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Recombinant Adeno-Associated Virus Type 2 Replication and Packaging Is Entirely Supported by a Herpes Simplex Virus Type 1 Amplicon Expressing Rep and Cap

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Recombinant adeno-associated virus (AAV) type 2 (rAAV) vectors have recently been shown to have great utility as gene transfer agents both in vitro and in vivo. One of the problems associated with the use of rAAV vectors has been the difficulty of large-scale vector production. Low-efficiency plasmid transfection of the rAAV vector and complementing AAV type 2 (AAV-2) functions (*rep* and *cap*) followed by superinfection with adenovirus has been the standard approach to rAAV production. The objectives of this study were to demonstrate the ability of a recombinant herpes simplex virus type 1 (HSV-1) amplicon expressing AAV-2 Rep and Cap to support replication and packaging of rAAV vectors. HSV-1 amplicon vectors were constructed which contain the AAV-2 *rep* and *cap* genes under control of their native promoters (p5, p19, and p40). An HSV-1 amplicon vector, HSV-RC/KOS or HSV-RC/*d*27, was generated by supplying helper functions with either wild-type HSV-1 (KOS strain) or the ICP27-deleted mutant of HSV-1, *d*27-1, respectively. Replication of the amplicon stocks is not inhibited by the presence of AAV-2 Rep proteins, which highlights important differences between HSV-1 and adenovirus replication and the mechanism of providing helper function for productive AAV infection. Coinfection of rAAV and HSV-RC/KOS resulted in the replication and amplification of rAAV genomes. Similarly, rescue and replication of rAAV genomes occurred when rAAV vector plasmids were transfected into cells followed by HSV-RC/KOS infection and when two rAAV proviral cell lines were infected with HSV-RC/KOS or HSV-RC/*d*27. Production of infectious rAAV by rescue from two rAAV proviral cell lines has also been achieved with HSV-RC/KOS and HSV-RC/*d*27. The particle titer of rAAV produced with HSV-RC/*d*27 is equal to that achieved by supplying *rep* and *cap* by transfection followed by adenovirus superinfection. Importantly, no detectable wild-type AAV-2 is generated with this approach. These results demonstrate that an HSV-1 amplicon expressing the AAV-2 genes *rep* and *cap* along with HSV-1 helper functions supports the replication and packaging of rAAV vectors in a scaleable process.

Recombinant adeno-associated virus (AAV) type 2 (rAAV) vectors have recently been shown to have great utility as gene transfer agents in vivo (19, 21, 22, 46). A major problem associated with the use of rAAV vectors has been the difficulty in producing large quantities of high-titer vector stocks (8, 9). The standard production protocol involves low efficiency transfection of plasmid DNA containing the AAV type 2 (AAV-2) *rep* and *cap* genes and a plasmid containing the rAAV provirus with inverted terminal repeats (ITRs). Cells are then superinfected with adenovirus (Ad) to provide essential helper functions required for rAAV production.

Alternative procedures have been developed to improve the efficiency of rAAV production by delivering *rep*, *cap*, and the Ad helper genes in novel ways. These technologies have included the generation of *rep*- and *cap*-inducible cell lines and plasmids expressing the essential Ad helper genes (8, 9, 41). Although these techniques have improved the yield of rAAV production, they have not been entirely satisfactory. Any procedure using transfection methods will not be efficient, will be variable in yield from preparation to preparation, and will be difficult to scale up to produce the large quantity of rAAV vector needed for clinical trials. The production of *rep*- and *cap*-inducible cell lines has been difficult, and the yield of rAAV produced from different clones is variable and does not exceed the efficiency of transfection methods (8, 9, 41). Production protocols for rAAV which use Ad and transfection of *rep* and *cap* and proviral rAAV plasmids also have the potential to generate wild-type (wt) AAV through recombination of the ITRs with *rep* and *cap* sequences. This leads to preferential amplification of the wt AAV genome over the rAAV genome.

AAV-2 infection results in the AAV-2 genome entering a nonproductive, non-progeny-producing latent state where the viral genome exists as a provirus integrated into the host cell's chromosomal DNA (7). Preferential integration of the AAV-2 genome seems to occur via site-specific, nonhomologous recombination in human cells at chromosome 19q13.3 (23–25, 36). A productive lytic cycle ensues in which AAV-2 DNA is replicated, amplified, and packaged into progeny virions only during coinfection of AAV-2 with the appropriate helper virus (Ad or herpesvirus), infection of a latently infected cell with helper virus, or infection of AAV-2 followed by UV irradiation (3, 32, 47). Infection of AAV-2 in the presence of DNA-damaging agents also promotes viral replication through the induction of cellular DNA repair pathways (47, 48). The AAV-2 DNA sequences, AAV-2 viral proteins, and helper virus genes which are required for productive AAV-2 infection have been identified and are used to produce rAAV vectors (2,

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4, 15, 28, 34, 43). The DNA sequences required for AAV replication which serve as origins of replication of the AAV genome and primers of second-strand synthesis are located in the ITRs of the AAV genome (35). These sequences must be located *cis* to the recombinant genome which is to be replicated and packaged, and this rAAV genome is usually introduced into cells by transfection. The AAV-2 Rep 78 and Rep 68 proteins, which direct replication of the genome from the ITRs, the viral Rep 52 and 40, which are necessary for efficient packaging, and the structural capsid proteins VP1, VP2, and VP3 are supplied in *trans* in the traditional packaging scheme, usually by transfection of Rep- and Cap-expressing plasmids (6, 33). Viral helper functions for AAV replication are usually supplied by Ad early gene expression of E1a, E1b, E2a, and E4 and by VA RNA after Ad infection (2, 4, 15, 34).

Ad has been the most thoroughly studied AAV-2 helper virus and the virus generally used to produce rAAV. The Ad helper functions required for AAV-2 or rAAV replication are probably not involved in AAV DNA synthesis directly. Instead, the Ad helper genes make AAV replication possible through regulation of cellular gene expression and regulation of *rep* expression (4). Attempts to construct Ad vectors which express AAV-2 replication genes have met with failure, presumably because the AAV-2 Rep proteins inhibit Ad replication (44).

Like Ad, herpes simplex virus type 1 (HSV-1) is a fully competent helper virus for AAV-2 replication and packaging (18, 28, 43). In contrast to Ad, however, the helper functions provided by HSV-1 are in large part due to the activities of replication proteins, not transcriptional regulators (43). The minimal set of HSV-1 genes required for efficient AAV-2 replication and encapsidation includes UL5, UL8, UL52, and UL29 (43). The genes UL5, UL8, and UL52 encode components of the HSV-1 helicase-primase complex (10). UL29 encodes a single-stranded-DNA binding protein (20). These four proteins essential for AAV-2 DNA replication are components of the HSV-1 core replication machinery along with the HSV-1 DNA polymerase (UL30), the polymerase accessory factor (UL42), and the origin binding protein (UL9) (5, 45). The genes UL5, UL8, UL52, and UL29 are transcribed early in infection preceding HSV-1 DNA replication and are absolutely required for HSV-1 DNA replication (31). It is interesting that AAV-2 replication and packaging can occur in the absence of HSV-1 DNA replication as long as HSV-1 early gene expression occurs (43).

In this report, we describe the development of the first system which provides the AAV-2 Rep and Cap proteins and the HSV-1 helper functions required for rAAV production in one agent. HSV-1 amplicon stocks which express Rep and Cap from their native promoters (p5, p19, and p40) and use wt HSV-1 (KOS strain) as the amplicon helper virus have been produced. (This HSV-1 amplicon with wt HSV-1 helper virus is referred to as HSV-RC/KOS.) To increase the yield of rAAV production and make the HSV-1 amplicon system a practical alternative to Ad systems for rAAV production, an HSV-1 amplicon system which expresses Rep and Cap from their native promoters and uses an ICP27-deleted HSV-1 virus, *d*27-1, as the amplicon helper virus was also generated. (This HSV-1 amplicon with *d*27-1 helper virus is referred to as HSV-RC/*d*27.) Using HSV-RC/*d*27 results in rAAV production with an efficiency equal to that of previously described transfection methods (12). Importantly, Southern blot and PCR analysis for the presence of wt AAV genomes has not detected any wt AAV following rAAV production using these amplicons. We believe that this system provides an alternative method to increase the scale of rAAV production to that required for preclinical and clinical trials utilizing rAAV vectors.

MATERIALS AND METHODS

Cell lines. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 10% heat-inactivated fetal calf serum (FCS). Vero cells were maintained in DMEM containing 5% FCS. V27, a Vero cell line derivative which expresses both the bacterial neomycin resistance gene, which confers resistance to the drug G418, and the HSV-1 protein ICP27 (30), was maintained in DMEM containing 10% FCS. All 293 cell lines were maintained in DMEM containing 10% FCS. Cells were cultured at 37°C in 5% CO_2.

