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Serotype-specific replicating AAV helper constructs increase recombinant AAV type 2 vector production

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Abstract

One of the major limitations of the use of adeno-associated virus (AAV) as a tool for gene therapy is the difficulty in providing sufficient quantities of the virus for pre-clinical and clinical trials. Here, we report a novel approach for amplifying AAV helper functions, which mimics the normal replication of wild type (wt) AAV resulting in a high yield of AAV vectors. Cotransfection of replicating but non-packaging AAV helper constructs in the presence of adenovirus (Ad) produces a high level of Rep and Cap proteins. Yield of AAV2/ GFP vector obtained from this helper DNA replication system was approximately 20-fold higher than traditional methods. Molecular analysis suggested that virus yield was associated with capsid protein production. The transfection ratio was optimized using these novel helper constructs, resulting in an additional 2-fold increase in vector yield without presence of replication competent AAV (rcAAV). This strategy supports development of AAV packaging systems that retain normal virus replication capability without helper virus encapsidation.
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Adeno-associated virus type (AAV) is a human parvovirus with a single-stranded DNA genome of approximately 4.68 kb. AAV has increasingly become an important gene therapy vector, largely because wild-type AAV (wtAAV) is not associated with any known human diseases and the viral genome has the capability to recombine site-specifically into the human genome at chromosome 19 qter13.4. Recombinant AAV (rAAV) genomes appear to persist in vivo primarily as episomes and are capable of long-term transgene expression (Schnepp et al., 2003). The lack of cell-mediated immune responses to rAAV transduced cells is another key reason for prolonged gene expression in vivo (Xiao et al., 1996). Additionally, rAAV can infect dividing and non-dividing cells in a broad range of tissues including muscle,

liver, brain and retina (Samulski et al., 1999; Stilwell et al., 2003). Phase I studies using AAV have demonstrated safe and persistent expression that is consistent with pre-clinical studies (Hildinger and Auricchio, 2004; Monahan and Samulski, 2000; Manno et al., 2003).

rAAV vectors are generally produced by cotransfection of the rAAV vector plasmid and the helper plasmid coding AAV *rep* and *cap* in the presence of a helper virus infection (Samulski et al., 1989). Several recent improvements to rAAV production have been reported such as using stable cell lines that contain a subset of the required viral genes, introducing AAV or the vector genome into producer cells via chimeric viral vectors, or by eliminating the need for helper virus infection through the use of cloned adenovirus genes (Clark et al., 1995; Conway et al., 1999; Feudner et al., 2001; Gao et al., 1998; Inoue and Russell, 1998; Liu et al., 1999; Matsushita et al., 1998; Qiao et al., 2002a, 2002b; Sollerbrant et al., 2001; Xiao et al., 1998; Zhang et al., 1999). Recently, Urabe et al. (2002) proposed using baculovirus as an alternative approach for producing high

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level of AAV vectors. Many of these approaches address large-scale production concerns. However, most research laboratories invest in optimizing vector cassettes as well as testing novel serotypes, which can be extremely laborious when establishing production cell lines (4–6 months) or recombinant viral packaging strategies. Additionally, in most cases, the production of rAAV is relatively inefficient when compared with the number of particles generated during a replicative wild-type virus infection. Under optimized conditions, the transient transfection procedure can yield a virus titer up to 10^{10} transducing units (TU) of rAAV per milliliter, which represents approximately 50–150 TU produced per transfected cell (Samulski et al., 1999). In contrast, wtAAV generates 1000–5000 TU per cell (Samulski et al., 1999). One major limitation of practical rAAV application has been the failure of current transient packaging systems to support sufficient amplification and expression of AAV *rep* and *cap* gene products during the vector packaging process. In order to improve the expression of Rep and Cap proteins, alternative replication systems have been exploited. Recombinant adenovirus (rAd) vectors carrying AAV helper functions have not been successful in rAAV vector production because AAV Rep proteins inhibit Ad replication. In order to address the need to amplify AAV helper functions for optimum rAAV vector production, inducible cell lines have been tested. Inducible amplification of chromosomal loci containing the simian virus 40 (SV40) replication origin in cells that express the SV40 large T antigen has been used to produce titers of virus 10 times higher than those produced by transfection methods. Virus produced in this manner is also apparently free of replication-competent AAV (rcAAV) (Inoue and Russell, 1998). Stable cell lines that have shown marked improvement in vector production all correlate with an uncharacterized Rep-dependent amplification of helper genes from the host chromosome (Blouin et al., 2004; Cao et al., 2002; Chadeuf et al., 2000; Fan and Dong, 1997; Gao et al., 2002b; Liu et al., 2000). All of these methods provide the additional helper replication needed during vector production. Unfortunately, the resulting amplification is not controlled and does not mimic the amplification typically seen with wtAAV infection. Here, we use a novel strategy that mimics wtAAV replication when supplying AAV helper genes *rep* and *cap* in trans to produce high titer rAAV (Fig. 1a). This strategy is based on the findings that the ITRs of AAV2 and AAV5 are only 60% identical and the *rep* from one serotype cannot initiate replication from the ITR of the other (Chiorini et al., 1999a, 1999b). In this system, the *rep* and *cap* genes from AAV2 are inserted between two ITRs from AAV5 and replication is supported by the addition of plasmids expressing type 5 Rep proteins. In turn, the type 2 Rep78 protein induces the replication of the rAAV vector carrying AAV2 ITRs, and Rep 52 can efficiently help to package this template into type 2 virions (Fig. 1a). When tested, this approach resulted in rAAV production 10- to 20-fold higher than traditional methods

that rely solely on the initial input amount of AAV helper constructs (Xiao et al., 1998).

A potential drawback with the use of serotype-specific replicating AAV helper constructs is the potential to cross package helper genomes into vector particles. To avoid production of replication-competent AAV particles through non-homologous recombination between serotype-specific helper plasmids (Allen et al., 1997; Cao et al., 2000; Grimm et al., 1998; Wang et al., 1998), we used the intron insertion mutant described by Cao et al. (2000) to increase the size of the AAV2 *rep+cap* cassette 2.35 kb above wild type packaging limits. While this modification had no effect on the amount of vector produced, it completely eliminated the presence of rcAAV. This study demonstrates that a significant increase in rAAV yield can be achieved by utilizing serotype-specific ITRs for replicating AAV helper constructs that more closely mimic the natural infection scheme of wtAAV.

