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Role for Highly Regulated *rep* Gene Expression in Adeno-Associated Virus Vector Production

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Recent success achieving long-term in vivo gene transfer without a significant immune response by using adeno-associated virus (AAV) vectors (X. Xiao, J. Li, and R. J. Samulski, J. Virol. 70:8098–8108, 1996) has encouraged further development of this vector for human gene therapy. Currently, studies focus on the generation of high-titer vectors by using the two-plasmid helper-vector system in adenovirus (Ad)-infected cells. To examine the effects of the AAV replication (*rep*) genes on recombinant AAV (rAAV) vector production, we have constructed a series of AAV helper plasmids that contain strong heterologous promoters in place of the endogenous p5 promoter. Although high-level *rep* gene expression was achieved, rAAV DNA failed to replicate in the absence of Ad infection. Moreover, unregulated overexpression of Rep78/68 led to substantially lower rAAV yields in the presence of Ad (10^{4-5} versus 10^{7-8}). In contrast, under similar conditions, reduced Rep78/68 expression resulted in much higher rAAV yields (10^9). Molecular characterization showed that overexpression of the *rep* gene decreased rAAV DNA replication and severely inhibited capsid (*cap*) gene expression. Interestingly, a reduced *rep* level enhanced *cap* gene expression and supported normal rAAV DNA replication. These studies suggest a critical role for regulated *rep* gene expression in rAAV production and have facilitated the development of a new AAV helper plasmid that increases vector production eightfold over currently used constructs.

Recombinant adeno-associated virus (rAAV) has many features of interest in the field of gene therapy (6, 14, 34, 50). The vector is based on a defective, nonpathogenic human parvovirus that can infect both dividing and nondividing cells without a marked tropism (12, 22, 32, 37, 52). In addition, the viral genome can stably integrate into a specific location within the host genome, facilitating long-term gene transfer (22, 23, 32, 37, 52). The production of rAAV utilizes a vector containing a transgene cassette flanked by the 145-bp inverted terminal repeats (ITR), which are the sole AAV *cis* sequences required for DNA replication, packaging, and integration (34, 40, 53). To produce rAAV particles, the AAV replication (*rep*) and capsid (*cap*) gene products are provided in *trans* from a different template, usually a helper plasmid (6, 14, 34, 40). The three viral coat proteins, VP1, VP2 and VP3, which are required for virion assembly, are derived from mRNA initiated at the p40 promoter, while the four overlapping nonstructural Rep proteins are essential for AAV DNA replication (3, 4, 6, 14, 34). Rep78 and Rep68 are expressed from unspliced and spliced transcripts, respectively, initiating at the p5 promoter, while Rep52 and Rep40 are similarly produced from transcripts initiating at the p19 promoter (3, 4, 6, 14, 34). Although Rep52/40 has been implicated in AAV single-stranded DNA formation (8) and gene regulation (2, 36), Rep78/68 appears to display all enzyme functions essential for AAV DNA replication (ITR binding, DNA helicase, and DNA site-specific nicking activity) (33). In addition to these functions, Rep78/68 both positively and negatively regulates AAV promoters (27, 36, 45) and represses numerous heterologous promoters (1, 17, 18, 20, 26).

rep gene expression appears to be critical for all steps of the AAV life cycle, including a latent state which occurs in the absence of a helper virus (3, 4, 40). Recently, Rep78/68 has also been associated with AAV site-specific integration (15, 24, 43, 48, 49). Repression of viral gene expression by Rep and host YY1 protein appears to be required for the establishment and maintenance of the latent state (27, 28, 36, 44). Such repression may be necessary to avoid the demonstrated cytostatic effect on the host cell by *rep* gene products (55). During a lytic infection, the AAV promoters, particularly p5, are transactivated by the adenovirus (Ad) E1A proteins and YY1 (29, 44). The p5 products positively regulate the p19 and p40 promoters, resulting in abundant production of Rep52/40 and viral capsid proteins (36). Early efforts to bypass AAV *rep* gene regulation by replacing the p5 promoter with the simian virus 40 (SV40) early promoter failed (25). Instead of constitutive Rep78/68 expression, the heterologous promoter unexpectedly behaved in the same manner as the endogenous p5 promoter: repressed in the absence and activated in the presence of Ad (25). While these studies were the first to suggest *rep* repression as a mechanism for regulating heterologous promoters, these findings also implied that AAV p5 products may be a rate-limiting factor in AAV production (25). Further efforts in this area have suggested that overexpression of Rep78/68 may increase rAAV vector yields (13).

Central to testing rAAV as an efficient delivery system is vector production. Although rAAV titers can approach wild-type (wt) levels after rounds of purification and concentration, the overall total yield is still substantially lower than that of wt AAV. To improve the current AAV packaging system and to elucidate the molecular mechanisms of the Rep proteins on rAAV production, we have constructed a series of AAV helper plasmids containing various strong heterologous promoters substituted for the AAV p5 promoter. When transfected into human 293 cells, these plasmids express high levels of Rep protein irrespective of Ad infection. Further characterization demonstrated that although Rep protein was expressed, rAAV DNA was unable to replicate in these cells in the absence of

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Ad coinfection. In addition, when these new constructs were compared to the helper plasmid pAAV/Ad, which contains an endogenous p5 promoter, the rAAV yields were much lower for the new helper plasmids in Ad-coinfected cells. Molecular analysis revealed less efficient rAAV DNA replication and lower capsid protein synthesis from these new constructs. Interestingly, a novel construct which reduced only Rep 78/68 expression resulted in an eightfold increase in rAAV yield. Our findings indicate that unregulated overexpression of Rep proteins adversely affects rAAV production and suggest a role for highly regulated *rep* gene expression in optimal rAAV production.