The UF2-293 cell line was generated by transfection of a 10-cm-diameter dish of 293 cells (American Type Culture Collection) with 10 μg of pUF2 DNA (49). The cells were then passaged in G418 (600 μg/ml; Gibco-BRL) for 3 weeks. Surviving cells were then subjected to fluorescence-activated cell sorting, using the absorption and emission spectrum of the humanized green fluorescent protein (hGFP) to isolate high-expressing cells (49). Cells considered high expressors were those that when on absorption of light of 395-nm wavelength emitted light of 509-nm wavelength at an intensity 125 times greater than the intensity of emission of similarly stimulated, nontransfected 293 cells. The high expressors were maintained in 600 μg of G418 per ml.

The GFP-92 cell line was created by infecting 293 cells with rAAVUF2. Cells were passaged in 200 μg of G418 per ml for 2 weeks and screened for GFP fluorescence. Colonies were isolated and analyzed by PCR, as described below, for the ability to produce rAAV when transfected with pIM45 DNA and superinfected with Ad type 5 (Ad5). A producer cell line was identified, and single clones were again isolated and analyzed for the ability to produce rAAV.

Plasmids. Plasmids pUF2, psub201, pIM45, and pRS5 have been previously described (12, 29, 33, 49). Plasmid pUF2 is a bicistronic vector containing the human cytomegalovirus (HCMV) major immediate-early (MIE) enhancer driving the hGFP-encoding gene (*hgfp*) and the HSV-1 thymidine kinase promoter driving a neomycin resistance gene inserted between AAV-2 ITRs. pRS5 and pIM45 are helper plasmids which supply Rep and Cap for generating rAAV. Plasmid pAV2 contains the AAV-2 genome (26). pAAV-lacZ is an HCMV MIE-driven *lacZ* reporter construct inserted between AAV-2 ITRs and is derived from psub201. pHSV-RC was used to generate the HSV-1 amplicons HSV-RC/KOS and HSV-RC/*d*27 and is a pUC19-based vector (Fig. 1). The *a* sequence contains the HSV-1 packaging signals and was cloned into the *Eco*RI site of pUC19. The *oriS* sequence contains an HSV-1 origin of replication (the internal *Sma*I fragment from the HSV-1 *oriS*) and was inserted at the pUC19-derived *Sma*I site to generate pHSV. To create pHSV-RC, the *rep* and *cap* genes from AAV-2 were isolated from psub201 by an *Xba*I digest and cloned onto the *Xba*I site of pHSV (Fig. 1). pHSV-gfp was constructed from pHSV and pCMV-gfp, a vector containing the HCMV MIE enhancer driving the wt *gfp* gene (Clontech). pCMV-gfp was *Not*I digested and Klenow enzyme blunted. This fragment was then cloned into the *Sph*I-digested and T4 polymerase-blunted pHSV to create pHSV-gfp. p43-hgfp is based on the pUF2 vector. The expression cassette from pCI (Promega) was isolated by a *Bam*HI-*Bgl*II digest and was cloned between the ITRs of *Bgl*II-digested pUF2 to create p43. The *hgfp* cDNA was isolated from pUF2 by a *Not*I digest and then cloned into the *Not*I site of p43 to create p43-hgfp. pCI-hgfp was created by cloning *hgfp* into the *Not*I site of pCI. The 115-bp deletion vector pCI-hgfpd was created by *Pfl*MI and *Pvu*II digestion of pCI-hgfp followed by T4 polymerase blunting of the overhanging ends and then self-ligation of the vector.

Transfections. Transfections for the rescue of rAAV genomes from plasmid pAAV-lacZ were done by using Lipofectamine (Gibco-BRL), following the manufacturer's protocol, 24 h after seeding 2×10^5 HeLa cells onto six-well plates. The UF2-293 cell line was generated by plating 10^6 293 cells onto a 10-cm-diameter dish followed by transfection with 10 μg of pUF2 24 h later. This transfection was done by precipitation of plasmid DNA with $CaCl_2$ (final concentration, 12.5 mM) in *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; final concentration, 25 mM). The transfected cells were incubated at 35°C in 3% CO_2 overnight. The transfected cells were rinsed once with phosphate-buffered saline (pH 7.4) and grown in DMEM with 10% FCS. To generate the first passage of the amplicons HSV-RC/KOS and HSV-GFP/KOS, 10^6 Vero cells were plated onto 10-cm-diameter dishes followed by transfection with 10 μg of pHSV-RC and 10 μg of HSV-1 (wt KOS strain) DNA or 10 μg of pHSV-gfp and 10 μg of HSV-1 (KOS) DNA by BES coprecipitation. To generate the first-passage HSV-RC/*d*27, 10^6 V27 cells were plated onto 10-cm-diameter dishes and transfected 24 h later with 20 μg of pHSV-RC DNA, using Lipofectamine. To produce rAAVUF2 from the GFP-92 cells by transfection, 2×10^6 cells were plated onto a 10-cm-diameter dish and transfected with 8 μg of pRS5 DNA, using Lipofectamine.

Viruses. HSV-1 (KOS) was propagated by infecting Vero cells (90% confluent in 175-cm^2 flasks) at a multiplicity of infection (MOI) of 0.1. Adsorption of virus was done for 45 min in reduced-serum (2% FCS) DMEM. After full cytopathic effect (CPE) was observed (usually 48 h postinfection), the cell pellet was collected by centrifugation (1,000 rpm for 10 min) and frozen and thawed three times. Cell debris was then removed by centrifugation (3,000 rpm for 5 min). *d*27-1 is an ICP27 deletion mutant of HSV-1 (KOS) and has been previously described (30). *d*27-1 was propagated as described for HSV-1 except that the complementing cell line, V27, was used. Ad5 (American Type Culture Collection) was propagated by infecting 293 cells (90% confluent, 15-cm-diameter dishes) at an MOI of 0.1. Ad5 was harvested as described for HSV-1 after full

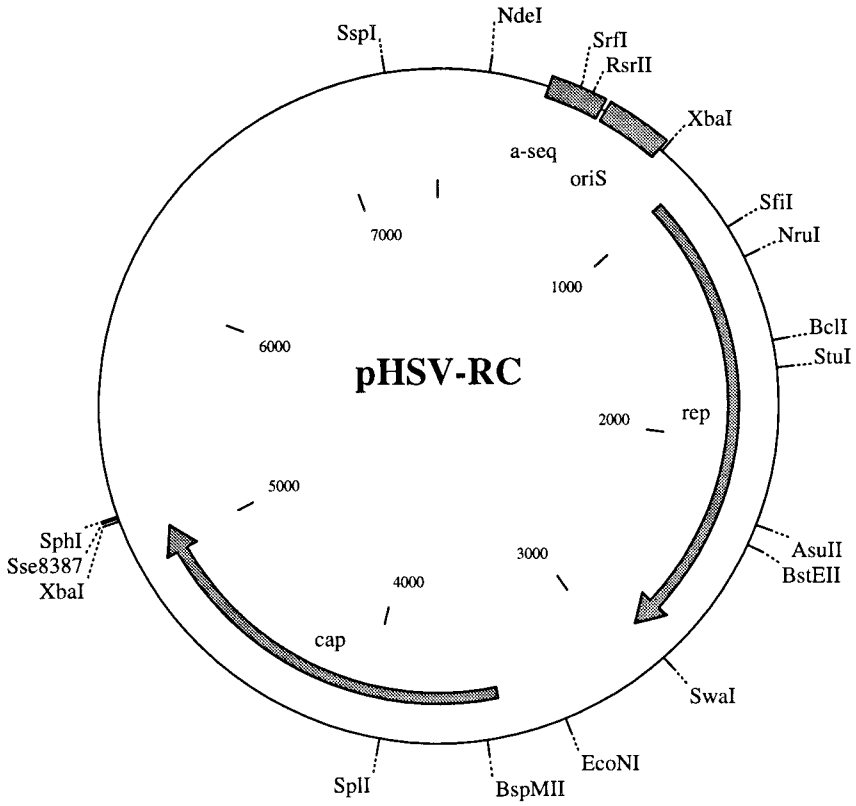

FIG. 1. Map of pHSV-RC which was used to generate amplicons that replicate and package rAAV virions. The plasmid is a pUC-based vector. The *a* sequence (*a*-seq) contains the HSV-1 packaging signals and is cloned into the *Eco*RI site. The 110 sequence contains an HSV-1 origin of replication and is the internal *Sma*I fragment from the HSV-1 *oriS*. The 110 sequence is inserted in the *Sma*I site. (The 110- and *a*-sequence-containing plasmid is pHSV.) rep and cap are the AAV-2 *rep* and *cap* genes isolated from psub201 by an *Xba*I digest and cloned into the *Xba*I site of pHSV to create pHSV-RC.

