T cells and grown at 37°C in 5% CO_2 to approximately 70% confluency ($\sim 1 \times 10^7$ cells). The cells were then infected with *rep*/*cap*- and AAVGFP-encoding HSV-1 viruses by replacing the full growth media with 8 ml serum-free DMEM containing the viruses at various MOIs. After 1 h, the cells were overlayed with 25 ml full growth media. After 48 h, the cells were harvested and pelleted by centrifugation (400 *g*, 10 min). The cell-free supernatant was removed and the pellet resuspended in 1 ml TMN buffer (50 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.15 M NaCl). The cells were lysed by three rounds of freeze/thawing, incubated with 50 U benzonase (Sigma) (37°C, 30 min) and clarified of cell debris by centrifugation (3000 *g*, 20 min). The cell-free lysate containing rAAV was aspirated, incubated at 55°C for 1 h to inactivate any helper HSV-1 and stored at –80°C.

Fluorescence assay to determine rAAV expression unit titre in cell lysates

293-T cells were grown to 70% confluency in 15 mm wells and transduced with heat-treated cell-free AAV preparations serially diluted in serum-free DMEM. The cells were overlayed with full growth media 30 min later and incubated for 48 h (37°C, 5% CO_2). The cells were analysed for GFP expression by fluorescence microscopy.

AAV capsid ELISA to determine rAAV capsid titre in cell lysates

A monoclonal antibody that is highly specific for AAV-2 capsids (A20)[8] was employed in an ELISA[16] to determine the capsid titre in cell-free rAAV preparations, generated from infection of producer cells by HSV/AAV hybrid vectors, following DNase treatment. The ELISA was performed according to the manufacturer's recommended protocol (Progen, Germany).

Rep protein detection by Western blot analysis

Cells (BHK, B130 and/or 293-T) were grown to 70% confluency in 35 mm tissue culture plates then infected with *repcap*-expressing HSV-1 viruses at the MOIs indicated. After 48 h, the infected cells were harvested in 2 × sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (125 mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.042% (w/v) bromophenol blue) and resolved by 10% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and probed for AAV rep proteins by standard immunoblotting techniques. The nitrocellulose membrane was blocked for 1 h (0.1% (v/v) Tween 20, 5% (w/v) skimmed milk in phosphate-buffered saline (PBS)), then incubated for 1 h with primary α-rep antibody (diluted 1:10 in 1 × PBS, 0.1% Tween 20) that recognizes all four rep proteins (monoclonal mouse α-replicase IgG1, clone 226.7 (Progen, Germany)) and similarly incubated with secondary antibody (horseradish peroxidase-conjugated rabbit α-mouse polyclonal antibody (Invitrogen), diluted 1:1000 in 1 × PBS, 0.1% Tween 20). The secondary antibody was detected by chemiluminescence and autoradiography.

Immunofluorescence assay

AAV-2 rep proteins were detected in 293-T cells by the immunofluorescence assay (IFA) following infection with *rep*/*cap*-expressing HSV-1 viruses. Cells were grown on 13 mm glass cover slides to 70% confluency and infected with 17 + /rc (MOI 0.1) or 17 + 27−/rc (MOI 10). At 12 h after infection, cells were fixed (4% paraformaldahyde in PBS, 1 h), washed twice in PBS and permeabilized (0.2% (v/v) triton X-100 in 1 × PBS, 2 min). Cells were washed twice with PBS and incubated with undiluted monoclonal mouse α-replicase IgG1 (clone 226.7 (Progen, Germany)) in a humidified chamber (1 h, 37°C). The cells were then washed three times in PBS and incubated with FITC-conjugated goat α-mouse secondary antibody (diluted 1:100 in PBS). The cells were then washed three times and the nucleus counterstained with 4′,6-diamidino-2-phenylidole (DAPI) diluted 1:1000 in PBS (2 min, room temp.) prior to mounting. Immunofluorescence was then analysed by microscopy at × 40 magnification.

Double-labelled IFA was performed on BHK cells infected with 17 + /rc (MOI = 0.1) and B130 cells infected with 17 + 27−/rc (MOI = 1) to detect both ICP8 and *rep* expression. Cells were treated as above except that after incubation with the α-rep primary antibody the cells were then washed and incubated with α-ICP8 antibody (Pab 3–83) diluted 1:50 in PBS. Additionally, the secondary antibody solution was a mix of FITC-conjugated goat α-mouse and TRITC-conjugated goat α-rabbit antibodies, both diluted 1:100 in PBS. Cells were counterstained with DAPI solution, mounted and analysed for immunofluorescence as above.