Results

Efficient replication of AAV2 rep and cap genes in the presence of pREP5 and adenovirus

To mimic replication conditions utilized by wtAAV for productive infection, we first constructed the plasmid pTR5/AAV2 with the *rep* and *cap* genes from AAV2 inserted between ITRs from AAV5 (Fig. 1b). To initiate replication of this hybrid plasmid, Rep protein from AAV5 was provided in a separate expression construct, pRep5. Forty-eight hours after cotransfection of 293 cells using pTR5/AAV2 with pRep5 in the presence of adenovirus, low-molecular weight DNA was recovered. The presence of AAV replicating templates was determined by Southern blot analyses. To differentiate the input plasmids from newly replicated DNA, Hirt DNA was digested with *Dpn*I endonuclease. AAV2 *rep* and *cap* helper plasmids were not replicated when only Rep5 or adenovirus was added singly (Fig. 2, lanes 10 and 11). However, in the presence of all required components (Rep5 and Ad), the AAV2 *rep* and *cap* helper plasmids were efficiently rescued and replicated (Fig. 2 lane 12). Only those constructs carrying the type 5 ITR were able to replicate in the presence of type 5 Rep protein, which is consistent with published studies. Replication of these hybrid substrates was identical to wtAAV both in yield and size (Fig. 2 lane 12). In order to reduce the risk of cross encapsidation of replicating helper constructs, we expanded the genome size by 2.35 kb above wtAAV using an intron approach described by Cao et al. (2000). Plasmid pTR5/AAV2/INS was constructed, in which a β-850 bp globin intron with a 1.5-kb lambda DNA fragment was inserted at position 654 of the AAV2 genome (Fig. 1b). This modification increased the total genome size between the two AAV5 ITRs to 6.8 kb, which is 2 kb beyond normal AAV2 packing limits. Cotransfection of pTR5/AAV2/INS
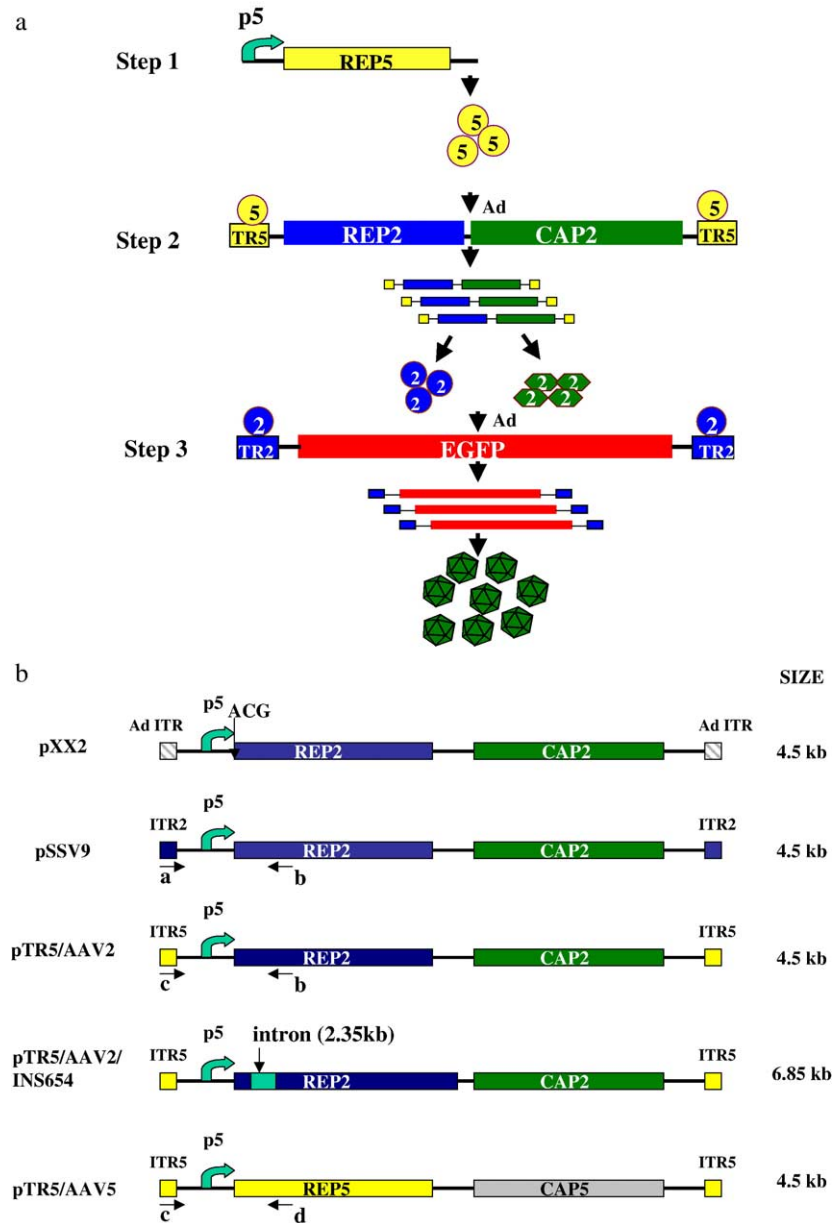
Fig. 1. (a) Strategy of helper DNA replication system to make rAAV virus. Step 1, AAV5 rep genome is driven by native p5 promoter to express REP5 protein. Step 2, REP5 protein interacts with AAV5 TR to initiate AAV2 helper genome (Rep and Cap genes) replication and induce REP2 and CAP2 expression. Step3, REP2 protein interacts with AAV2 TR of vector to initiate vector replication and helps package vector genome into capsids to form virions. (b) Diagram of plasmid constructs and PCR strategy for analyzing structures of AAV species. See Materials and methods for details. Primers are shown as arrows. Primers a, b, c and d are located at ITR2, rep2, ITR5 and rep5 genes, respectively.

with the required helpers (pRep5 and Ad) resulted in rescue and amplification of the oversized replicating AAV helper construct (6.8 kb) (Fig. 2 lane 16).