MATERIALS AND METHODS

AAV helper plasmid construction. Various AAV helper plasmids were constructed by standard methods (39). AAV/Ad is a previously published AAV packaging plasmid containing the entire AAV coding sequences including promoter p5 and has a molecular size of ca. 8.2 kb (40). Plasmid CMV/AAV also 8.2 kb (a kind gift from X. Zhou and N. Muzyczka, University of Florida), contains the entire AAV coding sequence, except that the AAV p5 promoter was substituted by a cytomegalovirus (CMV) immediate-early promoter (36). Plasmid pSV/AAV is a construct similar to pCMV/AAV except that an SV40 late promoter was substituted for the AAV p5 promoter. This plasmid is 7.8 kb. Plasmid HIV/AAV also contains the entire AAV coding sequences, except that the AAV p5 promoter was substituted by a human immunodeficiency virus (HIV) long terminal repeat promoter. The construct was made by three-fragment ligation. The first fragment was the *Ssp*I-*Hin*dIII fragment from pHIV-Rep (1), containing the complete HIV promoter and a portion of the *rep* gene. The second fragment was the *Hin*dIII-*Sna*BI fragment from psub201 (42), containing the rest of the *rep* gene and the entire *cap* gene along with the polyadenylation site. The third fragment was the *Ssp*I-*Sma*I fragment from Bluescript KS(+) (Stratagene), containing the plasmid origin and Amp^r gene. This construct is 7.6 kb. Plasmid ACG-2 is a variant of AAV/Ad containing an ATG-to-ACG mutation in the start codon of Rep78/68 (51), which reduces Rep78/68 protein synthesis, and is identical in size to the parental plasmid pAAV/Ad (8.2 kb). All the constructs were characterized by restriction analysis, and some were characterized by sequencing. The rAAV vector plasmid pdx31-LacZ was published previously (32). All plasmids were purified by double CsCl density gradient ultracentrifugation for transfection experiments (39).

Viruses and cells. rAAV vector was generated by calcium phosphate cotransfection methods as described previously (52). Briefly, human 293 cells were passed 1 day before transfection in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) with streptomycin and penicillin. At about 80% confluence, the cells were fed with 10 ml fresh Iscove's modified Dulbecco's medium (Gibco) containing 10% FBS without antibiotics 1 to 2 h before transfection. Plasmid DNA (25 μg) (vector plus helper at various ratios) was dissolved in 1 ml of 0.25 M $CaCl_2$ and then quickly mixed with 1 ml of 2× HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4 [pH 7.12]). The DNA complex was slowly added to the cells. After incubation for 8 h, the cells were fed with fresh Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS and antibiotics and infected with Ad5 (*dl*309) at a multiplicity of infection (MOI) of 2. Transfection efficiencies were monitored by staining a duplicate plate for β-galactosidase gene expression and counting the number of blue cells. At 2 days after Ad infection, the cells and media were collected and 0.1 ml of 1 M Tris-Cl (pH 8.5) was added to adjust the pH. Following four freeze-thaw cycles and removal of cell debris by centrifugation, the rAAV lysate was heated at 56°C for 30 min to inactivate the Ad and stored at −20°C before use.

The titers of AAV-LacZ were determined by counting the blue cells after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining following coinfection of 293 cells with various dilutions of the rAAV stocks and Ad *dl*309 at an MOI of 1 for 24 h. All experiments were done in triplicate to ensure reproducibility, and the average was taken for the final titer.

Assay of rAAV DNA replication. The rAAV DNA was recovered from the transfected cells by Hirt extraction (19) with slight modifications. Briefly, the cell pellet from 1/10 to 1/5 of a 10-cm dish was resuspended in 270 μl of 20 mM Tris-Cl–20 mM EDTA (pH 8.0) and lysed by addition of 30 μl of 10% sodium dodecyl sulfate (SDS). The cell lysate was incubated at 37°C for 1 h with 50 μg of proteinase K per ml and mixed with 80 μl of 5 M NaCl. After incubation on ice for more than 1 h, the cell lysate was centrifuged at 15,000 rpm at 4°C for 30 min in a Sorvall SS34 rotor. The supernatant was recovered and subsequently extracted with phenol, phenol-chloroform, and chloroform. Low-molecular-weight DNA was precipitated with an equal volume of isopropanol, rinsed with 70% ethanol, and redissolved in 50 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) containing 100 μg of DNase-free RNase per ml. The DNA was digested with *Dpn*I (New England BioLabs) and separated on a 1% agarose gel. DNA was blotted to a nylon membrane (GeneScreen Plus; DuPont). Southern analysis was performed with a *lacZ* DNA probe (a 2.1-kb *Cla*I/*Nde*I fragment) ^{32}P-labeled with 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 (21) with modifications. Briefly, the cell pellet from half of a 10-cm dish was lysed in 250 μl of RIPA buffer (10 mM Tris-Cl [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 being subjected to blocking in 10% nonfat dry milk in TBS buffer (50 mM Tris-Cl [pH 7.5], 200 mM NaCl) for 1 h, 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 monoclonal antibody (1F11) that recognizes all four Rep proteins; it was used at a 1:20 dilution (21). The primary antibody for Cap proteins is a guinea pig polyclonal antibody against AAV-2 (Braton Biotech, Inc.); it was used at a dilution of 1:400. Following primary antibody incubation and rinses, the membranes were incubated with the secondary antibodies at room temperature for 1 h. The secondary antibody for Rep is a goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma) at a 1:5,000 dilution. The secondary antibody for Cap is a rabbit anti-guinea pig conjugated with horseradish peroxidase; it was used at a 1:3,000 dilution. All the antibodies were diluted with 2% dry milk in TBS buffer. After three washes with TBS buffer containing 0.5% Tween 20 and one wash with TBS, the specific protein bands were visualized with chemiluminescence reagent (DuPont) and exposed to X-ray film.