In vivo gene delivery

In vivo gene delivery was achieved by injection of rAAV (in clarified cell lysate) directly into the striatum of rats. Animals were anaesthetized, the brain exposed, and 5 μl of rAAV virus suspension was injected into the striatum using a glass micropipette. The wounds were then closed and animals were allowed to recover. Virus suspension was used at 1×10^8 EU/ml. For the analysis of GFP expression, animals were anaesthetized, then perfused with 4% paraformaldehyde. The brain was further postfixed in 4% paraformaldehyde for 2 h, sectioned, and viewed by fluorescence microscopy.

Results

Generation of HSV/AAV hybrid vectors

The AAV *rep* and *cap* genes were inserted into the UL43 gene region of disabled (ICP27-deleted) and nondisabled HSV-1 viruses by homologous recombination resulting in viruses 17 + 27−/rc and 17 + /rc (Figure 1). Similarly, an AAVGFP vector genome was inserted into the US5 gene region of both replication competent and incompetent viral backbones, producing 17 + /AAVGFP and 17 + 27−/AAVGFP. This cassette was also inserted within the US5 region of 17 + /rc to produce 17 + /rc/AAVGFP. Correct insertion of the *repcap* and AAVGFP sequences within UL43 and US5, respectively, was confirmed by Southern blot analysis (data not shown).

Rep protein expression levels and intracellular localization

AAV-2 rep proteins are required during both AAV genome replication and capsid protein expression (in

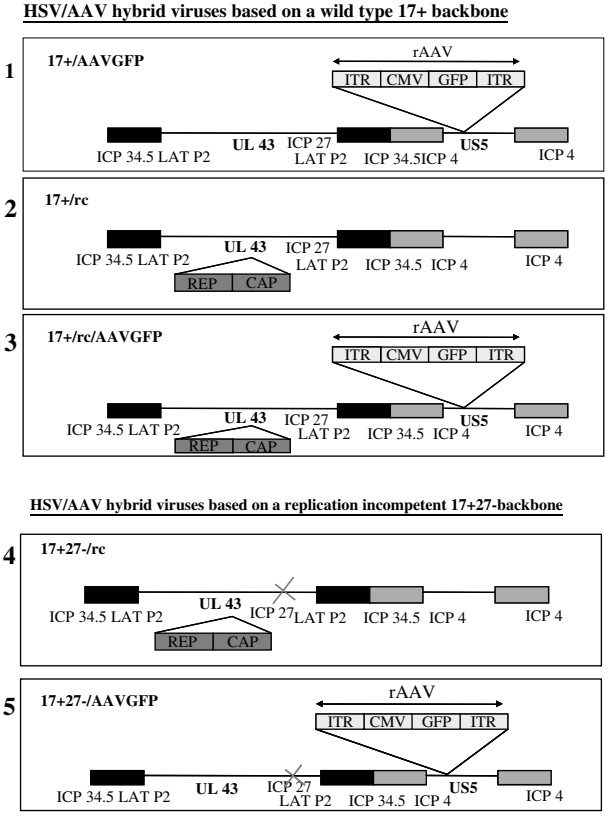

Figure 1 *Diagrammatic representation of the HSV/AAV hybrid virus constructs. Repcap was inserted into the UL43 gene region of HSV-1 by homologous recombination using pUL43/repcap linearized with SspI. Similarly, the AAVGFP vector genome and the mutant ICP27 gene were inserted using pAT15.3AAVGFP and p5Kmut27LAT, respectively, both linearized with XmnI. Viruses 1–3 were produced by standard transfection methods using a 17 + viral backbone on BHK cells, viruses 4 and 5 were similarly produced using a ICP27-deleted viral backbone on B130 cells.*

the context of the AAV genome under p40 regulation). Thus, in order for the AAVGFP vector genome to be rescued from the HSV backbone for packaging into infectious rAAV particles, the AAV *rep* proteins (78, 68, 52, and 40) must be expressed to sufficiently high levels for replication and be distributed within the nucleus of expressing cells.