Rep and Cap expression from replicating templates

Next, we compared the production of the Rep and Cap proteins of AAV2 from replicating and non-replicating substrates using western blot analysis. As described previously, AAV helper plasmid pXX2 generates fewer Rep78/68 proteins than wtAAV2 plasmid pSSV9, with Rep52/40 accumulating and representing the majority of Rep proteins expressed in the presence of Ad (Fig. 3a lane 2). Under non-replicating conditions for pTR5/AAV2, the ratio of Rep78/68 to Rep 52/40 was similar to that of pSSV9 in the presence of adenovirus (Fig. 3a lane 3 and lane 1, respectively). All Rep protein expression increased when pRep5 was supplied in trans (Fig. 3a lane 4). Surprisingly when the 2.35-kb intron was inserted into position 654 of the AAV2 genome, the pattern of Rep protein expression was dramatically changed (Fig. 3a lane 5). The ratio of Rep78/68 to Rep52/40 with pTR5/AAV2/INS was signifi-
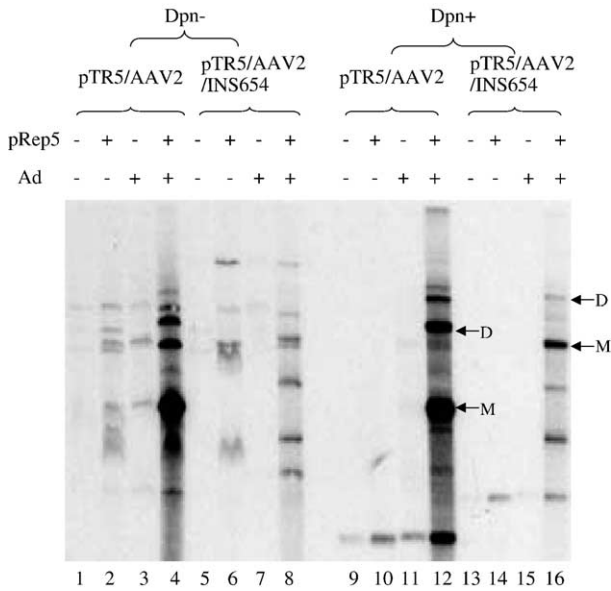
Fig. 2. Southern blot analysis of helper DNA replication. 5 μg of helper plasmids pTR5/AAV2 and pTR5/AAV2/INS were transfected into 293 cells with or without 5 μg pRep5 in the absence or presence of adenovirus infection. Low molecular DNA was recovered from the cells, digested with *Dpn*I, separated on 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a ^{32}p-labeled *rep/cap* probe made from the 4.5-kb *Xba*I fragment of pTR5/AAV2.
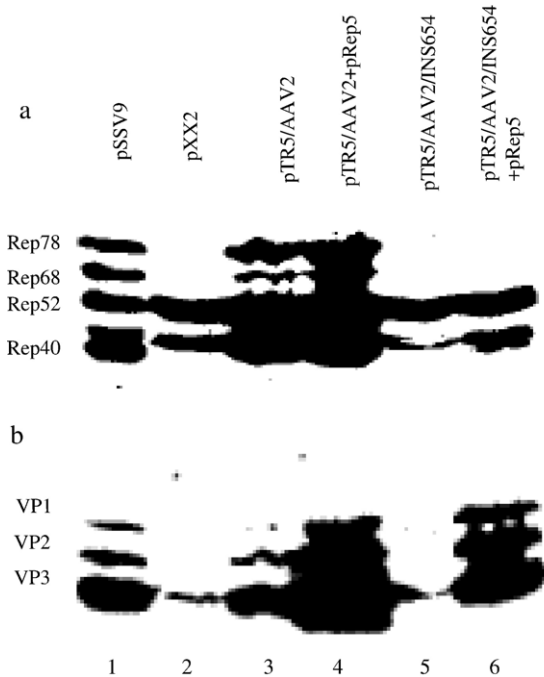

Fig. 3. Western blot analysis of rep2 and cap2 gene expression. 293 cells were transfected with 5 μg of different helper plasmids with or without 5 μg pRep5 in the presence of adenovirus. 48 h later, cells were harvested and 10 μl of each cell lysate were separated by PAGE (10% polyacrylamide), and transferred to a methylcellulose membrane. Western blot analysis was performed with the primary antibodies anti-Rep monoclonal antibody 1F11 (a) or anti-cap monoclonal antibody B1 (b), which recognize all four rep2 proteins or three cap proteins, respectively.

cantly decreased as compared with pTR5/AAV2 (Fig. 3a lane 5 and lane 3, respectively). These disparate levels were nearly identical in pattern to that seen with the previously published pXX2 helper, and strongly suggest that any modifications to the large Rep coding sequence dramatically influence protein expression level. Although the ratio varied with the use of the intron construct, the expression of Rep protein increased when Rep5 was supplemented in trans (Fig. 3a lane 6). The same membrane used in western analysis to determine Rep expression was used to evaluate the profile of Cap protein expression. Consistent with replication analysis, there were lower levels of VP1/VP2/VP3 from AAV2 helper constructs pXX2, pTR5/AAV2 and pTR5/AAV2/INS when only adenovirus was present (Fig. 3b, lanes 2, 3 and 5, respectively). The addition of pRep5 increased Cap protein expression significantly (Fig. 3b lanes 4 and 6). From these analyses, we have shown that the ability to replicate the AAV2 helper genes results in concomitant increase in Rep and Cap protein expression that is similar to wtAAV (Figs. 3a and b lanes 4, 6 and 1).

Replication of AAV2 rep and cap genes increases rAAV yield

To investigate whether the replication of the AAV helper *rep* and cap genes increases rAAV vector production, we used a recombinant AAV2-EGFP construct as a vector substrate and compared viral yields in the presence or absence of the replicating helper constructs described above. The transducing rAAV titer was assessed by GFP transduction of 293 cells. Forty-eight hours post-transfection and adenovirus co-infection, cells were harvested and subjected to three cycles of freeze-thaw. Non-replicating helper plasmids pTR5/AAV2 and pTR5/AAV2/INS produced comparable titers of vector to previously described pXX2 (Fig. 4). The vector yield increased 10- to 20-fold when helper constructs pTR5/AAV2 or pTR5/AAV2/INS were
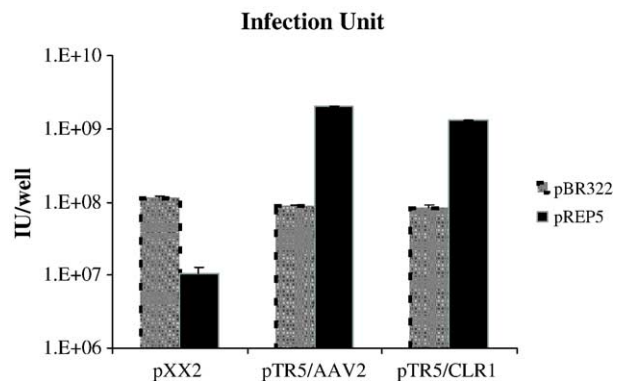

Fig. 4. The effects of helper DNA replication on rAAV2/GFP yields. 293 cells were cotransfected with 2.5 μg pTRUFR and 2.5 μg of different helper plasmids with or without 2.5 μg pRep5 in the presence of adenovirus at the MOI of 2. 48 h later, cells were recovered and frozen/thawed three times. Adenovirus was inactivated by a 30-min incubation at 56 °C. The supernatant of cell lysates was used to infect 293 cells to determine the transducing units (TU). The results were indicated as average value ± standard error from 4 different experiments.

complemented with pRep5 (Fig. 4). In contrast, the addition of pRep5 to pXX2 markedly reduced virus yield 10-fold (Fig. 4). To examine the molecular mechanism of increased virus yield in this helper replicating system, helper gene expression and the replication of helper genomes or vector genomes were assayed, as shown in Fig. 5. Virus production is positively associated with helper genome replication (Fig. 5d), which resulted in higher helper protein (Rep and Cap) expression (Figs. 5a and b), but not vector genome replication (Fig. 5c).