RESULTS

Efficient *rep* gene expression from heterologous promoters in the absence of Ad. To achieve high-level *rep* gene expression in an Ad-independent manner, we have constructed a number of AAV helper plasmids containing heterologous promoters substituted for the AAV p5 sequence. The heterologous promoters included the CMV immediate-early region, the HIV long terminal repeat, and the SV40 late promoter (Fig. 1). These sequences are among the strongest constitutive viral promoters commonly used and should express high levels of Rep78/68. A helper plasmid, pAAV/Ad (40), which retains the endogenous p5 promoter, was included in this study as a control. To determine the effect of low-level Rep expression on rAAV production, a novel plasmid, pACG-2 (50), was also constructed. This construct is identical to pAAV/Ad, except that a point mutation has converted the start codon of Rep78/68 from ATG into a less efficient ACG codon. AAV utilizes the ACG start codon for AAV Vp2 capsid production, which is expressed at low levels from the same mRNA that encodes the major capsid protein Vp3. Since this mechanism of regulation is used by AAV, it was expected that this point mutant would reduce Rep78/68 protein synthesis without altering AAV mRNA levels. The various AAV helper plasmids which carried specific promoter elements in place of the AAV p5 promoter differed at most by only 600 bp (pHIV/AAV, 7.6 kb) when compared to the parental plasmid, pAAV/Ad (8.2 kb).

Repression of AAV p5 (27, 28, 36) and heterologous promoters such as SV40 (26), HIV (1, 20), and CMV (17) by *rep* in the absence of Ad infection was overcome by using human 293 cells. E1A gene products have been shown to transactivate AAV p5 (44), CMV (16), and HIV (38) promoters. E1A gene products present in 293 cells will counteract the repression by AAV Rep proteins and thereby increase *rep* gene expression from these various promoters in the absence of Ad infection. The various AAV constructs were transfected into 293 cells with or without Ad infection and assayed for *rep* gene expression 48 h posttransfection by Western blot analysis with anti-Rep monoclonal antibody (21). From the experimental results shown in Fig. 2, several observations can be made. First, in the absence of Ad in 293 cells, Rep gene expression was detected from all five AAV constructs (Fig. 2, lanes 1 to 5). Plasmids pCMV/AAV and pHIV/AAV expressed extremely high levels of Rep78/68, whereas pSV/AAV and pAAV/Ad demonstrated more modest expression (compare lanes 3 and 4 with lanes 1

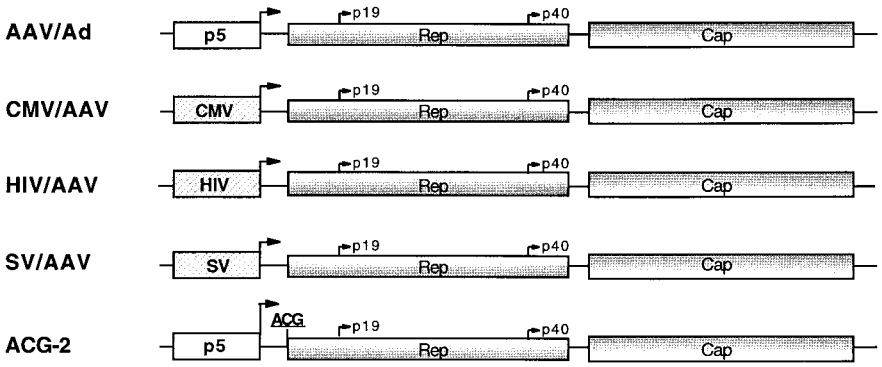

FIG. 1. Construction of AAV helper plasmids. Plasmids AAV/Ad and ACG-2 contain the endogenous p5 promoter (open boxes), while plasmids CMV/AAV, HIV/AAV, and SV/AAV contain the heterologous promoters (hatched boxes) replacing the original p5 promoter. All the constructs contain the same AAV coding sequences, i.e., the *rep* and *cap* genes (shaded boxes), except that construct ACG-2 has an ATC-to-ACG mutation at the translation initiation codon of Rep78/68 (see Materials and Methods for details).