HSV/AAV hybrid vectors were analysed for *rep* expression by Western blotting (Figure 2). *Rep* proteins were expressed at different levels depending on both the HSV backbone encoding *rep* and the host cells infected. High levels of all four *rep* proteins were observed in 293-T and BHK cells when infected with 17+27−/rc at various MOIs ranging from 0.1 to 25 (Figure 2, panel a). The highest levels of *rep* expression were observed in noncomplementing BHK cells at MOIs of both 1 and 5.

In comparison, relatively little expression of *rep* 78, 68, and 40 was detected in B130 cells infected with the same virus. This is in agreement with previous findings[14] and is thought to be due to the lytic replication of 17+27−/rc within B130s and particularly due to the function of ICP27 in virus replication (see Discussion). Surprisingly though *rep* 52 was well expressed in B130 cells as compared with that observed in 293-T and BHK cells. This increased expression of *rep* 52 has been observed elsewhere when adenovirus helper functions and *rep*/*cap* genes were supplied in the same plasmid by transfection.[3]

Nondisabled viruses 17+/rc and 17+/rc/AAV were similarly analysed for *rep* expression in both 293-T and BHK cells at MOIs ranging from 0.01 to 1 (Figure 2, panels b and c). These viruses undergo lytic replication in both cell types and thus only minimal *rep* expression was observed in 293-T cells when infected with either virus. Surprisingly, however, significantly higher levels of *rep* expression were observed in BHK cells following infection with 17+/rc and 17+/rc/AAV, with the highest levels of all four *rep* proteins detectable upon infection with 17+/rc/AAV at an MOI of 0.1. Use of such viruses on BHKs would therefore provide a fully replicative system that has production advantages over the use of the ICP27-deleted viruses in noncomplementing cells described above (see Discussion).

The *cap* expression was also investigated by Western blotting (data not shown). The pattern of *cap* expression was comparable to that of the *rep* expression in 293-T, BHK and B130 cells infected with either 17+/rc/AAV or 17+27−/rc. However, the *cap* expression in both 293-T and BHK cells infected with 17+/rc was significantly lower in comparison to the *rep* expression shown above.

The cellular localization of AAV *rep* proteins following expression from the HSV-1 backbones was also investigated by IFA of infected cells employing an α-rep antibody to detect all four *rep* proteins (Figure 3). The *rep* expression was detected by IFA in 293-T cells infected with either 17+27−/rc (panel a) or 17+/rc (panel b). Moreover, the areas of fluorescence corresponded to the cell nuclei as demonstrated by DAPI counterstaining (shown on the left of Figure 3). This confirmed that *rep* proteins are abundantly expressed by the viruses

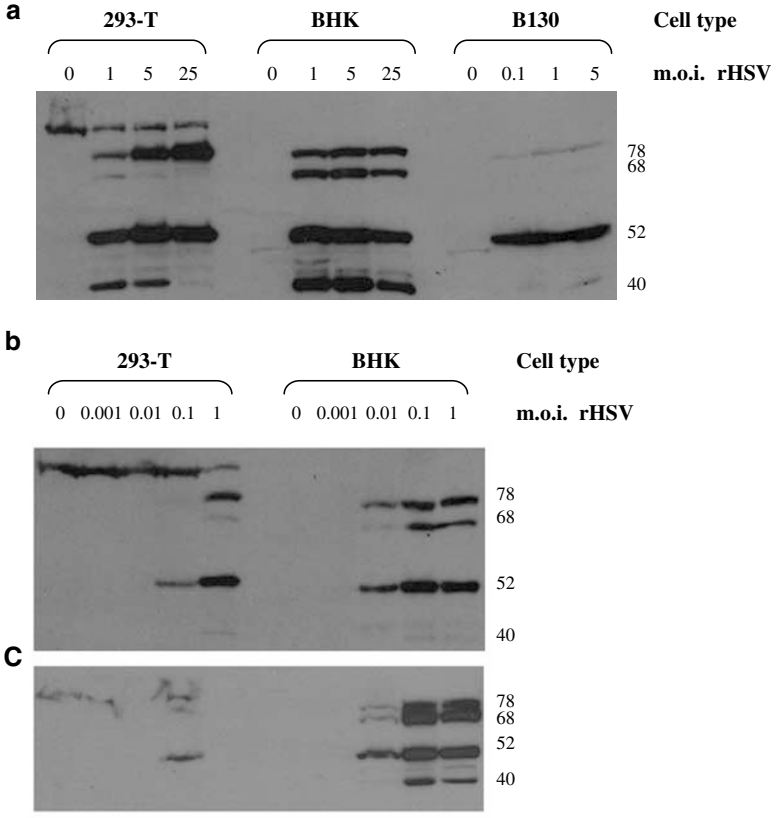

Figure 2 *Western analysis of AAV rep expression in 293-T, BHK and B130 cells 48 h after infection with various rep/cap-encoding HSV viruses over a range of moi. Panels a–c show expression of rep from 17+27−/rc, 17+/rc and 17+/rc/AAVGFP, respectively. The highest AAV rep expression was observed in BHK cells from the replication-incompetent 17+27−/rc virus at MOI of 1 and 5.*