CPE was observed (usually 72 to 96 h postinfection). AAV-2 was propagated by coinfection of 293 cells with AAV-2 (MOI of 200 particles per cell) and Ad5 (MOI of 0.1). AAV-2 lysates were prepared by freeze-thaw, and the Ad5 was heat inactivated by incubation at 55°C for 45 min.

HSV-1 (KOS) and *d*27-1 titers were determined by plaque-forming assays on Vero and V27 cells, respectively. Analysis of *d*27-1 stocks for the presence of wt HSV-1 was done by plaque assay on noncomplementing Vero cells (<100 PFU/ml detected). Ad5 titers were determined by plaque-forming assay on 293 cells. AAV-2 particle titers were determined by dot blot analysis as described below for recombinant genomes in the amplicon stocks.

HSV-RC/KOS was propagated by harvesting the cell pellet by centrifugation (1,000 rpm for 10 min) after full CPE was observed in the transfected cells. The cell pellet was frozen and thawed three times, and cell debris was removed by centrifugation (3,000 rpm for 5 min). One-fourth of the virus was then used to infect Vero cells (90% confluent in 175-cm^2 flasks) as previously described to generate the second passage of HSV-RC/KOS. One-fourth of the virus was used to infect Vero cells in 175-cm^2 flasks to generate each successive passage. HSV-RC/*d*27 was generated by superinfection of the pHSV-RC-transfected V27 cells with *d*27-1 virus 36 h posttransfection at an MOI of 2.5. The cell pellet was collected as previously described after full CPE was observed (72 h postinfection). Successive passages of HSV-RC/*d*27 were generated as described for HSV-RC/KOS except that the complementing cell line, V27, was used. Fourth-passage or greater amplicon stocks were used in the experiments described.

The helper virus titer in each amplicon stock was determined by a plaque-forming assay on the appropriate cell line (Vero cells for HSV-RC/KOS; V27 cells for HSV-RC/*d*27). The titers of HSV-1 in HSV-RC/KOS, in passages 2 through 6, varied between 1×10^8 and 3×10^8 PFU/ml. The titer of *d*27-1 in HSV-RC/*d*27, in passages 2 through 5, varied between 1×10^7 and 3×10^7 PFU/ml. HSV-RC/*d*27 was analyzed for the presence of wt HSV-1 by plaque assay on noncomplementing Vero cells (<100 PFU/ml detected). The titer of recombinant genomes (the *rep* and *cap* genome from pHSV-RC) in each amplicon stock was determined by dot blot analysis of the stocks. Aliquots of the virus were treated with DNase I (50 U) for 2 h at 37°C in DNase I buffer (final concentrations, 10 mM Tris [pH 7.4], 10 mM KCl, and 1.5 mM $MgCl_2$) and then treated with proteinase K (75 μg) for 2 h at 55°C in proteinase K buffer (final concentrations, 10 mM Tris [pH 7.4], 5 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS]). NaOH (final concentration, 0.12 N) was then added, and the samples were incubated at 65°C for 1 h. The samples were neutralized with NH_4OH (final concentration, 0.12 N) and were then transferred by using a vacuum apparatus to a nylon membrane which was first equilibrated in 1 N NH_4OH for 1 h. The slots were then washed with 50 μl of 2 N NH_4OH. A standard curve of serial dilutions of HSV-1 was processed and applied to the membrane in an identical fashion. A standard curve of serial dilutions of pHSV-RC was denatured, neutralized, and also applied to the membrane. The membrane was then incubated with prehybridization solution (1% SDS, 5 mg of nonfat dried milk per ml, 0.05 mg of heparin per ml, 0.2 mg denatured salmon sperm DNA per ml, 60 mg of polyethylene glycol 8000 per ml, 5× SSPE (750 mM NaCl, 50 mM Na_2HPO_4, 5 mM EDTA], 10% formamide) for 4 h at 60°C. The membrane was hybridized overnight at 60°C in the prehybridization solution with an [α-^{32}P]dATP-labeled, random primer-generated probe. The probe was generated from a 2.1-kb fragment of the *cap* gene isolated after *Kpn*I digestion of psub201. After hybridization, the membrane was washed twice in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C for 45 min. The membrane was exposed to film for 24 to 48 h at 70°C. The titer of recombinant genomes varied between 3×10^7 and 7×10^7 per ml for HSV-RC/KOS and from 1×10^7 to 3×10^7 per ml for HSV-RC/*d*27. The specificity of the probe for recombinant genomes was confirmed by demonstrating that the HSV-1 standard curve did not produce a signal when the membrane was hybridized with an [α-^{32}P]dATP-labeled, random primer-generated probe for the AAV-2 *cap* gene (data not shown). To verify that the HSV-1 DNA did transfer, the membrane was stripped by washing the membrane with 0.1× SSC–0.1% SDS at 100°C and then rehybridized with an [α-^{32}P]dATP-labeled *oriS* DNA probe. The membranes were then processed as described above.

The packaging, purification, and titering of rAAVlacZ have been described previously (19). rAAVUF2 was prepared from six 175-cm^2 flasks of UF2-293 cells. Flasks were infected with HSV-RC/KOS (MOI of the HSV-RC/KOS was two recombinant genomes per cell and 2.5 PFU of HSV-1 per cell) when the cells were 90% confluent (10^8 cells in total). The total number of cells in the preparation was determined by counting the number of cells present on a similarly prepared flask, using a hemocytometer. Forty-eight hours later (after development of full CPE), the cells were centrifuged for 10 min at 1,000 rpm. The cell pellet was then frozen and thawed three times, and cell debris was removed by centrifugation at 3,000 rpm for 10 min. The sample was heat inactivated for 1 h at 55°C and DNase I treated for 1 h at 37°C in DNase I buffer. Virus was purified on an isopycnic CsCl gradient as described previously (19). Fractions of 100 μl were collected, the refractive index was determined for each fraction, and each

fraction was then analyzed for the presence of rAAVUF2 by infecting HeLa cells in the presence of Ad5 and directly observing the cells by fluorescence microscopy for the presence of hGFP expression 36 h later (2×10^5 HeLa cells were plated onto six-well plates 24 h before coinfection with 1 μl of each CsCl fraction and Ad5 [MOI of 2].) Positive fractions were pooled and dialyzed overnight against 4 liters of 10 mM Tris–1 mM EDTA (pH 7.4). The presence of infectious rAAVUF2 was determined by replication assay as described below. No contaminating HSV-1 was detected in a Vero cell plaque assay (<10 PFU/ml).

rAAVUF2 was prepared from GFP-92 cells by one of three methods. Thirty 10-cm-diameter dishes were seeded with 2×10^6 cells, and 24 h later the cells were either transfected with pRS5 as described above or infected with HSV-RC/*d*27 (MOI of HSV-RC/*d*27 was one recombinant genome per cell and 1 PFU of *d*27-1 per cell). The total cell number in each preparation was determined by counting the cells on identically seeded dishes, using a hemocytometer. For the transfection method, the transfection solution was removed 8 h later, and Ad5 (MOI of 2.5) was added to the cells in DMEM with 10% FCS. One group of dishes that was infected with HSV-RC/*d*27 was superinfected with wt HSV-1 (MOI of 1) 12 h later. The cells were collected after full CPE had developed and processed as described above.

Replication assays. Rescue and replication of rAAV genomes from transfected plasmids, producer cell lines, or infected rAAV particles were demonstrated by first seeding 2×10^5 HeLa cells onto six-well plates or 10^6 HeLa cells onto 10-cm-diameter dishes. After 24 h, the cells were either mock transfected, mock infected, transfected with an rAAV plasmid, infected with AAV-2, infected with rAAV, or subjected to a combination of these treatments (as described in the figure legends). After an additional 24 h, the cells were either mock infected, infected with HSV-1, infected with *d*27-1, or infected with one of the amplicons (as described in the figure legends). Cells were harvested 36 h later and centrifuged for 5 min at 2,000 rpm. Medium was removed, and low-molecular-weight DNA was isolated from the pellet by Hirt extraction (14). Five or 10 μg (as indicated in the figure legends) of Hirt-extracted DNA was loaded per lane on an 0.8% agarose gel and run at 25 V for 12 h. DNA from the gel was transferred to a nylon membrane by Southern blotting. The nylon membrane was then prehybridized and hybridized as described above. The different templates used to generate the [α-^{32}P]dATP-labeled probes were a 3.3-kb *lacZ* DNA fragment, a 4.4-kb AAV-2 DNA fragment (used in Fig. 2 to detect wt AAV), and a 700-bp *hgfp* DNA fragment. The membranes in Fig. 3, 4, 6, and 9 were stripped as described above and reprobed for the presence of replicating wt AAV genomes, using an [α-^{32}P]dATP-labeled probe generated from a 2.1-kb fragment of the AAV-2 *cap* gene. For the *Dpn*I assay, 10 or 20 μg (as indicated in the figure legends) of Hirt-extracted DNA was extensively digested with *Dpn*I (100 U) for 24 h, ethanol precipitated, and run on an 0.8% agarose gel for 12 h at 25 V. Densitometry measurements of the replicative forms (monomers) in Fig. 6 were performed by using Mac BAS v2.2 software.