and 5). The lower expression from the SV40 promoter in 293 cells is most probably due to SV40 enhancer repression by E1A proteins as previously described (16, 47). As expected, plasmid pACG-2 generated the smallest amount of Rep78/68 (lane 2). Second, in the presence of Ad and as expected from previously published work (44), the levels of Rep78/68 appeared to be the same as those of the minus-Ad extracts for pCMV/AAV, pHIV/AAV and pSV/AAV (compare lanes 3 to 5 with lanes 8 to 10). However, for pAAV/Ad and pACG-2, the Rep78/68 levels appeared to be slightly lower after Ad infection, implying further modulation of AAV gene regulation by Ad coinfection. Third, although Rep 52/40 expression was not altered after Ad infection, the ratio of Rep78/68 over Rep52/40 was significantly different among the various constructs (Fig. 2). The helper plasmids with strong heterologous promoters, such as pCMV/AAV and pHIV/AAV, demonstrated obvious abnormal Rep78/68-to-Rep52/40 ratios (Fig. 2, lanes 3 to 5 and 8 to 10), while pAAV/Ad expressed p5 and p19 products at roughly a 1:1 (lanes 1 and 6). For pACG-2, the levels were less than 1 (lanes 2 and 7), suggesting that p19 products (Rep52/40) were made at higher levels than were p5 products (Rep78/68).

Expression of *rep* gene was not sufficient to replicate rAAV DNA. Since we demonstrated that high levels of Rep protein can be expressed independent of Ad infection, we next assayed for rAAV DNA replication under these conditions. Plasmids pCMV and pHIV, which demonstrated the highest expression of Rep78/68, were tested in cotransfection experiments with a rAAV-LacZ vector plasmid as a replication substrate (32). As a control, the same constructs were assayed for rAAV-LacZ replication in the presence of Ad infection.

At 48 h posttransfection, low-molecular-weight DNA was recovered and analyzed by Southern blotting. To distinguish between input plasmid and newly replicated DNA, samples were treated with *Dpn*I endonuclease or left untreated. An autoradiograph of the experiment is shown in Fig. 3. Resistance to *Dpn*I digestion suggested that in the presence of Ad infection, Rep proteins from pCMV/AAV and pHIV/AAV successfully replicated the rAAV-LacZ vector DNA (Fig. 3, lanes 3 and 4 [pCMV/AAV] and lanes 7 and 8 [pHIV/AAV]). However, sensitivity to *Dpn*I digestion in the absence of Ad infection (lanes 1 and 2 [pCMV/AAV] and lanes 5 and 6 [pHIV/AAV]) suggested that rAAV vector DNA failed to replicate even though abundant Rep proteins were produced from these helper constructs (Fig. 2, lanes 3 and 4). Similarly, cotransfection of the rAAV-LacZ vector plasmid with other helper plasmids, such as pAAV/Ad and pSV/AAV, in the absence of Ad infection also failed to replicate the vector DNA (data not shown). These results support the conclusion that constitutive Rep gene expression is not sufficient to mediate AAV DNA replication and that other Ad helper functions,

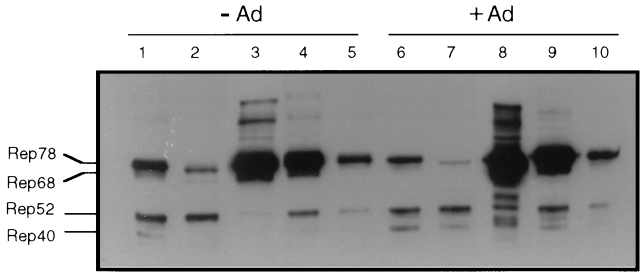

FIG. 2. Western blot analysis of *rep* gene expression from various AAV helper plasmids. 293 cells were transfected with plasmid AAV/Ad (lanes 1 and 6), ACG-2 (lanes 2 and 7), CMV/AAV (lanes 3 and 8), HIV/AAV (lanes 4 and 9), and SV/AAV (lanes 5 and 10) in the absence or presence of Ad infection. Samples of cell lysates were separated by PAGE (10% polyacrylamide). Western blot analysis was performed with an anti-Rep monoclonal antibody, which recognizes all four Rep proteins (21).

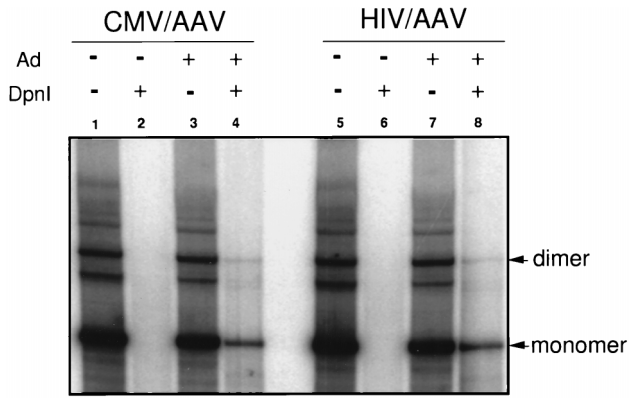

FIG. 3. Southern blot analysis of rAAV DNA replication. AAV vector plasmid pdx31-LacZ was cotransfected into 293 cells with helper plasmids CMV/AAV or HIV/AAV in the absence or presence of Ad infection. Low-molecular-weight DNA was recovered from the cells and separated on 1% agarose gel without or with prior *Dpn*I digestion. Southern blot analysis was performed with a ^{32}P-labeled *lacZ* probe (a 2.1-kb *Cla*I-*Nde*I fragment).