constructed and that appropriate intracellular localization is achieved, and is necessary for the replication of rAAV.

Effect of rep expression on HSV replication

The *rep* protein expression is known to inhibit maturation of adenoviral replication centres[17] and is thought to do so by repression of the E1a, E2a, and E4 gene promoters.[18] Whether or not a similar interaction occurs between AAV and HSV was investigated in two ways. If the *rep* gene products were inhibiting early HSV gene expression, one would anticipate either an absence or reduction in size of viral plaques upon infection of complementing cells with a *rep*/*cap*-expressing HSV virus. However, no reduction in plaque size was visible in BHK cells infected with 17+/rc or in B130 cells infected with 17+27−/rc in comparison with similar non-*rep*/*cap*-expressing viruses over a period of 4 days (data not shown)

ICP8 is a single-stranded DNA-binding protein recruited to herpesvirus DNA replication centres in the nucleus.[11] We therefore used ICP8 as a marker for the detection of replication centres within cells infected with *rep*/*cap*-expressing herpesviruses by IFA (Figure 4). ICP8 expression was detected using a TRITC-conjugated secondary antibody in B130 cells infected with 17+27−/rc at an MOI of 1 and in BHK cells infected with 17+/rc at an MOI of 0.1 (centre of panels a and b respectively). The majority of ICP8 was localized to the nucleus as shown by DAPI counterstaining (left of panels a and b). The level of brightness was not diminished in comparison to BHK cells infected with a 17+ wild-type virus not expressing *rep* (panel c). The *rep* protein expression from 17+27−/rc and 17+/rc was also detected in the nuclei of these cells (right of panels a and b, respectively), albeit to a lesser extent than that observed in similarly infected 293-T cells (Figure 3). This, therefore, further suggests that HSV replication functions are not inhibited by the AAV *rep* proteins, as is further evidenced by the fact that the titres of recombinant HSV generated are not reduced following insertion of the *rep*/*cap* expression cassettes.

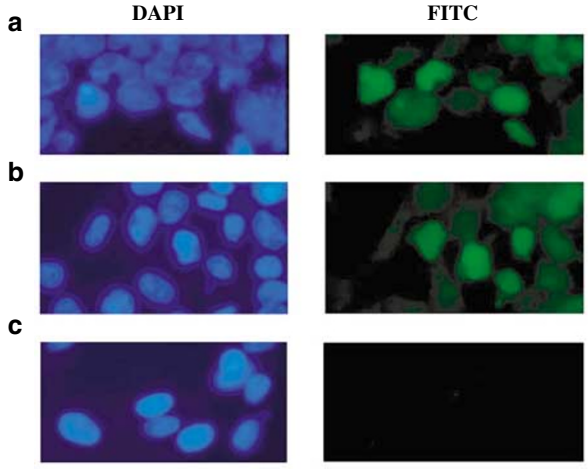

Figure 3 *Detection of AAV rep in 293-T cells by IFA following infection with AAV2-rep-expressing HSV viruses. At 12 h after infection 293-T cells were fixed and permeabilized for IFA before being incubated with a primary mouse α-rep monoclonal antibody then incubated with an FITC-conjugated goat α-mouse antibody. The cells were then DAPI counterstained and viewed by fluorescence microscopy (magnification ×40). Panels a and b show IFA carried out on cells infected with 17+27−/rc and 17+/rc, respectively. AAV2 rep can be observed in green on the right of each panel corresponding to DAPI-stained cell nuclei visible on the left. The negative control is shown in panel c where IFA was performed on uninfected 293-T cells.*

Infectious rAAV is produced from both replicating and nonreplicating HSV/AAV hybrid vectors

To establish whether rAAV was more efficiently produced from a replicating or nonreplicating HSV/AAV