PCR assays. Samples from clarified cell lysates (70 μl from 7 ml for detection of rAAVUF2 made from the cell line UF2-293 with HSV-RC/KOS, 2 μl from 3 ml for detection of rAAVUF2 made from the GFP-92 cell line with HSV-RC/*d*27, and 100 μl from 3 ml for wt AAV detection) were treated with 50 U of DNase I for 2 h at 37°C in DNase I buffer and then proteinase K digested in proteinase K buffer for 2 h at 55°C. The samples were then phenol and chloroform extracted, ethanol precipitated, and centrifuged at 14,000 rpm for 30 min at 4°C to pellet the DNA. The DNA pellet was rinsed once with 70% ethanol, dried, and reconstituted in distilled water (dH_2O). An aliquot of this sample (1 μl from 20 μl for rAAVUF2 analysis and 9 μl of 10 μl for wt AAV analysis) was used in the PCRs. PCRs were carried out in a 50-μl volume, and PCR products (15 μl) were analyzed on 2% agarose gels at 100 V. For the quantitative-competitive PCR (QC-PCR), the products were analyzed on 2% agarose gels for 3 h at 50 V. A Stratagene Eagle Eye detection system was used to record the images.

The primers used to detect rAAVUF2 particles anneal to the coding region of *hfgp* and generate a 700-bp product. The *hgfp* sense primer was 5′-ATGAG CAAGGGCGAGGAACTGTTC-3′; the *hgfp* antisense primer was 5′-TCACTT GTACAGCTCGTCCATGCC-3′. The positive control was 200 pg of p43-hgfp. The PCR conditions were 4 min at 94°C, 25 cycles of 60 s at 94°C, 30 s at 60°C, 60 s at 72°C, and then 4 min at 72°C.

The primers used to detect the presence of wt AAV anneal to the ITR *D* sequence and to the *cap* coding sequence and generate a 370-bp product. The *D*-sequence primer was 5′-CTCCATCACTAGGGGTTCC-3′; the *cap* primer was 5′-CTTCATCACACAGTACTCCACGGG-3′. The positive controls were serial dilutions of pAV2. The PCR conditions were identical to those used with the *hgfp* primers except that 30 cycles were completed. Typically 10 fg of pAV2 could be detected by PCR amplification after ethidium bromide staining.

A particle count of rAAVUF2 was determined by QC-PCR and was based on the determination of the amount of rAAVUF2 template present in a sample through comparison with a known quantity of internal control standard. The internal control for the QC-PCRs, pCI-hgfpd, was identical to the *hgfp* sequence to which the primers annealed and amplified except that an internal deletion was made as described above. The *hgfp* primers generate a 585-bp product when pCI-hgfpd is used as the template. A constant amount of rAAVUF2 DNA was added to each QC-PCR mixture (1 μl), and the amount of internal control was varied to produce a standard curve (see figure legends for the exact amount of pCI-hgfpd added to each reaction). The amount of rAAVUF2 template present was then determined by identifying the amount of internal control DNA that had to be added which would give full-size and deleted PCR products of equal intensity after ethidium bromide staining. The number of single strand template genomes present (the number of particles) was then calculated.

The PCR detection of rAAVUF2 particles does not give a false-positive result under the conditions used. As a negative control for the specificity of the PCR analysis to detect actual rAAV particles and not residual DNA template from undigested cellular DNA, 10^8 GFP-92 cells were pelleted and reconstituted in 1 ml of DMEM. The cells were then frozen and thawed three times. The cell debris was removed by centrifugation at 3,000 rpm for 10 min, and DMEM was added to the lysate so that the final volume was 1 ml. One hundred microliters of this sample was DNase I and proteinase K treated, phenol and chloroform extracted, precipitated, and reconstituted in 20 μl dH_2O. Five microliters (out of 20 μl) of the negative control did not give a detectable PCR product when the *hgfp* primers and PCR conditions that were used for all *hgfp* PCRs were used for 30 amplification cycles.

Statistical analysis. Multiple-comparison analysis of variance was performed by using the Fisher test (Statview 4.5 statistical software). A P value of less than .05 was considered a statistically significant difference.

RESULTS

An HSV-1 amplicon which contains the AAV-2 *rep* and *cap* genes, an HSV-1 origin of replication, and HSV-1 packaging sequences can be constructed. The expression of Rep 78 or 68 has been shown to inhibit the replication of DNA viruses. Rep proteins interact with Ad and cellular DNA replication proteins in viral replication centers and disrupt their subsequent formation and function (44). Expression of Rep proteins also inhibits HSV-1-induced cellular DNA amplification and HSV-1 viral DNA replication itself (13). It was possible that the expression of Rep could have interfered with HSV-1 DNA replication to such an extent that creation of amplicon stocks of reasonable titer would not be possible. Similar problems have been observed by multiple investigators attempting to create a recombinant Ad vector expressing Rep. To determine if an amplicon system which expressed Rep could be created, a plasmid which expresses Rep from the p5 and p19 promoters, pHSV-RC, was constructed (Fig. 1). When pHSV-RC was cotransfected with HSV-1 (KOS) DNA into Vero cells, it took 48 h longer for induction of full CPE than when HSV-1 DNA and pUC19 or when HSV-1 DNA and pHSV-gfp (a non-Rep-expressing control amplicon plasmid) were transfected (7 days for full CPE versus 5 days). In subsequent passages (passages 2 through 6), no difference was seen in the time course of CPE for the different amplicon stocks (48 h for full CPE). Also, the titers of plaque-forming wt HSV-1 and recombinant genomes in the different passages did not vary a great deal (wt HSV-1 titer varied from 1×10^8 to 3×10^8 PFU/ml; recombinant genome dot blot titer varied from 3×10^7 to 7×10^7 genomes/ml [data not shown]). A HSV-1 amplicon expressing Rep which extends transgene expression from the amplicon in dividing glioma cells has also recently been described by another group (18).

An HSV-1 amplicon expressing Rep from the p5 and p19 promoters and made with wt HSV-1 helper virus (HSV-RC/KOS) can support rescue and replication of rAAV genomes. The HSV-1 amplicon had to be able to rescue and replicate rAAV genomes efficiently if the HSV-1 amplicon system expressing Rep and Cap was to successfully package rAAV genomes into virions. Rescue and replication of rAAV genomes by HSV-RC/KOS requires the appropriate expression of Rep from p5 and p19 promoters that are in a different genomic structural context than they are in the AAV-2 genome. Additionally, expression of Rep from the amplicon genome has to be appropriately timed with HSV-1 early gene expression so that rAAV replication could proceed as does AAV-2 replication. The ability of HSV-RC/KOS to replicate rAAV genomes introduced into cells by infection of rAAV virions, by transfection of plasmids, or when maintained as proviral rAAV genomes integrated into cellular chromosomal DNA was analyzed.