TABLE 1. Comparison of rAAV titers by different helper plasmids

Helper plasmid	Vector/helper ratio	rAAV titer	
		TU/plate[a]	TU/cell[b]
CMV/AAV	3:1	2.0×10^5	0.04
	1:1	8.5×10^4	0.02
	1:3	8.5×10^4	0.02
HIV/AAV	3:1	1.4×10^5	0.03
	1:1	8.5×10^4	0.02
	1:3	1.2×10^5	0.02
SV/AAV	3:1	8.3×10^6	1.6
	1:1	6.0×10^6	1.2
	1:3	5.5×10^6	1.1
AAV/Ad	3:1	8.5×10^7	17
	1:1	1.4×10^8	28
	1:3	1.0×10^8	20
ACG-2	3:1	5.0×10^8	100
	1:1	1.1×10^9	220
	1:3	8.0×10^8	160

[a] The rAAV-LacZ yields were mean values from three experiments performed with a 10-cm plate of human 293 cells. The transducing units (TU) were determined by infecting 293 cells with Ad at an MOI of 1 and various dilutions of heat-inactivated rAAV virus stocks. After X-Gal staining, each blue cell was translated into 1 TU.

[b] The number of transducing units (TU) produced per cell was obtained by dividing the titers (total TU) from each 10-cm plate by the total number of 293 cells (approximately 5×10^6).

besides the constitutive expression of E1 in 293 cells, are required.

Overexpression of the *rep* gene inhibits rAAV titers. The above experiments demonstrated that the new helper constructs can obtain high levels of Rep proteins, which are functional for AAV replication only in the presence of Ad coinfection. To further characterize these helper constructs for rAAV production, the yield of vector particles generated after transfection experiments was measured. Previously, we observed that different transfection methods, such as liposome treatment or calcium phosphate coprecipitation, require various vector-to-helper-plasmid ratios (30). For example, a 1:1 ratio of rAAV vector to helper plasmid (pAAV/Ad) resulted in optimal rAAV yield when we used the calcium phosphate transfection method with 293 cells (52). The calcium phosphate method was used to measure the efficiency of the new helper plasmids in this experiment. Three different vector-to-helper ratios (3:1, 1:1, and 1:3), covering a ninefold range, were tested. At 8 h posttransfection, the medium was changed and the cells were infected with Ad5 *dl*309 and incubated for additional 48 to 60 h until the full cytopathic effect was observed. Since approximately 10 to 30% of the rAAV viruses generated are released in the culture medium, the cells were harvested together with the medium before the particle number was assayed for. After four freeze-thaw cycles and removal of cell debris, the lysates were heated at 56°C for 30 min to inactivate any residual Ad and subjected to titer determination on 293 cells by staining for β-gal activity (see Materials and Methods).

As shown in Table 1, helper plasmids pCMV/AAV and pHIV/AAV generated the lowest rAAV yields, even though these constructs produced the highest Rep78/68 levels. Plasmid pSV/AAV resulted in rAAV yields higher than those from pCMV/AAV and pHIV/AAV but significantly lower than those from pAAV/Ad. This is an interesting observation since both of these plasmids produced similar levels of Rep78/68 (Fig. 2, compare lanes 1 and 6 with lanes 5 and 10). The overall yields of rAAV generated from pCMV/AAV, pHIV/AAV, and pSV/AAV were not dramatically affected by the ninefold range of vector-to-helper ratio (Table 1), suggesting that the rate-limiting factor is not merely the level of Rep protein or the quantity of helper plasmid but some fundamental difference between pAAV/Ad and the other three helper plasmids (presumably *cis* sequences). As possible explanations for the decrease in vector yield, pCMV/AAV and pHIV/AAV overexpression of Rep78/68 may have inhibited other genes, both viral (AAV and/or Ad) and cellular. In addition, the substitution of the p5 promoter may have removed essential *cis*-acting regulatory functions required for appropriate p19 and p40 expression (31, 36).

TABLE 2. Effects of Ad infection time on rAAV yields

Time of Ad infection[a]	rAAV titer (TU)[b] for:	
	AAV/Ad	HIV/AAV
Before	1.7×10^8	1.5×10^7
Same	2.5×10^8	1.7×10^7
After	1.9×10^8	1.8×10^5

[a] Ad infection was carried out at three different time points, 1 h before, simultaneously with, and or 8 h after DNA transfection, for the three individual samples of a given helper plasmid.

[b] The rAAV-LacZ yields were obtained from a 10-cm plate of 293 cells. The transducing units (TU) were determined by infecting 293 cells with Ad at an MOI of 1 and various dilutions of heat-inactivated rAAV virus stocks. After X-Gal staining, each blue cell was translated into 1 TU.

This hypothesis is supported by the results obtained with helper plasmid pACG-2, which produced the highest rAAV titers (Table 1). This construct retained the p5 promoter sequences but reduced Rep78/68 expression through inefficient translational initiation (Fig. 1 and 2, lanes 2 and 7). The average rAAV yield from this plasmid was increased eightfold compared to that from its parental plasmid, AAV/Ad. The results suggested that unregulated overexpression of Rep78/68 may have a negative effect while lower but sufficient Rep78/68 expression can generate higher rAAV yields.