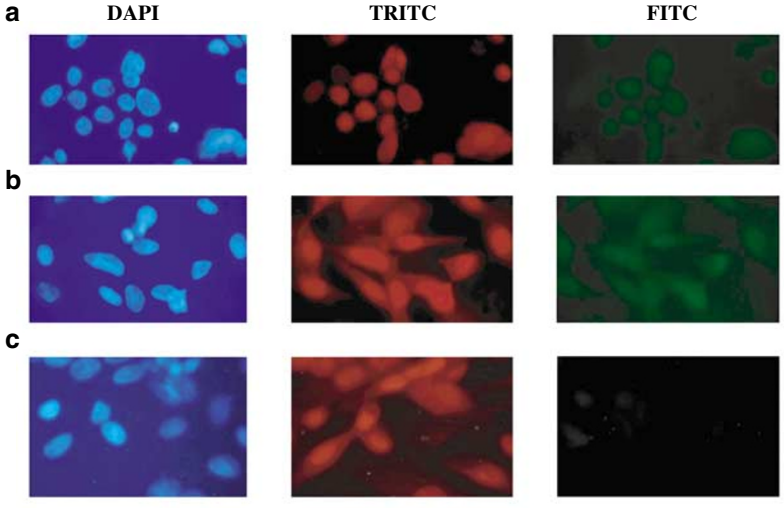

Figure 4 *IFA for the detection of mature HSV-1 replication centres and AAV rep in B130 and BHK cells following infection with AAV-rep-expressing HSV-1 viruses. At 12 h following infection, cells were fixed and permeabilized for IFA, then incubated with a primary mouse α-rep monoclonal antibody and a rabbit polyclonal α-ICP8 antibody. The cells were then incubated with an FITC-conjugated goat α-mouse antibody and a TRITC-conjugated goat α-rabbit antibody. The cells were then DAPI counterstained and viewed by fluorescence microscopy (magnification ×40). Panel a shows IFA carried out on B130 cells infected with 17+27−/rc (MOI of 1) and panel b shows IFA performed on BHK cells infected with 17+/rc (MOI of 0.1). IFA was also performed on BHK cells infected with non-rep-expressing HSV-1 (Panel c). ICP8 can be observed in red in the middle of each panel corresponding to DAPI-stained cell nuclei visible on the left. Weak rep expression is visible in green on the right of panels a and b.*

hybrid system, we performed double infections on both BHK and B130 cells with 17 + 27−/rc and 17 + 27−/AAVGFP at MOIs ranging from 0.1 to 10 (Figure 5). The resulting cell-free rAAV preparations were analysed for capsid titre by ELISA[16] using the antibody A20 specific only for AAV-2 capsids[8] and expression unit titre by titration onto 293-T cells and counting GFP-positive cells by fluorescence microscopy in wells containing 10–100 GFP-expressing cells. Infectious rAAV was produced in both replicating and nonreplicating system as observed by GFP expression in 293-T cells. Potent GFP expression is shown in Figure 5 (panel a) where rAAV vector produced from 17 + 27−/rc and 17 + 27−/AAVGFP on BHK cells at MOI 10 is used to transduce 293-T cells at a 1:1000 dilution. Observing GFP expression by fluorescence microscopy in 293-T cells transduced with rAAV-clarified cell lysates was the preferred method for determining EU titres, albeit at lower dilutions than those shown in Figure 5 (panel a). Clarified cell lysates from an infection of only 17 + /AAVGFP, or 17 + 27−/AAVGFP, were included as negative controls in the determination of rAAV EU titre. No GFP expression was observed in either of these cell lysates (data not shown). Both capsid titre and EU titre were found to be highest when non-complementing BHK cells were infected with 17 + 27−/rc and 17 + 27−/AAVGFP at a joint MOI of 1, reaching 1.55×10^{12} and 4×10^{8} respectively from approximately 1×10^{7} cells.

Infectious rAAV was also purified from BHK cells following double infection with the nondisabled HSV/AAV hybrid vectors 17 + /rc and 17 + /AAVGFP at MOIs ranging from 0.01 to 1 (Figure 5, panel c). Capsid titres reached 3×10^{9} in BHK cells infected at a joint MOI of 1 and were typically 10-fold lower than those obtained when using HSV/AAV hybrid vectors deleted for ICP27 on the ICP27-complementing cell line B130 (Figure 5, panel b). Expression unit titres were also lower than those observed in panel b (ICP27-viruses) reaching a maximum yield of 1.5×10^{5} from BHK cells infected at a joint MOI of 1.