WT-like virus is generated from plasmid pTR5/AAV2

Previous studies have demonstrated the transcapsidation of type 5 TR in AAV2 virions (Chiorini et al., 1999b). Based on these data, we expected pTR5/AAV2, but not pTR5/AAV2/INS, to be encapsidated in type 2 shells. Using PCR analysis, we assayed for transcapsidation of AAV helper constructs as well as DNA recombinants that may have been generated from these replicating helper and vector constructs (Fig. 1b). The primers used to carry out these analyses are described in Fig. 1b. After transfection of pTR2-EGFP, pTR5/AAV2 and pRep5, the cell lysate was used to infect 293 cells in the presence of Ad for amplification of wt-like AAV virus. The Hirt DNA was extracted after each cycle and used as the template in PCR analysis. Our results showed the presence of AAV2 wt-like recombinants (TR2/AAV2) after two passages of vector cell lysate (Fig. 6a, lane 2 and 3). Compared to control wtAAV2 amplification, we estimated about 1000 TU of wt-like recombinants after 3 cycles of cell passage (compare Fig. 6a

lanes 2, 3 and 6–9). We also detected hybrid type 5 wt-like recombinants (TR5/Rep5Cap2) packaged in AAV2 virion shells, but only after hybridization of PCR materials (Fig. 6a lanes 15–17, data not shown). This suggests a reduced frequency of recombination events between Rep2 and Rep5. As expected from previous studies, we were able to detect transcapsidated TR5/AAV2 templates in type 2 virion shells (Fig. 6a lanes 10–12).

Plasmid TR5/AAV2 with intron reduces the generation of rcAAV particles

Previous studies have determined that an AAV2 genome size above 5.5 kb results in little to no encapsidation (Dong et al., 1996). Cao et al. (2000) used an intron approach to increase the helper plasmid size to reduce wt-like replicating helper encapsidation. The pTR5/AAV2/INS helper used in these studies carried an insertion of 2.35 kb. When assaying for wt-like recombinants (both type 2 and type 5) using PCR-specific primers, we were unable to detect PCR products from primers TR2/Rep2 and TR5/Rep5 (Fig. 6b, lanes 4 to 6 and 23 to 25). The only PCR products observed were amplified with primers from TR5 and Rep2 (Fig. 6b, lanes 13–15). The size of the product obtained using those primers indicates a deletion of the intron on the Rep2 coding cassette, generating fragments similar in size to wt-like. However, based on PCR band intensity, the production of TR5/AAV2 virion templates gradually decreased in each successive passage (Fig. 6b, lanes 13–15). This suggests that a reduction in size in order to achieve efficient encapsidation may result in virus with a compromised
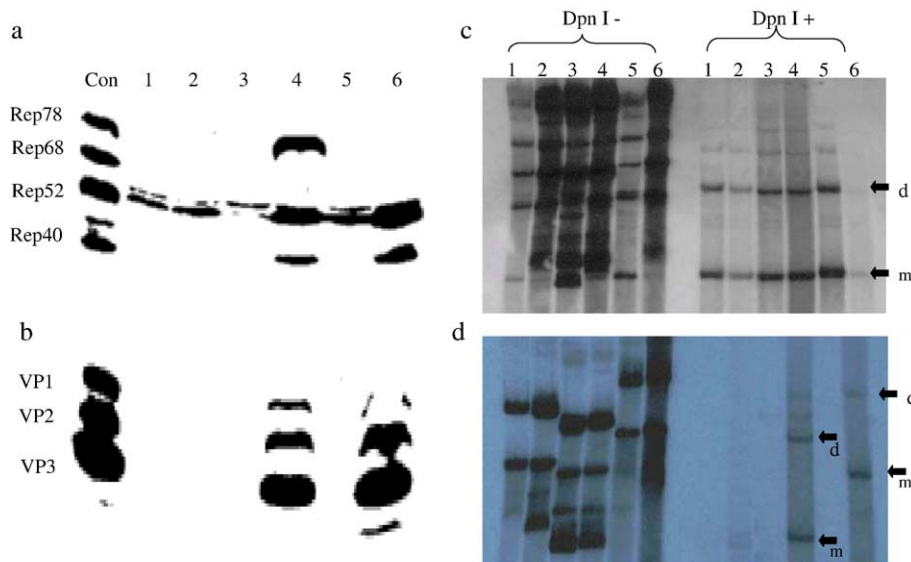

Fig. 5. The expression of viral proteins and the replication of helper DNA and transgene in the vector production system. 48 hours after transfection with 2.5 μg of each plasmid, 293 cells were collected and divided, aliquot of cells was suspended in RIPA medium for Western blot analysis to examine the expression of the rep2 and cap2 genes. The other aliquot of cells was used to extract the low molecular DNA to measure the replication of helper DNA and vector transgene EGFP using the probes rep2/cap2 and EGFP (a fragment of Bgl II), respectively. (a) The expression of rep proteins. (b) The expression of cap proteins. (c) Vector EGFP replication. (d) Helper DNA replication. Con: pSSV9 transfection, 1: pXX2, 2: pXX2+pRep5, 3: TR5/AAV2, 4: pTR5/AAV2+pRep5, 5: pTR5/AAV2/INS, 6: pTR5/AAV2/INS+pRep5.
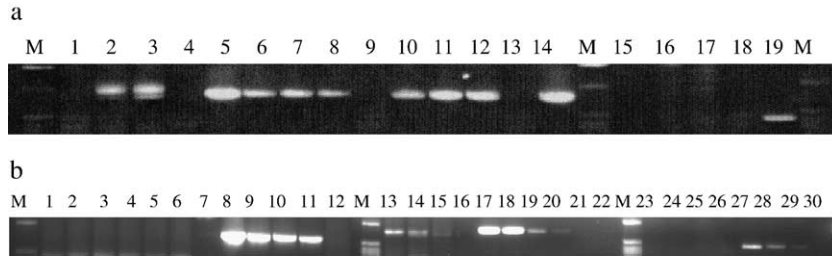
Fig. 6. Detection of replication competent AAV by PCR method in the virus preparations with helper pTR5/AAV2 or pTR5/AAV2/INS. 1 ml of cell lysate of virus preparation with helper pTR5/AAV2 or pTR5/AAV2/INS and pTR2-EGFP and pRep5 was used to infect 293 cells in the presence of adenovirus at MOI of 5 to amplify the rcAAV for total 3 cycles. Hirt DNA was extracted for PCR assay after each cycle of amplification. Panel a: M: DNA molecular marker, 1: 1st cycle's DNA amplified with primers a/b; 2: 2nd cycle's with primer a/b; 3: 3rd cycle's with primer a/b; 4: Hirt DNA from negative control with 293 cells only infected with adenovirus with primer a/b; 5: positive control with 1 ng pSSV9 with primer a/b; 6: 3rd cycle's Hirt DNA infected with 1000 TU wtAAV2 amplified with primer a/b. 7: 3rd cycle with 100 TU wtAAV2 with primer a/b; 8: 3rd cycle with 10TU wtAAV2 with primer a/b; 9: 3rd cycle with 1TU wtAAV2 with primer a/b; 10. 1st cycle of Hirt DNA transfected with pTR5/AAV2 amplified with primer c/b; 11: 2nd cycle with primer c/b; 12: 3rd cycle with primer c/b; 13: negative control with primer b/c; 14: 1 ng pTR5/AAV2 amplified with primer c/b; 15: 1st cycle with primer c/d; 16: 2nd cycle with primer c/d; 17: 3rd cycle with primer c/d; 18: negative control; 19: 1 ng pTR5/AAV5 with primer c/d. Panel b: Primers a/b were used for samples 1–12; primers c/b for samples 13–22; primer c/d for 23–30. Samples 1–3 were from the cycle 1–3 Hirt DNA transfected with pXX2 to make virus. Samples 4–6, 13–15 and 23–25 from the cycle 1–3 Hirt DNA transfected with pTR5/AAV2/INS with pRep5 to make virus. Samples 7, 16 and 26 are negative controls. Samples 8, 17 and 27 were 1 ng of plasmids pSSV9, pTR5/AAV2 and pTR5/AAV5, respectively. Samples 9–12 represent the Hirt DNA from 3rd cycle infected with 1000, 100, 10 and 1TU wt AAV2, respectively. Samples 18–22 were 10, 1, 10^{-1}, 10^{-2} and 10^{-3} pg plasmid pTR5/AAV2, respectively. Samples 28–30 were 10, 1 and 10^{-1} pg plasmid pTR5/AAV5, respectively.