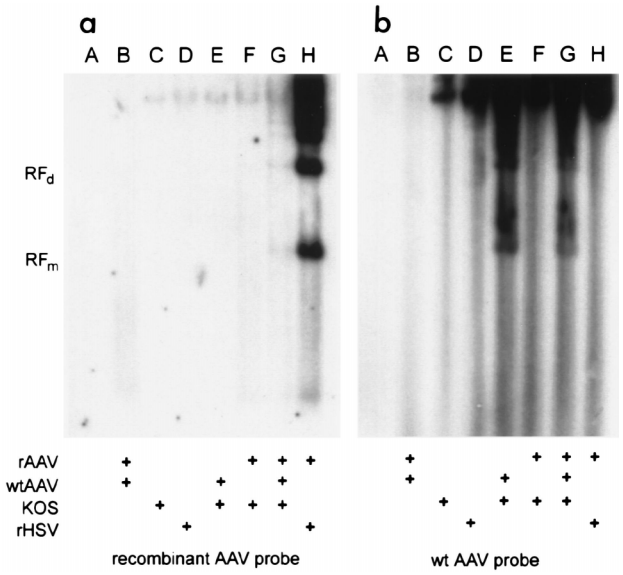

FIG. 2. Replication of rAAV genomes was produced by HSV-RC/KOS. HeLa cells (2×10^5) were seeded onto six-well plates. After 24 h, designated wells were infected with rAAVlacZ (5×10^5 particles), AAV-2 (MOI of 100 particles), or both. After 24 h, designated wells were infected with HSV-1 (KOS strain, MOI of 2) or HSV-RC/KOS (MOI of HSV-RC/KOS [rHSV] was one recombinant genome per cell and 2 PFU of HSV-1 per cell). The wells were scraped, and the cells were collected and centrifuged (2,000 rpm, 5 min) after 36 h. Medium was removed, and the low-molecular-weight DNA in the pellet was isolated by Hirt extraction. Hirt-extracted DNA (5 μg per lane) was loaded on an 0.8% agarose gel and run for 12 h at 25 V. DNA from the gel was transferred to nylon membrane by Southern blotting. (a) Membrane hybridized with a [α-^{32}P]dATP-labeled *lacZ* DNA probe, 24-h exposure; (b) membrane hybridized with a [α-^{32}P]dATP-labeled AAV-2 DNA probe, 12-h exposure.

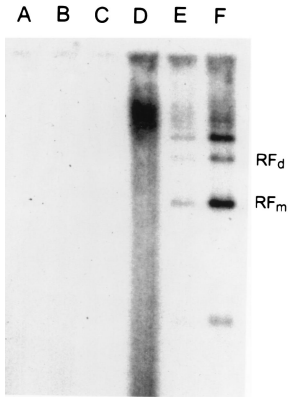

FIG. 3. Replication and rescue of rAAV genomes from transfected plasmids is produced by HSV-RC/KOS. HeLa cells were seeded onto six-well plates (2×10^5); 24 h later, the cells were either mock transfected (lanes A and C), transfected with 3 μg of pAAVlacZ (lanes B, D, and E), or infected with rAAVlacz (5×10^5 particles; lane F). The cells were either mock infected (lanes A and B), infected with wt HSV-1 (MOI of 2; lane D), or infected with HSV-RC/KOS (MOI of the HSV-RC/KOS was one recombinant genome per cell and 2 PFU of HSV-1 per cell; lanes C, E, and F) 24 h later. Cells were collected 36 h later and centrifuged for 5 min at 2,000 rpm. Medium was removed, and low-molecular-weight DNA was isolated from the pellet by Hirt extraction. Hirt-extracted DNA was extensively digested with *Dpn*I (100 U) for 24 h, ethanol precipitated, and analyzed on an 0.8% agarose gel for 12 h at 25 V. Ten micrograms of DNA was analyzed in all lanes except lane D, in which 20 μg of DNA was analyzed. DNA was transferred to a nylon membrane by Southern blotting. The membrane was hybridized with an [α-^{32}P]dATP-labeled *lacZ* DNA probe and exposed for 24 h.

The ability of HSV-RC/KOS to replicate and amplify an rAAV genome, rAAVlacZ, after rAAV infection was examined (Fig. 2). HeLa cells were either mock infected (lane A) or infected with various combinations of rAAVlacZ, AAV-2, HSV-1, or HSV-RC/KOS (lanes B through H). In this assay, replicative intermediates of rAAV, the double-stranded monomers (RF_m), double-stranded dimers (RF_d), and higher-molecular-weight replicative forms, indicate successful replication. Positive replication was observed in lanes in which low-molecular-weight DNA was analyzed from cells coinfected with rAAV, AAV-2, and HSV-1 (positive control [lane G]) or coinfected with rAAV and HSV-RC/KOS (lane H). Replicative forms of rAAV were not detectable in any of the negative control lanes (lanes A through F). These data illustrate that HSV-1 gene expression and Rep expression from an HSV-1 amplicon are temporally and quantitatively appropriate for the task of replicating rAAV genomes introduced into cells by viral infection. In addition, the intensity of the RF_m and RF_d in lane H compared to lane G suggests that Rep expression from an amplicon in the presence of HSV-1 coinfection supports rAAV replication more efficiently than coinfection of AAV-2 and HSV-1 at similar MOIs. This may be due to the absence of replication-competent AAV-2 in HSV-RC/KOS. Replication-competent AAV-2 would successfully compete with rAAV for replication machinery and lead to a decrease in rAAV replication (9).

One demonstration that wt AAV is not generated and amplified by the HSV-1 amplicon expressing Rep is shown in Fig. 2b. Only in the lanes in which Hirt-extracted DNA was analyzed from cells coinfected with AAV-2 and HSV-1 are RF_m and RF_d of wt AAV observed when probed for *rep* and *cap* sequences (lanes E and G). In addition, a 7-day exposure of the film did not reveal any replicative forms of wt AAV in any additional lanes (data not shown). Normally, replicative forms of wt AAV are observable after 48 h exposure of the film.

The ability of HSV-RC/KOS to rescue and replicate rAAV genomes from different rAAV templates was also evaluated (Fig. 3 and 4). The data indicate that HSV-RC/KOS was able to rescue and replicate rAAV genomes from transfected plasmids (Fig. 3). HeLa cells were either not transfected (lanes A and C), transfected with pAAVlacZ (lanes B, D, and E), or infected with rAAVlacZ (5×10^5 particles) (lane F) and incubated for 24 h. Plasmid pAAVlacZ contains an HCMV MIE-driven *lacZ* expression cassette flanked by ITRs. The cells were either mock infected (lanes A and B), infected with HSV-1 (lane D), or infected with HSV-RC/KOS (lanes C, E, and F). After 36 h, low-molecular-weight DNA was isolated from the cells by Hirt extraction and extensively digested with *Dpn*I. *Dpn*I will not digest newly replicated rAAV which is not methylated at *Dpn*I sites after replication in eukaryotic cells. The RF_m and RF_d were readily observed in lane F, the positive control for rescue and replication of rAAV genomes. Rescue and replication of *Dpn*I-resistant rAAV genomes from transfected plasmids was also observed in lane E, where pAAVlacZ transfection was followed by HSV-RC/KOS superinfection. Replicative forms of rAAV are not observed in any of the negative control lanes. The nonspecific hybridization at the top of lane D resulted from overloading this sample in the attempt to detect replicative forms of rAAV.

HSV-RC/KOS was also proven to be able to rescue and amplify proviral rAAV genomes that were chromosomally integrated in the cell line UF2-293 (Fig. 4). This cell line was either mock infected (lane A), infected with HSV-1 (lane B), or infected with HSV-RC/KOS (lane C). The replicative monomers and dimers indicative of rAAV rescue and replication were seen only in the lane containing Hirt-extracted DNA from the UF2-293 cells infected with HSV-RC/KOS. Rescue of rAAV genomes from the UF2-293 cells was not due to latent wt AAV infection of the cells, which could supply Rep in *trans*.

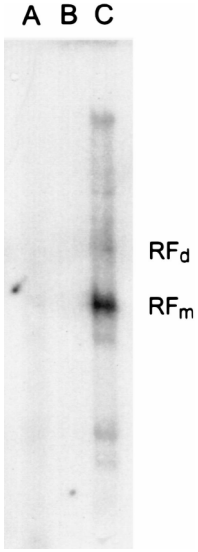

FIG. 4. HSV-RC/KOS is capable of rescuing and replicating rAAV genomes from an integrated cell line. Dishes (10-cm-diameter) were seeded with 1.5×10^6 UF2-293 cells. After 24 h, the cells were mock infected (lane A), infected with HSV-1 (MOI of 2; lane B), or infected with HSV-RC/KOS (MOI of the HSV-RC/KOS was one recombinant genome per cell and 2 PFU of HSV-1 per cell; lane C). Plates were scraped 36 h postinfection. Cells were centrifuged (5 min, 2,000 rpm), and the medium was discarded. Low-molecular-weight DNA was isolated from the pellet by Hirt extraction. Hirt extracted DNA (10 μg per well) was analyzed on an 0.8% agarose gel for 12 h at 25 V. DNA was transferred to a nylon membrane by Southern blotting. The membrane was hybridized with an [α-^{32}P]dATP-labeled *hgfp* DNA probe and exposed for 24 h.

Replicative forms of rAAV were not observed in lane B, in which Hirt-extracted DNA was analyzed from HSV-1-infected UF2-293 cells. If the UF2-293 cells were latently infected with wt AAV, rescue and replication of rAAV genomes would be observed in this lane. In addition, stripping of the membrane and reprobing for wt AAV replicative forms with an [α-^{32}P]dATP-labeled *cap* probe did not reveal any wt AAV replicative forms after exposure of the film for 7 days (data not shown). HSV-RC/KOS was also able to rescue and replicate rAAV proviral genomes from GFP-92 cells in a similar assay with similar controls for detecting the presence of wt AAV replication (data not shown).