Higher yields of both rAAV capsids and expression units were obtained from BHK cells singularly infected with a nondisabled HSV/AAV vector encoding both *rep/cap* and the AAVGFP vector genome (17 + /rc/AAVGFP) at MOIs ranging from 0.01 to 1 (Figure 5, panel d). Capsid titres were typically 1×10^{10} at all three MOIs, while the expression unit titre peaked at 9×10^{6} at an MOI of 1. No rAAV was detected in the lysates of producer cells infected with either 17 + /AAVGFP or 17 + 27−/AAVGFP alone or coinfected with a control HSV-1 virus not expressing *rep/cap* (data not shown). Similarly, the lysates of producer cells singularly infected with *repcap*-expressing HSV-1 viruses contained only empty AAV capsids as detected by ELISA (data not shown). No GFP fluorescence was observed upon titration of the cell lysate controls onto 293-T cells. Recombinant AAV vector stocks were also analysed for the presence of infectious HSV-1 virus. No HSV-1 plaques were detected on complementing cells 72 h after incubation with the rAAV vector stocks (data not shown).

rAAV vectors produced from HSV/AAV hybrid vectors are functional for gene delivery in vivo

In addition to determining optimal conditions for rAAV production, it was also necessary to determine whether or not rAAV vectors produced by the HSV/AAV hybrid vector system described here could be used for *in vivo* studies. Clarified cell lysate containing approximately

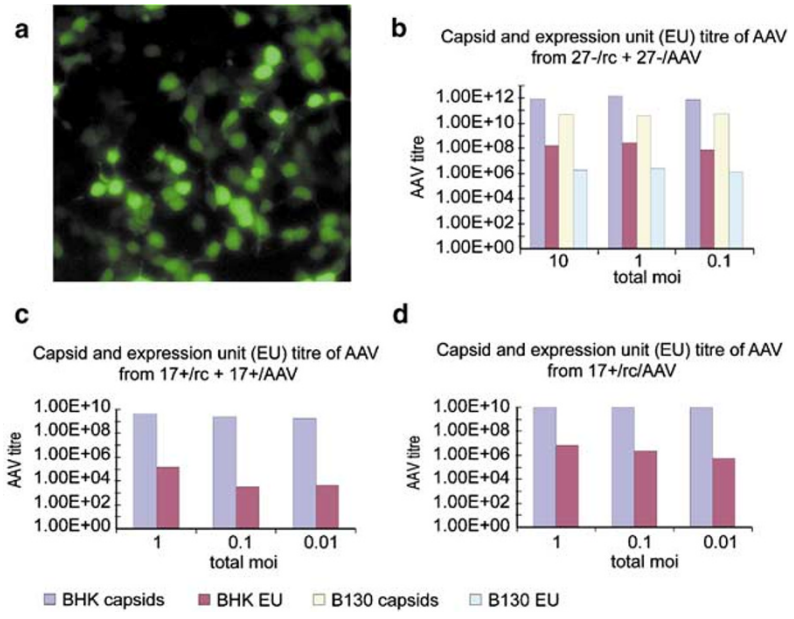

Figure 5 *rAAV was produced to varying titres from BHK and B130 cells by either a single or double infection of HSV/AAV hybrid vector(s) over a range of MOIs (approximately 1–2 × 10⁷ cells were used per infection). The cells were harvested 48 h after infection, subjected to three rounds of freeze/thawing, treated with endonuclease (1 h, 37°C) and the cell debris removed by centrifugation (4000 g, 20 min). After heat inactivation (1 h, 55°C) the cell free supernatant was titred onto 293-T cells and GFP expression was observed 48 h later using fluorescence microscopy. Panel a: rAAVGFP (clarified cell lysate, 1:1000 dilution) produced from a double infection of BHK cells with 17 + 27−/rc and 17 + 27−/AAVGFP was able to transduce 293-T cells and efficiently express the eGFP transgene. Panels b–d: rAAVGFP titres (in clarified cell lysate) from infection with (b) 17 + 27−/rc and 17 + 27−/AAV, (c) 17 + /rc and 17 + /AAV, and (d) 17 + /rc/AAV.*