ability to propagate due to inability of Rep2 proteins to efficiently nick TR5 sequences (Fig. 6b, lanes 13–15).

Optimizing vector ratio increases the vector production

As described above, we observed a 10-fold decrease in vector yields from AAV helper plasmid pXX2 in the presence of the Rep5 protein. This suggested that an excess of Rep protein may be deleterious to optimum vector production. In attempt to avoid this concern, we transfected different ratios of pTR5/AAV2/INS to pRep5 with a fixed amount of vector DNA template (2.5 μg). Higher titers of vector were generated when less replicating helper plasmid and pRepTR5 construct were used (Fig. 7). The optimal ratio (2.5:1:1) of transgene to pRep5 to pTR5/AAV2/INS was determined, which resulted in a 2- to 3-fold increase in titer over the standard ratio of 1:1:1. We also analyzed the replication of helper genomes and vector transgenes when different ratios of pRep5 to pTR5/AAV2/INS were used (Fig. 8). When the concentration of helper plasmid was fixed, increasing amounts of pRep5 resulted in more efficient replication of helper construct pTR5/AAV2/INS (Fig. 8 panel c: lanes 1–3 and 4–6). Interestingly when 2.5 μg of helper DNA was used, replication of transgene was increased by lowering the amount of pRep5 DNA (Fig. 8, panel d, lanes 1–3). However, when lower amount (1 μg) of helper DNA was used, the replication of transgene was similar regardless of the amount of pRep5 DNA (Fig. 8, panel d, lanes 4–6). In addition, when the pRep5 construct concentration was fixed (2.5 μg), an increase in helper plasmid negatively affected vector replication (Fig. 8, panel d: lane 1 vs. lane 4). It was implicated that transgene replication did not correlate with increasing
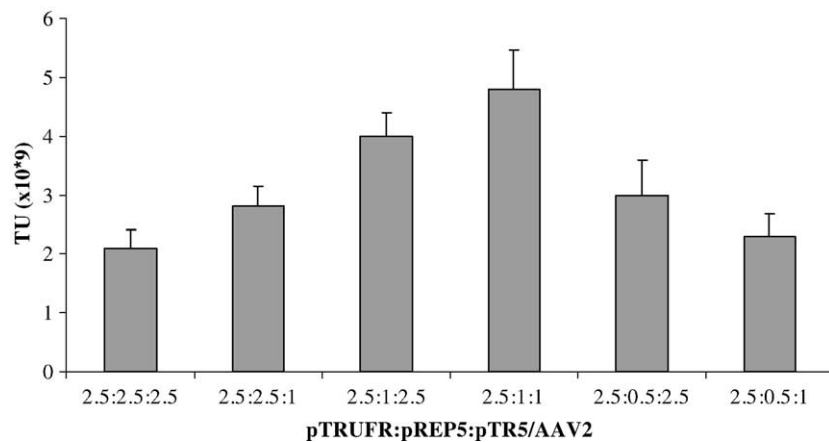

Fig. 7. The effect of optimal ratio of pRep5 plasmid to helper plasmid pTR5/AAV2/INS on vector yield. The data represented the average value ± standard error of three different experiments.
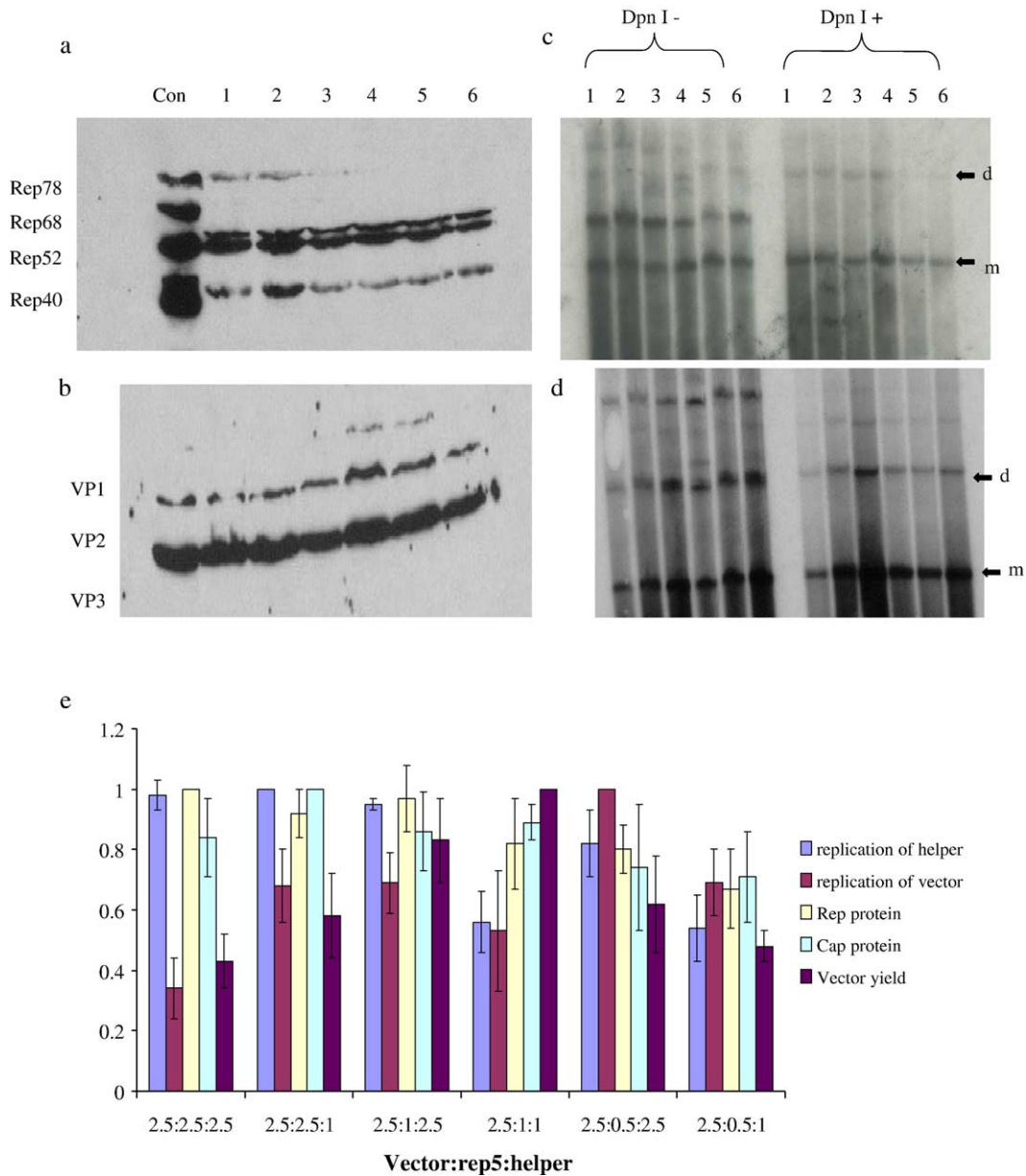
Fig. 8. The expression of helper DNA *rep* and cap and replication of helper DNA and transgene GFP at different ratio of pRep5 and pTR5/AAV2/INS. Panel a, the expression of Rep proteins; Panel b, the expression of cap proteins; Panel c, helper DNA replication; Panel d, the transgene replication. Panel e, plot of the relationship between virus yield and rep/cap protein production or the replication of helper gene/vector gene. Con: Control with pSSV9. 1: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 2.5 μg:2.5 μg; 2: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 1 μg:2.5 μg. 3: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 0.5 μg:2.5 μg. 4: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 2.5 μg:1 μg. 5: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 1 μg:1 μg. 6: the ratio of pRep5 to pTR5/AAV2/INS for transfection is 0.5 μg:1 μg.