These observations are not in agreement with a previous report (13), where an AAV helper plasmid (pRS5) containing the same HIV long terminal repeat showed a 7.5-fold increase in rAAV virus yield over that from pAAV/Ad. In our experiments, the helper construct pHIV/AAV gave rise to much lower rAAV yields than did pAAV/Ad. One important variable which may explain this difference is the addition of Ad relative to transfection. In our experiments, Ad infection was carried out 8 h after transfection instead of 1 h before as described by Flotte et al. (13). In an attempt to better explain these differences, we tested this possibility by infecting the cells with Ad at 1 h before, simultaneously with, or 8 h after plasmid transfection. The cells were harvested 48 h after the Ad infection, and the rAAV titers were measured. The results shown in Table 2 indicated that when pAAV/Ad was used as the helper plasmid, less-than-twofold differences in rAAV titers were observed when the time of Ad infection was varied (Table 2). However, the addition of Ad prior to pHIV/AAV transfected plates was important (Table 2). rAAV titers were about 100-fold higher (1.5×10^7 to 1.7×10^7 transducing units/10-cm plate) when Ad infection was carried out 1 h before or simultaneously with rather than 8 h after transfection (1.8×10^5 transducing units/10-cm plate) (Table 2). This implies that the presence of Ad gene expression before the onset of AAV Rep gene expression has a significant effect on rAAV production when pHIV/AAV is used as a helper plasmid. Regardless, the

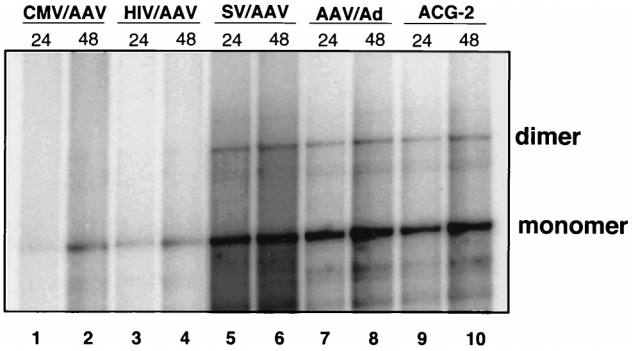

FIG. 4. Comparison of rAAV DNA replication in cells transfected with different helper plasmids. AAV vector plasmid pdx31-LacZ was cotransfected into 293 cells with helper plasmids CMV/AAV, HIV/AAV, SV/AAV, AAV/Ad, and ACG-2 in the presence of Ad infection. Low-molecular-weight DNA was recovered from the cells posttransfection 24 h (lanes 1, 3, 5, 7, and 9) or 48 h (lanes 2, 4, 6, 8, and 10). Southern blot analysis was performed with a ^{32}P-labeled *lacZ* probe (a 2.1-kb *Cla*I-*Nde*I fragment).

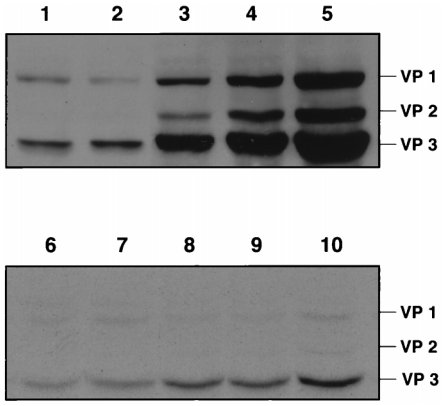

FIG. 5. Western blot analysis of *cap* gene expression from different AAV helper plasmids. AAV vector plasmid pdx31-LacZ was cotransfected into 293 cells with helper plasmids CMV/AAV (lanes 1 and 6), HIV/AAV (lanes 2 and 7), SV/AAV (lanes 3 and 8), AAV/Ad (lanes 4 and 9), and ACG-2 (lanes 5 and 10) in the presence (lanes 1 through 5) or absence (lanes 6 through 10) of Ad infection. Samples of cell lysates were separated by PAGE (10% polyacrylamide). Western blot analysis was performed with an anti-Cap polyclonal antibody which recognizes all three Cap proteins.

best titers of pHIV/AAV were still about 10-fold lower than that of pAAV/Ad. Since pAAV/Ad contains Ad ITRs flanking AAV genes, the Ad ITR sequences may influence AAV gene expression and explain the difference in vector yield that we observed. To examine this possibility, the AAV gene cassette was deleted from pAAV/Ad and replaced by pHIV/AAV. This construct, like pAAV/Ad, now has HIV/AAV sequences flanked by the Ad ITRs. This new variant of pHIV/AAV/Ad did not produce better rAAV yields despite the addition of the Ad ITRs (data not shown). The discrepancy between our results and those of others (13) can be only partially attributed to the difference in the helper plasmids. However, based on the results we observed concerning the time of addition for Ad infection with pHIV/AAV, this may be the rate-limiting factor which influences rAAV yields when strong constitutive promoters such as the HIV long terminal repeat are used. In addition, one may need to consider the numerous variables used during rAAV production, such as different lots of 293 cells, Ad strain, and various transfection and infection methods, to make direct comparisons.