5×10^5 rAAV expression units (in a volume of 5 μl) produced from a double infection of BHK cells with 17 + 27−/rc and 17 + 27−/AAVGFP was injected into rat striatum using a glass micropipette. The brain was later sectioned and analysed for GFP expression around the site of injection (Figure 6). At 4 days postinjection, no GFP expression was visible as would be expected with infectious HSV-1 (data not shown). Marker gene expression in the rat striatum from variously disabled HSV-1 backbones (including ICP27-deleted viruses) within 4 days postinjection has been previously demonstrated in our laboratory.[19] At 1 month postinjection, however, large numbers of neurons expressing GFP were evident, which were not evident in sections from negative control animals. This is further confirmation that AAV-mediated transduction has occurred as HSV-mediated transduction would be expected to be evident at early time points, unlike the case with rAAV. These results are comparable to *in vivo* studies performed using rAAV vectors produced by conventional methods.[2,20,30]

Discussion

Recombinant AAV vectors are highly promising gene therapy tools, which may be useful in the treatment of such diseases as haemophilia,[7] cystic fibrosis,[5] α1-antitrypsin deficiency,[5] malignant brain tumours,[21] and for gene transfer to the eye.[22] In contrast to recombinant adenovirus vectors, the lack of inflammatory toxicity has allowed these vector studies to progress into phase II trials in the case of cystic fibrosis and phase I trials in α1-antitrypsin deficiency and haemophilia. Despite these early successes, however, concern has been expressed over the technical difficulties in preparing large quantities of high-titre rAAV in sufficient quantities for routine clinical use.[23,24]

We have demonstrated here the first example of rAAV production from HSV/AAV hybrid vectors where all the components necessary for rAAV production are contained within the HSV helper genome(s), thus allowing rAAV vector production from a single infection step. The greatest rAAV capsid and expression unit yield was obtained in BHK producer cells coinfected with replication competent 17 + 27−/rc and 17 + 27−/AAVGFP hybrid vectors at an MOI of 1. This was in accordance with *rep* expression levels, as observed by Western blotting, and their subsequent distribution within the nucleus of both BHK and 293-T cells. The rAAV production was found to be decreased when BHK cells were infected with the same virus combination at a 10-fold higher and 10-fold lower MOI. At an MOI of 10, the producer cells are likely to have suffered from the toxic effects of HSV endogenous proteins, thereby reducing the cells capacity to support AAV genome packaging, and at an MOI of 0.1, not all the producer cells will have been doubly infected by both 17 + 27−/rc and 17 + 27−/AAVGFP and thus yielded less rAAV.

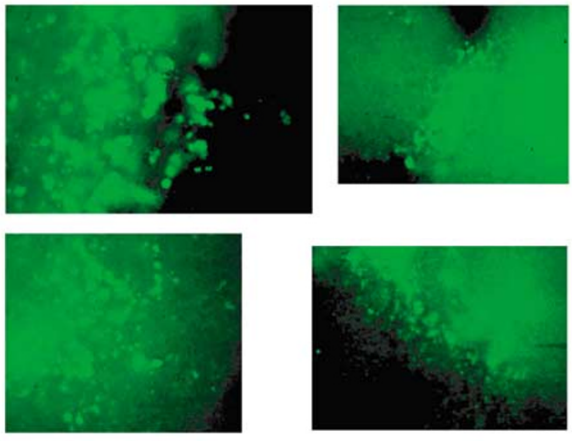

Figure 6 *Fluorescence micrograph showing in vivo GFP expression from rAAV. rAAV in clarified cell lysate was injected into rat brain using a glass micropipette. A volume of 5 ml containing 5×10^5 rAAV expression units was transferred into the striatum, which was analysed for transgene expressing 1 month postinjection. GFP expression is visible at the site of injection and spreading outward from the needle tract. Magnification ×20 (top left panel) and ×10 (all other panels).*

The HSV replication was not shown to be inhibited by the *rep* expression as demonstrated by HSV-1 plaque assays and nuclear localization of HSV replication centres in cells infected with HSV expressing AAV *rep* proteins. The HSV-1 UL29 gene product (ICP8) has been identified as one of the HSV-1 helper genes required for AAV replication[25] and interestingly Ward *et al*[26] suggest that the UL29 gene product may in fact work together with AAV *rep*68. As ICP8 is a single-stranded DNA-binding protein, Ward and co-workers propose that while the AAV rep protein exerts helicase activity upon chromosomally integrated AAV DNA, ICP8 may act to prevent reannealing of complimentary strands. Similarly, the adenovirus helper gene E2a is a single-stranded DNA-binding protein and may act in a similar manner, although once expressed, the AAV *rep* proteins are reported to exert an inhibitory effect on the E2a expression and adenoviral DNA replication in general,[18,27] which was not seen here.