amounts of helper plasmid or pRep5 DNA input (Fig. 8, panel e). The highest amounts of pRep5 and pAAV2/AAV2/INS DNA input initiated the lowest replication of transgene EGFP (Fig. 8, panel d, lane 1, panel e). The expression of *rep* and *cap* was also examined. Rep2 and Cap2 expression was enhanced with higher levels of pRep5 DNA input, which seems to be positively associated with virus vector yield if the inhibition effect of Rep proteins (rep2 + rep5) for virus production is considered. Taken together, these data may suggest that an optimal balance among the production of Rep/Cap, virus yield, and the potential negative effect of Rep5 can

be achieved by carefully controlling input ratios that subsequently respond to amplification and coordinated expression of AAV2 Rep/Cap and rAAV DNA replication and encapsidation (Fig. 7 and Fig. 8 panel e).

Discussion

We have demonstrated that an AAV2 *rep* and *cap* helper genome can be efficiently amplified when using ITRs from AAV5, with rep5 supplied in trans. Replication of the helper

genes correlated with increased AAV2 Rep and Cap protein production, which in turn acted positively on rAAV2 vector genomes replicating and packaging similarly to a wt AAV infection. Using this system, we observed a 10- to 20-fold increase in vector production when compared to established methods (Xiao et al., 1998). Insertion of an intron into the AAV2 rep gene dramatically decreased the production of rcAAV particles without decreasing the titer of rAAV production. We also demonstrated that the optimal ratio of vector to Rep5 to AAV2 helper plasmids is 2.5:1:1.

Among the 9 serotypes of AAV, AAV5 is unique in that it is the only dependent parvovirus originally isolated from a patient sample instead of from laboratory stocks of adenovirus (Bantel-Schaal and zur Hausen, 1984; Georg-Fries et al., 1984). Similarity between AAV2 rep ORF and the rep sequences from serotypes 1, 3 and 4 suggests that AAV5 rep is more divergent (90% and 67% homologous, respectively) (Chiorini et al., 1997; Gao et al., 2002a, 2004; Muramatsu et al., 1996; Rutledge et al., 1998; Srivastava et al., 1983; Xiao et al., 1999). In addition to divergency at the protein level, the ITR sequence of AAV5 is only 58% identical to AAV2's ITR sequence (Chiorini et al., 1999a, 1999b; Srivastava et al., 1983). Although the rep binding element (RBE) motif repeat of GAGC/T is conserved in the ITRs of AAV5 and AAV2, the downstream terminal resolution site (trs) sequence has only weak homology between the two serotypes and a different spacing between these elements (18 bp and 13 bp, respectively) (Chiorini et al., 1999a, 1999b; Srivastava et al., 1983). In vitro replication experiments determined that trans complementation does not occur at the level of DNA replication between the respective Rep proteins and the divergent ITR sequences, although these proteins will bind to either ITR with similar efficiency (Chiorini et al., 1999a). Using these features, we designed AAV2 helper constructs with type 5 ITR (pTR5/AAV2) that could rescue and replicate only in the presence of AAV5 Rep protein in Ad co-infected cells (Fig. 2). In addition to selected amplification of the AAV2 hybrid helper construct in the presence of Rep5, we observed that the type 2 Rep and Cap protein patterns were similar to that of wtAAV2 infection. Recent studies characterizing high titer packaging cell lines have determined that the integrated rep and cap coding sequences are typically amplified from the host chromosome by an unknown mechanism (Blouin et al., 2004; Chadeuf et al., 2000; Liu et al., 2000). Consistent between our study and those of high titer packaging cell lines is the ability to amplify the AAV helper genes rep and cap. Using the replicating-dependent AAV helper DNA system described in this study, we obtained about 1000–2000 TU/cell. This resulted in a 10- to 20-fold increase over non-replicating helper pTR5/AAV2 or pXX2.