HSV-RC/KOS can successfully replicate and package rAAV at low efficiency. To determine if HSV-RC/KOS could replicate and package rAAV particles and to measure the efficiency of the process, the particle titers of rAAVUF2 prepared from UF2-293 cells by using HSV-RC/KOS were determined by QC-PCR (Fig. 5). The number of particles produced per cell was 2.3 ± 0.3. The number of rAAVUF2 particles produced per cell was 100-fold lower than the number of particles usually produced per cell by transfection methods using Ad superinfection (data not shown).

An HSV-1 amplicon expressing Rep from the p5 and p19 promoters and made with *d*27-1 helper virus (HSV-RC/*d*27) can support rescue and replication of rAAV genomes. The efficient replication of rAAV genomes in a lytic cycle by HSV-RC/KOS is clearly shown (Fig. 2 to 4). Packaging of rAAV genomes by HSV-RC/KOS is extremely inefficient, however. The initial choice of wt HSV-1 as the helper virus to generate HSV-RC/KOS was made because it can supply the necessary functions (early gene expression) required for AAV-2 production. Unfortunately, HSV-1 induces CPE in infected cells much more rapidly than a similar infection with Ad. The rapid time course of host cell death probably limits the amount of rAAV that can be produced from each cell. Full CPE of host cells was consistently observed within 36 to 48 h after infection with HSV-1, compared to 72 to 96 h after Ad infection at the same MOIs (data not shown). The rapidity of CPE after HSV-1 infection is due, in part, to the toxicity of HSV-1's immediate-early (IE) gene products which are expressed within 2 h after infection and quickly alter the host cell's macromolecular synthesis machinery (16, 17). Host cell transcription, RNA splicing and protein synthesis are all perturbed by IE gene products of HSV-1 and contribute to the rapid CPE (16, 17).

Another reason for the inefficiency of rAAV particle production by HSV-RC/KOS is the inhibition of host cell mRNA splicing by ICP27 (37). ICP27 expression would also interfere with the appropriate splicing of the AAV late p40 transcripts which encode Cap proteins. Decreased synthesis of Cap messages would limit the production of rAAV particles.

To increase the yield of rAAV produced per cell, a Rep- and Cap-expressing amplicon was made by using the defective HSV-1 virus, *d*27-1. *d*27-1 has a deletion in ICP27 and was chosen for several reasons (30). Although the other IE proteins are still expressed in *d*27-1 and the vector still induces CPE, ICP27 itself is toxic to cells, and therefore elimination of ICP27 should reduce toxicity of the defective vector compared to wt HSV-1 (17). The ICP27 protein is also implicated in the inhibition of mRNA splicing, and the *d*27-1 strain should permit more efficient and accurate splicing of the late p40 transcripts encoding Cap proteins and increase rAAV particle yield per cell. In addition, ICP27 is involved in the down regulation of HSV-1 early gene expression. ICP27 mutants overexpress the early gene products of HSV-1 such as ICP8, and it is these early gene products that are essential for AAV-2 productive infection (27, 30, 43). Overexpression of early gene products may result in an increase in the yield of rAAV particles produced.

To determine if an HSV-1 amplicon expressing Rep and Cap and made with *d*27-1 helper virus could support replication and packaging of rAAV particles, HSV-RC/*d*27 was produced and tested in a replication assay (Fig. 6). The capability of HSV-RC/*d*27 to rescue and replicate chromosomally integrated rAAV provirus from the cell line GFP-92 was demonstrated (lane D). Coordinated expression of Rep from the amplicon and early genes from *d*27-1 allows replication of rAAV. Wild-type levels of HSV-1 DNA synthesis and HSV-1 late gene expression are clearly not required for rAAV replication, in agreement with previous reports for AAV-2 replica-

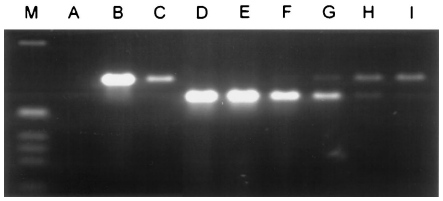

FIG. 5. QC-PCR determination of the particle titer of rAAVUF2 prepared from the cell line UF2-293 by using HSV-RC/KOS. UF2-293 cells (10^8 cells) were infected with HSV-RC/KOS. After full CPE occurred, the cell pellet was harvested and then frozen and thawed three times. The cell lysate was then clarified, and an aliquot (1/100 of the volume of the cell lysate) was DNase I and proteinase K treated, phenol and chloroform extracted, and precipitated in ethanol. Aliquots (1 μl) of the reconstituted DNA pellet (1/20 of the volume) were then analyzed by QC-PCR. For the control lanes, either no DNA template (lane A), 100 pg of p43-hgfp (lane B), 1 μl of rAAVUF2 DNA (lane C), or 50 pg of pCI-hgfpd (lane D) was added to the reaction mixture. For each QC-PCR, 1 μl of viral template and various amounts of internal control DNA template (pCI-hgfpd) were added. The amounts of internal control template were 5 pg, 1 pg, 500 fg, 100 fg, and 20 fg (lanes E, F, G, H, and I, respectively). A 1-kb ladder was run in lane M.

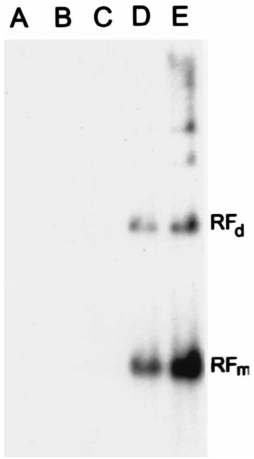

FIG. 6. Rescue and replication of rAAV genomes from an integrated cell line is produced by HSV-RC/*d*27. Plates were seeded with 2×10^5 GFP-92 cells per well. After 24 h, the cells were mock infected (lane A), infected with wt HSV-1 (MOI of 1; lane B), infected with *d*27-1 (MOI of 1; lane C), infected with HSV-RC/*d*27 (MOI of HSV-RC/*d*27 was one recombinant genome per cell and 1 PFU of *d*27-1 per cell; lane D), or infected with HSV-RC/*d*27 (MOI of the HSV-RC/*d*27 was one recombinant genome per cell and 1 PFU of *d*27-1 per cell) and 12 h later superinfected with HSV-1 (MOI of 1; lane E). Plates were scraped 36 h postinfection. Cells were centrifuged (5 min, 2,000 rpm), and the medium was discarded. Low-molecular-weight DNA was isolated from the pellet by Hirt extraction. Hirt-extracted DNA (10 μg per well) was analyzed on an 0.8% agarose gel for 12 h at 25 V. DNA was transferred to a nylon membrane by Southern blotting. The membrane was hybridized with an [α-^{32}P]dATP-labeled *hgfp* DNA probe and exposed for 24 h.

tion (43). Addition of wt HSV-1, which would provide ICP27 and allow HSV-1 DNA replication and expression of late genes to occur, does however increase rAAV DNA replication 2.7-fold, as determined by densitometry measurements (lane E).

To analyze if HSV-RC/*d*27 was sufficient not only to replicate but also to package rAAV in the absence of wt levels of HSV-1 DNA synthesis and late gene expression, GFP-92 cells were infected with different control viruses (Ad5, wt HSV-1, and *d*27-1 [Fig. 7, lanes D, E, and F, respectively]) or HSV-RC/*d*27 (lane G) or were transfected with pRS5 (which supplies Rep and Cap) and superinfected with Ad5 (positive control [lane H]). HSV-RC/*d*27 was sufficient to produce DNase I-resistant, PCR-detectable rAAV genomes from rAAVUF2 particles (lane G). This result supports the report that neither HSV-1 DNA synthesis nor late gene expression is necessary for efficient AAV-2 particle production (43).

The GFP92 cell line was not latently infected with wt AAV, as demonstrated by the absence of RF_m and RF_d in lanes B and C of Fig. 6. If GFP-92 cells were latently infected with wt AAV, replication of rAAV genomes would have occurred when the cells were infected with HSV-1 or *d*27-1 alone. Replicative forms of wt AAV were not detected when the membrane was stripped and probed for wt AAV sequences with an [α-^{32}P] dATP-labeled *cap* DNA probe after a 7-day exposure, either (data not shown). In addition, no PCR-detectable rAAV genomes were present after the cells were infected with any of the control viruses—Ad5, HSV-1, and *d*27-1 (Fig. 7, lanes D to F).