Surprisingly in the virus containing both *rep* and *cap* and an integrated rAAV construct, the rAAV was not lost by replicational excision following the *rep* expression as might have been expected. Southern blot has shown the double-insertion viruses to be stable following serial passage without the appearance of a virus population not containing the integrated rAAV (not shown).

In addition to using a nonreplicating, ICP27-deleted, HSV/AAV hybrid vector system in the production of rAAV, the work reported here is the first to demonstrate that a fully replication competent HSV/AAV hybrid system can be used for rAAV production. Here, it was found that BHK cells gave better results than 293-T cells as have been used in previous work in the area. Using a lower input MOI of virus could have production advantages, as scale-up would deplete HSV/AAV hybrid stocks less rapidly, but at present results in a lower per cell yield of rAAV. This reduction in yield may be due to two main reasons. First, replication competent HSV spreads rapidly through BHK cell monolayers causing toxicity and lysis in infected cells within 24–36 h. Thus, the yield of AAV vector stocks produced using our replicating HSV/AAV vectors might be further improved using both producer cells and incubation temperatures that are suboptimal for efficient growth of HSV, thus allowing more time for AAV vector genome rescue and packaging. Additionally, the ICP27-deleted

viruses were most effective as a helper for AAV production when infecting noncomplementing producer cells, which may relate to one of the usual functions of ICP27 in the virus life cycle, namely the inhibition of pre-mRNA splicing in the host cell. The other main function of ICP27 is to activate the expression of late genes from the viral genome during virus replication. Prior to AAV *rep* and *cap* expression, AAV RNA must be spliced (probably by the host cell) in order to provide mRNAs for the expression of the four rep proteins (rep78, rep68, rep52, and rep40) and the three capsid proteins (VP-1, VP-2, and VP-3). Thus, it was not surprising that AAV production was enhanced in the absence of ICP27, as in the presence of ICP27 splicing of the AAV transcripts would be expected to be inhibited thereby reducing the levels of the AAV *rep* and *cap* proteins. If the ICP27 gene were to be mutated in such a way as to reduce the ICP27 protein's ability to inhibit host pre-mRNA splicing while still allowing the promotion of late gene expression, then one might anticipate a greater infectious rAAV yield from a replicating HSV/AAV hybrid vector production system. Soliman *et al*[28] have previously described three ICP27 mutants (vBS3.3, 4.3 and 5.3) with an activity as described above (ie a reduced ability to inhibit splicing) and we are currently working on incorporating these mutants into the HSV/AAV hybrid vectors described in conjunction with various different producer cell lines to improve the yield of infectious rAAV vector stocks from replicating HSV/AAV hybrid vectors.

AAV is efficient at transducing postmitotic cells, neurons being particularly susceptible. It was therefore necessary to demonstrate that rAAV vector produced by the transfection-free method described here could be used *in vivo* for gene transfer to the brain, as are rAAV vectors produced by traditional transfection methods. We found that at 1 month postinjection, rAAV in heat-treated clarified cell lysate, free from infectious HSV-1, had successfully transduced neurons of rat striatum, demonstrating that rAAV vectors produced by our transfection-free process are fully biologically active and can be used for the same *in vivo* applications as rAAV vectors produced by conventional means.

This paper therefore describes an improvement in rAAV production technology, in terms of simplicity and scalability, in comparison to the production methods currently used. In comparison to other methods based on HSV, it is the first in which all functions are supplied by the helper virus(es), rather than also requiring a stably transfected cell line or transient transfection. The most efficient rAAV production process described relies on the use of nonreplicating HSV/AAV hybrid vectors that requires significant quantities of virus stock (due to the necessary MOI). Ideally, an HSV-based rAAV system for large-scale production would use a lower MOI. The use of lower MOIs requires the use of the replicative versions of the viruses described, which while effective for production are of lower efficiency than the nonreplicating viruses. Work therefore is underway to improve the efficiency of the replicative system further, as discussed above, through the use of ICP27 mutants in which pre-mRNA splicing is not inhibited. This is anticipated to improve efficiency through increased expression of the AAV rep and cap proteins, which are expressed from mRNA transcripts that are multiply spliced.

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