Overexpression of the *rep* gene inhibited rAAV DNA replication and *cap* gene expression. To explore why overexpression of Rep78/68 resulted in a lower yield of rAAV particles, we examined two essential steps, viral DNA replication and capsid protein synthesis. To examine rAAV replication, the rAAV-LacZ plasmid was cotransfected with various AAV helper plasmids and then subjected to Ad infection. At 24 and 48 h posttransfection, low-molecular-weight DNA was isolated and *Dpn*I digested and then subjected to Southern analysis with ^{32}P-labeled *lacZ* probe. The experimental result indicated that rAAV DNA replication was significantly lower when pCMV/AAV (Fig. 4, lanes 1 and 2) and pHIV/AAV (lanes 3 and 4) were used as helper plasmids, compared to pSV/AAV, pAAV/Ad, and pACG-2 (lanes 5 through 10). PhosphorImager quantitation of the monomer bands at the 48-h time point revealed approximately 80% reduction in vector DNA replication when pCMV/AAV and pHIV/AAV were compared to pAAV/Ad. On the other hand, no significant difference between pSV/AAV, pAAV/Ad, and pACG-2 was found (lanes 5 through 10 and data not shown). Interestingly, rAAV DNA replication was not reduced in pACG-2-transfected cells, although the Rep78/68 levels were considerably lower (Fig. 2, lane 7). Since pCMV/AAV and pHIV/AAV expressed the highest levels of Rep78/68 (Fig. 2, lanes 8 and 9), which resulted in the lowest levels of rAAV DNA replication (Fig. 4, lanes 1 through 4), this suggested that Rep78/68 are not rate-limiting factors, but instead may interfere with vector DNA replication. The mechanism of inhibition, however, remains unknown.

Since capsid proteins are essential for particle formation, we also examined the accumulation of these proteins from the various helper plasmids. At 48 h posttransfection, cells were harvested and analyzed by Western blotting with an anticapsid polyclonal antibody. The results are shown in Fig. 5. In the absence of Ad infection, all helper plasmids generated low but detectable levels of capsid proteins, consistent with published observations showing that Ad infection enhances AAV capsid gene expression (34). In the presence of Ad infection, pACG-2-transfected cells synthesized and/or accumulated the highest level of capsid proteins (Fig. 5, lane 5) while pCMV/AAV and pHIV/AAV produced the lowest levels (lanes 1 and 2). From these results, we observed a negative correlation between high Rep78/68 levels (Fig. 2, pCMV/AAV [lane 8] and pHIV/AAV [lane 9]) and efficient capsid gene expression (Fig. 5, pCMV/AAV [lane 1] and pHIV/AAV [lane 2]). For pACG-2, we observed just the reverse; i.e., low *rep* expression (Fig. 2, lane 7) resulted in the highest capsid gene expression (Fig. 5, lane 5). While pSV/AAV replicated rAAV DNA to similar levels to those of pAAV/AD and pACG-2 (Fig. 4, lanes 5 to 10) and expressed AAV capsid gene products at a higher level than did pCMV/AAV and pHIV/AAV (Fig. 5, compare lane 3 to lanes 1 and 2), these capsid gene products had reduced levels compared to those from helper plasmids pAAV/Ad and pACG-2 (Fig. 5, compare lane 3 to lanes 4 and 5). From this analysis, we saw a direct correlation between the amount of Rep protein produced, the level of AAV capsid expression (Fig. 5), and the yield of rAAV (Table 1), with pACG-2 expressing the lowest levels of Rep, the highest levels of capsid protein, and the best yields of rAAV, followed by pAAV/AD, pSV/AAV, pHIV/AAV, and pCMV/AAV.

DISCUSSION

We have demonstrated that high-level Rep78/68 expression can be achieved in an Ad-independent manner by replacing the p5 promoter of AAV with strong heterologous promoters such as those of CMV and HIV. However, only in the presence of

Ad infection do these proteins replicate AAV DNA. In addition, reduced levels of Rep 78/68 appear to be sufficient for normal levels of rAAV DNA replication, and they produce higher-titer vector than does the conventional helper construct pAAV/Ad, possibly by increasing the level of AAV capsids.

Previous work has revealed that AAV uses cellular machinery for DNA replication (3, 33). The only viral proteins required in the process are the AAV p5 products Rep78 and/or Rep68 (33). Elimination of Rep52/40 has minimal effect (8). In our transfection experiments, generation of *rep* gene products did not lead to successful AAV DNA replication in the absence of Ad infection. We did observe partial DNA replication, as illustrated by the presence of trace amounts of *Dpn*I-resistant DNA fragments smaller than the expected monomer size (data not shown). The lack of replication cannot be attributed to cells not cycling, since the cell population in the transfection experiments was rapidly growing and unsynchronized. The failure to detect any full-length replicated AAV DNA suggests, that Ad helper functions besides E1 are required.