HSV-RC/*d*27 can replicate and package rAAV as efficiently as standard methods. To determine if HSV-RC/*d*27 could package rAAV as efficiently as transfection methods, larger-scale production of rAAVUF2 was attempted. GFP-92 cells (at 60% confluence) were either transfected with pRS5 (and then superinfected with Ad5) or infected with HSV-RC/*d*27 (with and without superinfection with wt HSV-1). The number of particles produced per cell by each of the methods was determined by QC-PCR (Fig. 8). Particle production for the different methods from five experiments is presented in Table 1. Although transfection with pRS5 followed by superinfection with Ad did yield a mean rAAV particle production per cell slightly higher than when HSV-RC/*d*27 was used, analysis of variance indicates that this is not a statistically significant difference (pRS5 plus Ad versus HSV-RC/*d*27 plus wt HSV-1, $P = 0.50$; pRS5 plus Ad versus HSV-RC/*d*27, $P = 0.10$). Also, although the replication of rAAV is increased after superinfection of HSV-RC/*d*27 with wt HSV-1, this method did not provide a statistically significant increase in rAAV particle production over the use of HSV-RC/*d*27 without wt HSV-1 superinfection (HSV-RC/*d*27 plus wt HSV-1 versus HSV-RC/*d*27, $P = 0.29$). It is notable that these experiments were done at 60% cellular confluence 24 h after seeding to maximize transfection efficiency. Cell confluence could likely be increased to 90% as would be done during rAAV production with these amplicons without affecting the yield per cell, thereby improving overall yield per experiment.

The rAAVUF2 generated by a Rep- and Cap-expressing amplicon is infectious. rAAVUF2 prepared from the amplicon system was heated to inactivate the helper virus, purified on an isopycnic CsCl gradient, and analyzed for its ability to transduce cells as measured by replication competence following transduction of HeLa cells (43). The replicative forms indicative of infectious rAAV were produced after the cells transduced with rAAVUF2 were superinfected with HSV-RC/KOS (Fig. 9). The RF_m and RF_d were probably not due to transduction of the cells with a recombinant HSV vector that was generated through a recombination event of the amplicon or HSV-1 helper virus with the proviral rAAVUF2. A recombinant HSV-1 vector would not be infectious after prolonged heat inactivation and purification on a CsCl gradient. The rAAVUF2 produced by infecting the GFP-92 cells with HSV-RC/*d*27 was also infectious after heat inactivation of helper virus and CsCl gradient purification (data not shown).

HSV-RC/*d*27 does not generate wt AAV during the production of rAAV. A PCR assay was used to detect the generation of wt AAV during production of rAAV by using the HSV-1 amplicons. Primers that anneal to the *D* sequence and *cap* sequence of AAV-2 will produce a product after PCR amplification only if wt AAV is present. Product was not detected in any of the experiments. (Data from experiments 1 and 2 are

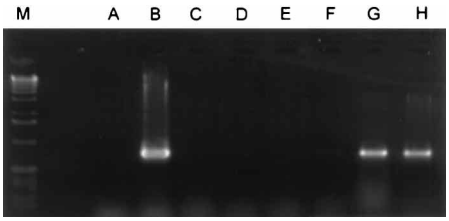

FIG. 7. The HSV-RC/*d*27 amplicon is able to generate rAAVUF2 DNase I-resistant particles from the cell line GFP-92. GFP-92 cells (2×10^5) were plated onto six-well dishes. After 24 h, the cells were either not infected or transfected (lane C), infected with Ad5 (MOI of 2; lane D), HSV-1, (MOI of 1; lane E), *d*27-1 (MOI of 1; lane F), or HSV-RC/*d*27 (MOI of HSV-RC/*d*27 was one recombinant genome per cell and 1 PFU of *d*27-1 per cell; lane G), or transfected with pRS5 DNA (2 μg) and superinfected with Ad5 8 h later (MOI of 2; lane H). The cells were scraped and pelleted after full CPE was observed. The cell pellet was then frozen and thawed three times in 100 μl of DMEM and clarified. An aliquot of the clarified lysate (10 μl) was then DNase I and proteinase K treated, phenol and chloroform extracted, and ethanol precipitated. The DNA was pelleted and reconstituted in 20 μl of dH_2O. An aliquot (2 μl) was then added to the 50-μl PCRs. An aliquot of PCR products (15 μl) was analyzed on a 2% agarose gel at 100 V for 30 min. For the controls, either no DNA template (lane A) or 200 pg of p43-hgfp (lane B) was added to the PCR.

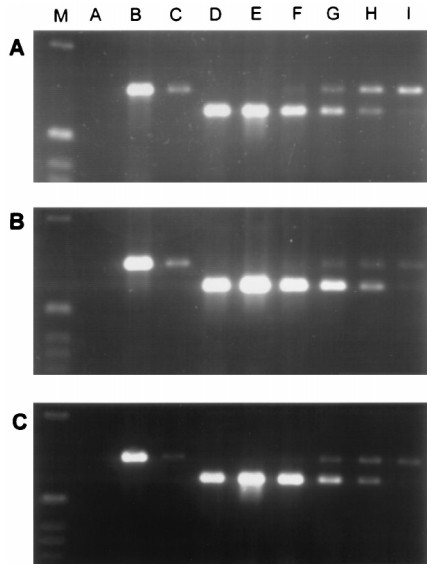

FIG. 8. The particle titers of rAAVUF2 prepared from the cell line GFP-92 by the transfection method (transfection of the Rep- and Cap-expressing plasmid pRS5 followed by superinfection with Ad5 [A]), by infection with the amplicon HSV-RC/*d*27 (B), or by infection with the amplicon HSV-RC/*d*27 followed by superinfection with wt HSV-1 (C) are comparable. In experiment 1, 6.5×10^7 GFP-92 cells were in each preparation. After full CPE occurred, the cell pellet was harvested and then frozen and thawed three times. The cell lysate was then clarified, and an aliquot (1/1,500 of the volume of the cell lysate) was DNase I and proteinase K treated, phenol and chloroform extracted, and precipitated in ethanol. Aliquots of the reconstituted DNA pellet (1 μl, 1/20 of the total volume) were then analyzed by QC-PCR. For the control lanes, either no DNA template (lane A), 100 pg of p43-hgfp (lane B), 1 μl of rAAVUF2 DNA (lane C), or 50 pg of pCI-hgfpd (lane D) was added to the reaction mixture. For each QC-PCR, 1 μl of viral template and various amounts of internal control DNA template (pCI-hgfpd) were added. The amounts of internal control template were 100 pg, 25 pg, 5 pg, 1 pg, and 200 fg (lanes E, F, G, H, and I, respectively). A 1-kb ladder was run in lane M.

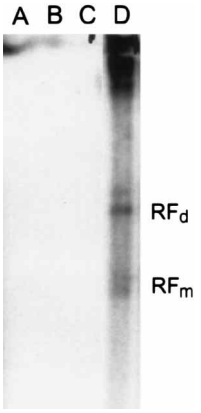

FIG. 9. The rAAVUF2 prepared by the amplicon system is infectious. The rAAVUF2 was prepared from six confluent 175-cm^2 flasks of UF2-293 cells (10^8 cells). Flasks were infected with HSV-RC/KOS (MOI of the HSV-RC/KOS was two recombinant genomes per cell and 2.5 PFU of wt HSV-1 per cell). After 48 h, rAAVUF2 was collected, and helper virus was heat inactivated for 1 h at 55°C and CsCl gradient purified as described in the text. The purified rAAVUF2 (5×10^5 particles) was added to 2×10^5 HeLa cells seeded into six-well plates 24 h earlier (lanes A, C, and D), or the cells were mock infected (lane B). After 24 h, the cells were either mock infected (lane A), infected with HSV-1 (MOI of 2.5; lane C), or infected with HSV-RC/KOS (MOI of the HSV-RC/KOS was two recombinant genomes per cell and 2.5 PFU of HSV-1 per cell; lanes B and D). Cells were scraped 36 h later and pelleted by centrifugation (2,000 rpm, 5 min). Low-molecular-weight DNA was isolated by Hirt extraction. Hirt-extracted DNA (10 μg per lane) was analyzed on an 0.8% agarose gel for 12 h at 25 V. DNA was transferred to a nylon membrane by Southern blotting. The membrane was probed with an [α-^{32}P]dATP-labeled *hgfp* DNA probe and exposed for 24 h.

presented in Fig. 10; data from experiments 3 through 5 are not shown). A sensitivity of detection of 10 fg of pAV2 in the PCR assay would indicate that there is less that 1 wt AAV particle per 2×10^6 rAAV particles. In addition, the membranes from Fig. 3, 4, 6, and 9 were stripped and reprobed for the replicative forms of wt AAV, using an [α-^{32}P]dATP-labeled *cap* DNA probe. After exposure of the films for 7 days, no replicative intermediates of wt AAV were observed (data not shown).