Further characterization of this AAV production system determined that AAV2 DNA can be packaged into AAV5 capsids, although the Rep5 protein is not able to nick TR2 to initiate replication (Chiorini et al., 1999a, 1999b). We also found that AAV5 DNA can be packaged by AAV2 capsids (data not shown). Thus, pTR5/AAV2 can be packaged into AAV2 capsids to make recombinant virus in the replication system. The presence of this kind of recombinant virus was demonstrated when 293 cells were infected with vector preparations from three cycles of virus passaging. TR5/AAV2 genome containing viruses were detected after 3 passages by PCR analysis with one primer on TR5 and another primer on Rep2. Viruses with this type of genome are replication deficient, so more attention should be paid to replication competent virus production through either homologous or non-homologous recombination (Allen et al., 1997; Grimm et al., 1998; Wang et al., 1998). Since pTR5/AAV2 contains the full genome of AAV2, minus the ITRs, and there is 58% homology between ITRs from AAV5 and AAV2, as well as 67% homology between the rep2 and rep5 genes, there is a possibility of homologous recombination to form virus with TR2/AAV2 genome when pTR5/AAV2 is transfected into cells with a transgene cassette containing ITRs from AAV2 (Chiorini et al., 1999b; Srivastava et al., 1983). Several investigations have found that 0.0001%–10% of rcAAV can be produced through the non-homologous recombination between helper and transgene cassettes in the preparations of vectors using traditional methods (Allen et al., 1997; Cao et al., 2000; Wang et al., 1998). In the present study, the existence of rcAAVs was detected by PCR amplification of the low molecular weight DNA from the infection of rAAV vectors and adenovirus. In the PCR system, the forward primer comes from an ITR and the reverse primer is derived from a rep gene sequence. This assay is quite sensitive, and has previously been used to show that as little as one TU can be detected in this way (Cao et al., 2000; Clark et al., 1999; Gao et al., 1998). In our experiment, as a standard control, 10 TU wtAAV2 can be amplified by PCR from three cycles of virus passaging in 293 cells. In addition, approximately 500 copies of pTR5/AAV2 and 5000 copies of pTR5/AAV5 were detectable using this PCR method. This assay requires the rcAAV to possess both functional ITRs and an intact rep gene. All three kinds of rcAAV (TR5/AAV2, TR2/AAV2 and TR5/Rep5cap2) were found in the amplified PCR products following transfection with pTR2-EGFP, pTR5/AAV2 and pRep5, which indicates that the recombination may occur between rep2 and rep5, TR2 and TR5, or AAV2 repcap and vector EGFP.

Some groups have attempted to solve the rcAAV production problem when making rAAV by modifying the TR2 or rep2 genome or by splitting rep and cap into different constructs. Wang et al. reported that the 10 nucleotides in the AAV D sequence distal to viral hairpin structures are involved in recombination events leading to rcAAV generation. They further suggest that the first 10 nucleotides in the D sequence proximal to the AAV hairpin structures are essential for successful replication and encapsidation of the viral genome (Wang et al., 1998). Following from those conclusions, the combined use of

rAAV plasmids lacking the distal 10 nucleotides in the D sequence and helper plasmids lacking the adenovirus ITRs led to complete elimination of replication-competent wtAAV-like particles in recombinant vector stocks (Wang et al., 1998). This contribution is significant with respect to eliminating rcAAV; however, there is concern that this alteration may influence the level of vector replication and efficiency of vector packaging. A second approach developed to control rcAAV production uses heterologous promoters for driving the *rep* and the *cap* genes (Allen et al., 1997; Flotte et al., 1995). However, with this method, rcAAV particles can still be detected in large-scale preparations (Allen et al., 1997; Flotte et al., 1995). Splitting the *rep* gene and the *cap* gene into different vectors is yet another approach to reducing rcAAV production. Establishing AAV *rep* and *cap* cell lines may significantly reduce the generation of rcAAV (Gao et al., 1998). This logic is based on the fact that considerably lower copies of integrated helper genome are available for recombination. Generally, these approaches, while successful in reducing rcAAV, also resulted in lower titer of rAAV. New improvements in packaging cell lines have suggested that this approach is clearly viable for high titer, scalable production. One advantage of the system described in this study is the fact that many labs rely on a transient vector production system as the means to define the final vector cassette to use in establishing a packaging cell line. We feel this replicating helper system enhances the ease of establishing optimized AAV vectors for preclinical studies. Recently, Cao et al. (2000) explored a new approach to reduce the generation of rcAAV particles using intron-mediated mutagenesis of the AAV genome. The additional intron in the AAV coding region sufficiently increased the AAV genome to a size beyond the packaging capacity and effectively reduced the potential of generating rcAAV particles (Cao et al., 2000; Dong et al., 1996). No effect on the efficiency of the helper plasmid to support rAAV replication and packaging was observed in these experiments. We used the same approach by inserting the 850 bp β-globulin intron with 1.5 kb lambda DNA fragment into the *rep* gene of pTR5/AAV2 at position 654 to construct pTR5/AAV2/INS. A primary difference was the ability to replicate the pTR5/AAV2/INS helper construct in trans using pRep5 expression. The transgene of pTR5/AAV2/INS effectively replicated after cotransfection with pRep5 in the presence of adenoviruses. However, the expression of rep78 and rep68 was decreased, consistent with the finding of Cao et al. (2000) who suggested that the 1.5-kb lambda DNA inserted into the *rep* gene inadvertently disrupted the efficacy of the intron. Previous studies have suggested that a reduction of rep78/68 protein expression is associated with an increase of vector yield (Li et al., 1997). In our experiments, pTR5/AAV2/INS can induce yields of vectors similar to pTR5/AAV2 helper. The primary advantage of the INS modified helper is the dramatic reduction in rcAAVs. However, we did observe cross packaging of hybrid-virions that were replication-

deficient (TR5/AAV2). It is not clear how to classify these putative rcAAV since they are not a product of recombination and do not have ability to propagate in the vector preparation.

With traditional plasmid transfection method, only 10^3 to 10^4 rAAV particles are generated per cell (Samulski et al., 1999). Recently, Chadeuf et al. demonstrated that 10- to 100-fold more particles per cell could be produced with the stable HeLa *rep-cap* cell line since the integrated *rep-cap* genes were rapidly amplified (Blouin et al., 2004; Chadeuf et al., 2000). However, the amplification of the *rep-cap* genes is not controllable in the cell lines, and there is potential risk of helper virus contamination, such adenovirus and herpes. In this system, the *rep* and *cap* expression can be regulated by input of pRep5 (Fig. 8e). With the modulation of the ratio of helper plasmid to pRep5, the virus yield can be further increased. In these experiments, the vector yield is associated with *rep2* and *cap2* expression and is not greatly influenced by helper plasmid replication and/or vector replication when judged solely by final viral yield (Fig. 8e). Although we did observe that the helper *rep* and *cap* gene replication is dependent on the total input of pRep5 and helper plasmid (Fig. 8e). However, the optimum vector replication did not appear to be related with the expression of Rep2 protein, replication of helper templates and/or input of pRep5 (Fig. 8e). All of these observations strongly suggest that the intricate relationship between trans and cis-acting elements remains to be defined.

In conclusion, high-titer rAAV vectors generated by replication of helper *rep* and *cap* genes that mimic the production of wtAAV as described represents a novel method for generating sufficient rAAV for preclinical and small Phase I clinical trials. Increasing the size of the replicating helper plasmid can dramatically decrease the generation of rcAAV. The primary objective of this study was to generate AAV Rep/Cap helper constructs that mimic wtAAV protein levels during a productive infection. While we have begun to approach this objective, room for further improvement still exists. More importantly, using this approach, we have begun to determine some of the critical relationships between Rep/Cap protein levels, levels of replicating viral templates and the efficiency of virion production.

Materials and methods

Plasmids

The plasmid XX2 is a previously published AAV packaging plasmid containing the entire AAV coding sequences with an ATG-to-ACG mutation in the start codon of rep 78/68, reducing Rep78/68 protein synthesis (Xiao et al., 1998). Its molecular size is 8.2 kb. pTR5/AAV2 was constructed by inserting the entire AAV2 coding sequence from an *Xba*I digestion of pSub201 between the two ITRs

of AAV2 in the plasmid p7D05. pTR5/AAV2/INS was constructed by inserting the *xba*1 digested fragment containing the insertion of 850 bp human β-globin intron and 1.5 kb lambda DNA fragment at position 654 of AAV genome from pCLR1–1.5 kb between the two ITRs of AAV5 (Fig. 2) (Cao et al., 2000). pTR5/AAV5 was constructed by inserting the AAV5 *rep* and *cap* genes from pAAV5-2 between two ITRs of AAV5. pAAV5-2 was digested with *Pvu*I and *Msc*I to delete the *cap* gene, blunted and ligated to clone pRep5 in which rep5 gene is driven by native AAV5 p5 promoter. pAAV5-2 and p7D05 were kindly provided by Dr. Kotin (NIH), pCLR1-1.5 was a generous gift from Dr. Xiao (University of Pennsylvania).

Viruses and cells

rAAV was made by calcium phosphate cotransfection methods as described previously (Xiao et al., 1996). 1×10^6 human 293 cells were seeded on 10 cm plate 24 h before transfection in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) with streptomycin and penicillin. The cells were refreshed with 10 ml DMEM containing 10% FBS without antibiotics 1–2 h before transfection. Plasmids were dissolved in 0.5 ml of 0.25 M $CaCl_2$ and then quickly mixed with 0.5 ml of 2× HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM $NaHPO_4$ [pH 7.07]). The DNA precipitate was slowly added to the cells. Meanwhile the cells were infected with Ad5 (dl309) at a multiplicity of infection (MOI) of 2. Forty-eight hours after transfection, the cells were collected and divided into three aliquots. One aliquot of cells and media was subjected to three cycles of freeze–thaw and briefly centrifuged to remove cell debris, heated at 56 °C for 30 min to inactivate the Ad and stored at −20 °C before use. The other aliquots of cells were used for Hirt DNA extraction and Western blot of Rep and Cap proteins.

Assay of DNA replication

DNA was recovered from transfected cells by Hirt extraction with slight modifications (Hirt, 1967). The cell pellet from 1/3 of a 10-cm plate 293 cells was resuspended in 728 μl of suspension buffer (20 mM Tris–HCl, 20 mM EDTA[pH 8.0]) and lysed by adding 46 μl of 10% sodium dodecyl sulfate (SDS). The cell lysate was mixed with 218 μl of 5 M NaCl, placed overnight at 4 °C, and centrifuged at 15 000 rpm at 4 °C for 30 min. The supernatant was harvested and extracted with phenol/chloroform, and chloroform. Low-molecular weight DNA was precipitated by the addition of an equal volume of isopropranol, rinsed with 70% ethanol, and redissolved in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA [pH 8.0]) containing 100 μg/ml of DNase-free RNase. The DNA was digested with *Dpn*I (New England Biolabs) and separated on a 0.8% agarose gel. DNA was transferred to a nylon membrane. Southern blot analysis was performed with a ^{32}p-labeled

probe by using a random primer kit (Boehringer Mannheim Biochemicals) as recommended by the manufacturer.

Western analyses of AAV Rep and Cap proteins

Western blots of AAV proteins were carried out by previously published methods with modifications (Hunter and Samulski, 1992). The cell pellet from 1/2 of a 10-cm plate was lysed in 250 μl of RIPA buffer (10 mM Tris–HCl [pH 8.2], 1% Triton X-100, 1% SDS, 150 mM NaCl). The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) and transferred to a nitrocellulose membrane. After blocking with 10% nonfat dry milk in TBS buffer (50 mM Tris–HCl [pH 7.5], 200 mM NaCl) overnight at 4 °C, the membranes were incubated at room temperature for 1 h with primary antibodies in TBS containing 0.5% Tween-20. The primary antibody for Rep is a mouse monoclonal antibody (1F11) that recognizes all four Rep proteins; which is used at a 1:20 dilution. The primary antibody for Cap proteins is B1 which recognizes all three Cap proteins at a dilution of 1:100. Following primary antibody incubation and washing, the membranes were incubated with the secondary antibody, a goat anti-mouse antibody conjugated with peroxidase (Sigma) at a 1:2000 dilution, at room temperature for 1 h. All antibodies were diluted in TBS buffer with 2% dry milk. After washing, an enhanced chemiluminescence kit (Amersham) was used for detection. In order to directly compare the relationship between Rep and Cap protein expression, the same membrane was used for both primary antibody hybridization with the primary antibody of 1F11 or B1 after stripping the membrane with 0.2 M NaOH.

rcAAV assay and wtAAV titer determination

The infectious rcAAV or wt AAV was measured with infection amplification assay. One ml of the cell lysate of rAAV preparations was used to infect 293 cells of 10 cm plate in the presence of adenovirus infection at the MOI of 5. The cells were harvested 48 h after infection and subjected to 3 freeze–thaw cycles. Ad was inactivated by heat treatment at 56 °C for 30 min. 1 ml cell lysate was used to infect 293 cells with adenovirus for the second cycle of amplification. Infection was repeated for a total of three cycles and after each cycle of infection low molecular weight DNA was extracted using the Hirt method. The rcAAV was qualitatively determined by PCR analysis of Hirt DNA. Three pairs of primers were used for this analysis (Fig. 1b). The first pair of primers, primer a from AAV2 ITR and prime b from AAV2 Rep were used to examine the wt AAV2-like particles. The second pair, primer c from AAV5 ITR and primer b were used to detect the hybrid TR5/AAV2 virion production. The third pair, primer c and primer d from AAV5 Rep were used to analyze the existence of wtAAV5-like viruses. In the control experiment, wt AAV2 viruses were used to infect 293 cells of 10 cm plate at different

transducing units (1, 10, 100, 1000) and co-infected with adenoviruses dl309 at MOI of 5. Three cycles later, Hirt DNA was extracted and PCR analysis was used to determine the threshold of PCR sensitivity. The PCR primer positions are indicated in Fig. 1b and sequences are shown as following: a: AGAGAGGGAGTGGCCAACTCC; b: ATTCTCTTTGTTCTGCTCCTGCG; c: AGAGTGCCACACTCTCAAGCAAGG; d: ACTGCACAAAGAATTTGGACTCCTGC. The sizes of PCR products with primers a/b, c/b and c/d are 830 bp, 813 bp and 420 bp, respectively.

rAAV titer determination

The rAAV infectious titer was determined using green fluorescent protein (GFP) as the reporter gene. Serial dilutions of the rAAV preparations were used to infect 293 cells in the presence of adenovirus at an MOI of 5 for 24–48 h. Each dilution was performed in triplicate. The average number of cells expressing GFP at the highest dilution was accounted as the final titer.

Blot density assay

For Western blot and replication assay, the density of Rep52, Cap VP3 and monomer in different samples were measured with Personal densitometer SI (Molecular Dynamics, Inc. Sunnyvale, CA). The value of samples was calculated by comparison with the highest density in the same panel.

Acknowledgments

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