DISCUSSION

Gene transfer agents based on rAAV vectors have been used extensively for in vivo marker gene transduction studies and therapeutic gene transfer studies (1, 11, 19, 21, 22, 46). A clinical trial whose goal is the correction of cystic fibrosis transmembrane regulator defects in the nasal epithelia of patients with cystic fibrosis has used rAAV vectors. Many other preclinical and clinical applications of rAAV vectors are planned. Clearly one of the most promising gene transfer agents developed to date is the rAAV vector.

A major drawback in the use of rAAV vectors for gene transfer studies in vivo and their eventual clinical application has been the difficulty in producing large quantities of rAAV vector. For the therapeutic correction of some diseases, it is estimated that 10^{14} rAAV particles will have to be administered per patient. This will require the culture of more than 10^{12} cells to produce the quantity of rAAV vector that will be needed to therapeutically treat each patient. The use of transfection methods on this scale of rAAV production is extremely problematic, costly, and time-consuming.

The development of a packaging system that can provide all helper functions needed for rAAV production from an rAAV proviral cell line would greatly facilitate the large-scale production of rAAV. Transfection procedures will not be required, and the proviral cell line could be grown in large quantities at high densities in commonly used equipment. Superinfection of this culture with the amplicon described here would then produce quantities of rAAV not attainable by transfection. Additionally, a second amplicon containing the rAAV provirus could be used to introduce the rAAV genome into cells. This approach would obviate a continual problem of spontaneous deletions in the AAV ITR when rAAV plasmids are grown in bacteria.

For this purpose, we have developed HSV-1 amplicons which, with their helper viruses, were able to supply all *trans* factors and helper functions required for rAAV packaging. An HSV-1-based helper system to generate rAAV has not been previously reported. In this report, these HSV-1 amplicons

TABLE 1. Particle production by different methods

Expt no.	Total no. of cells (10^7)	rAAV particles/cell with indicated method		
		Transfection, pRS5 + Ad	Infection, HSV-RC/*d*27	Infection + superinfection, HSV-RC/*d*27 + wt HSV-1
1	6.5	400	150	200
2	6.4	150	200	500
3	4.6	700	300	350
4	5.2	850	500	700
5	5.9	800	450	650
Mean ± SEM	5.7	580 ± 250	320 ± 150	480 ± 175

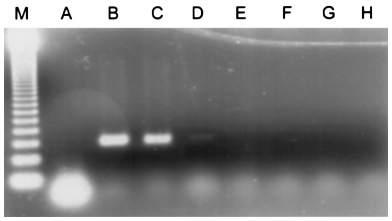

FIG. 10. Particles of wt AAV were not detectable in any rAAVUF2 preparations generated with HSV-RC/*d*27. An aliquot of the clarified cell lysate from GFP-92 cells infected with HSV-RC/*d*27 or HSV-RC/*d*27 plus wt HSV-1 (1/30 of the volume of the cell lysate; experiment 1 and 2) was DNase I and proteinase K treated, phenol and chloroform extracted, and precipitated in ethanol. Aliquots of the reconstituted DNA pellet (9 μl, 90% of the total volume) were then analyzed for the presence of wt AAV. DNA template was not added to the PCR analyzed in lane A. A standard curve of 1 pg, 100 fg, and 10 fg of pAV2 DNA was added to the PCRs analyzed in lanes B, C, and D, respectively. Aliquots from the PCR using DNA from experiment 1, HSV-RC/*d*27 and HSV-RC/*d*27 plus HSV-1, and experiment 2, HSV-RC/*d*27 and HSV-RC/*d*27 plus HSV-1, were analyzed in lanes E, F, G, and H, respectively. A 123-bp DNA ladder was run in lane M. Unincorporated primers are visible are below the 123-bp marker in lane A. The other 1 μl from the DNA samples was analyzed for the presence of rAAVUF2 DNA by using the *hgfp* primers to ensure that DNA was present in the samples (data not shown).

have been shown to be capable of rescuing and replicating all forms of rAAV genomes, including rAAV genomes introduced into cells by infection of rAAV virions, rAAV genomes transfected into cells on plasmids, and proviral rAAV genomes integrated into cellular chromosomal DNA.

The HSV-1 amplicons HSV-RC/KOS and HSV-RC/*d*27 (with their helper viruses) were able to replicate and amplify all forms of proviral rAAV. One possible application of the amplicons could be their use in replication center assays or to detect the presence of episomal or integrated proviral rAAV in cells previously infected with rAAV. The use of HSV-RC/KOS or HSV-RC/*d*27 would eliminate the need for coinfection of cells with wt AAV and Ad and help standardize problematic assays, which are difficult to reproduce. Eliminating the use of wt AAV is also desirable since it would reduce the likelihood of wt AAV contamination of viral preparations and cells.

A proviral cell line was able to produce rAAV vector when infected with HSV-RC/KOS. However, although HSV-RC/KOS could express all of the helper functions needed for rAAV production, this system was extremely inefficient. A defective HSV-1 vector, *d*27-1, which overexpresses the HSV-1 helper genes required for AAV replication, was then used to make the second Rep- and Cap-expressing amplicon, HSV-RC/*d*27. The amplicon system HSV-RC/*d*27 was shown to be capable of providing all of the helper functions required for rAAV replication and packaging. Infection with HSV-RC/*d*27 was capable of producing rAAV particles as efficiently as transfection methods. The rAAV virus produced by the HSV-1 amplicons was infectious after heat inactivation of helper virus and CsCl gradient purification. Finally, wt AAV was not detected in any of the HSV-1 amplicon-produced rAAV preparations.

Purification of rAAV intended for clinical trials should be facilitated by the described amplicons. HSV-1 is a large enveloped virus greater than 200 nm in diameter (31). The HSV-1 virion is extremely sensitive to heat and chemical inactivation. Additionally, size exclusion chromatography is extremely effective at eliminating HSV-1 virions from the rAAV preparations (data not shown). This is probably due to the large size difference between the AAV capsid (20-nm diameter) and HSV-1 virion. Chromatographic methods have already been developed to increase the efficiency of rAAV production by eliminating the need for CsCl gradients (38). Size exclusion chromatography could easily be added to these production processes.

Additional HSV-1 amplicon constructs and helper viruses are under development to further increase the efficiency of rAAV production from an HSV-1 amplicon system. Substitution of heterologous promoters such as the human immunodeficiency virus long terminal repeat or the HCMV IE promoter to drive Rep or Cap expression has been shown to increase the production of rAAV in transfection systems (12, 42). Constructs in which Rep and Cap are expressed from these promoters could easily be incorporated into an amplicon plasmid. Another approach would be to use HSV-1 viral promoters with VP16-response elements in them such as the HSV-1 IE-110 promoter to drive Cap expression. The transactivating properties of the HSV-1 virion factor VP16 would increase Cap expression and increase rAAV production. Amplification of rAAV virions from a cell lysate by using an HSV-1 amplicon system may also be possible. Proviral cell lines and large-scale transfections would not be needed. Stepwise coinfections could then be used to amplify the rAAV vector as is commonly done for other recombinant viruses that can replicate in complementing cell lines.

The subcellular localization of AAV-2 or rAAV genomes and Rep proteins during wt HSV-1 coinfection has not yet been investigated as it has for Ad and AAV coinfection (44). The effects of Rep proteins on HSV-1 prereplication and replication centers have also not been studied. Clearly, Rep proteins do not disrupt HSV-1 replication as completely as they affect Ad replication. One member of the herpesvirus family, human herpesvirus 6, actually encodes and expresses a functional Rep homolog (39, 40). In contrast, Rep proteins potently disrupt the replication of Ad, and this has made the production of p5-driven *rep* recombinant Ad unsuccessful to date. The creation of a recombinant Ad with inducible *rep* expression has also been problematic. While Rep proteins have been shown to decrease HSV-1 viral DNA replication, they clearly do not preclude construction of HSV-1 amplicons which express functional Rep proteins.

A great deal of effort has been expended on improving the efficiency of rAAV production. The HSV-1 helper functions that permit AAV-2 replication have been largely ignored, however. The use of HSV-1 amplicons, which express AAV-2 Rep and Cap proteins for the production of rAAV vectors, is unique and has broad implication for rAAV vector biology. The effects of Rep proteins on the replication of different DNA viruses are intriguing and may offer insight into fundamental differences between Ad and herpesvirus replication.

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