The dependence on Ad infection for AAV replication is also in agreement with in vitro replication experiments (35, 47b). In one study, AAV DNA replication required not only purified baculovirus expressed Rep78 or Rep68 but also Ad-infected HeLa cell extract (35). In the other study, full-length linear AAV DNA replication was 50-fold greater in extracts from Ad-infected cells (47b). There are several potential explanations for the inability to replicate AAV DNA in vivo in Ad-uninfected cells. These range from inefficient DNA synthesis initiation on plasmid substrates to a lack of critical cellular factors. However, our in vivo observations suggest that more than 50% of the rAAV plasmid DNA recovered from pCMV/AAV- and pHIV/AAV-transfected cells was converted into the monomer-sized replicative intermediate molecule, suggesting that the first step was not rate limiting (Fig. 2) (53). This result suggests that Rep proteins are functional for resolution of the AAV plasmid but cannot facilitate the next step in viral DNA replication. It may be that in the absence of Ad, Rep proteins mediate the inhibition of DNA replication in these cells (54, 55). Our DNA replication data from different helper plasmids offer indirect evidence supporting this notion. Overexpression of Rep proteins resulted in much reduced AAV DNA replication compared to that for helper plasmids that expressed normal to lower levels of Rep (Fig. 5). In addition, the E4 region of Ad facilitates viral DNA replication and transport of AAV mRNA during productive infection (7, 41). Recently, E4 ORF6 was shown to enhance rAAV vector second-strand DNA synthesis (10, 11) and to inhibit the functions of p53, which is involved in cell cycle regulation (9). These results, in addition to the fact that Ad infection may induce DNA replication activity and create an environment more suitable for AAV replication (3, 33), provide a suitable explanation for the inability to replicate AAV when only Rep gene products are expressed.

One of the primary objects of this study was to test if overexpression of Rep proteins can increase rAAV vector yields. The rationale was based on the notion that Rep gene products in a permissive infection can positively regulate endogenous AAV promoters (27, 28, 36), which, in turn may increase viral production. Although high-level *rep* gene expression was achieved and the Rep proteins were functional in the presence of Ad infection, the rAAV yields showed a negative correlation with the levels of Rep gene expression. For example, plasmids pCMV/AAV and pHIV/AAV expressed the highest level of p5 products and generated the lowest rAAV yields (about 1,000-fold lower than those from pAAV/Ad). pACG-2 expressed the lowest level of p5 products but produced the highest rAAV yield. Southern analysis revealed less efficient vector DNA replication when helper plasmids containing strong heterologous promoters expressing Rep were used. Interestingly, Western blot analysis also showed reduced Cap gene expression when Rep protein expression was high. Reduction of DNA replication and reduced capsid expression appeared to have an additive effect on vector yield (Table 1).

Although the detailed molecular mechanisms behind the effects of *rep* gene expression on rAAV vector production remain to be investigated, several possible explanations can be deduced based on our observations and prior published results. First, as previously described, the Rep protein has pleiotropic effects on cellular as well as AAV gene expression. In the latent state, *rep* gene expression in host cells can cause growth inhibition and cell cycle arrest (55). Since AAV replication relies on cellular enzymes, overexpression of Rep78/68 may inhibit host DNA replication machinery and consequently inhibit AAV synthesis. In addition, it has been shown that Rep proteins can inhibit the AAV p40 promoter in 293 cells in the absence of Ad infection (46). Normally, Rep protein enhances p19 and p40 promoters during a productive infection (36). Inhibition of p40 mRNA at the translation level by Rep proteins in 293 cells has also been previously reported (46). Sufficient Rep to transduce the p19 and p40 promoters but reduced enough that capsid mRNA translation is not effective may be the single underlying reason for why the mutant helper pACG-2 resulted in the highest rAAV titer. All or part of these explanations may be directly related to the results we observed in this study. Second, AAV can inhibit Ad growth during coinfection (5). Overexpressed Rep proteins therefore may reduce the helper functions provided by Ad. This suggestion is indirectly supported by the observation that Ad infection prior to pHIV/AAV plasmid transfection generated much higher rAAV yields. Since pHIV/AAV expresses constitutively high levels of AAV Rep proteins, one would need to preinfect the cells with Ad to obtain efficient Ad gene expression. The increase we observed in rAAV titer when Ad was introduced prior to transfection of this AAV helper plasmid also provides a working explanation for the differences between our observations with the helper construct pHIV/AAV and those of previously published studies with a similar plasmid (13). In this scenario, early Ad infection would provide essential helper functions before Rep inhibition. Since Rep proteins are nonstructural and most of their functions are catalytic and regulatory, excess Rep proteins may not be required for efficient virus production. This hypothesis is supported by results obtained with our ACG-2 mutant helper construct described below.

Finally, substitution of p5 promoters with strong heterologous promoters may abolish the temporal regulation of Rep gene expression. Muzyczka and his colleagues (31, 36) have recently demonstrated that the p5 promoter, as a *cis*-acting element, exerts positive effect on both p19 and p40 but negatively autoregulates its own transcription in the presence of Ad infection. Moreover, Rep78/68 as a *trans* factor, in the presence of Ad, inhibits the p5 promoter and enhances the p19 and p40 promoters (36). Therefore, substitution of the p5 promoter by heterologous sequences may not only alter the transcriptional regulation of Rep78/68 but also abolish the Rep-mediated *cis*-acting functions required for p19 and p40, hence disrupting the delicate balance required for optimal vector production. This hypothesis seems to be best illustrated by the mutant helper plasmid pACG-2 that we constructed. While all the *cis* sequences required to regulate p5, p19, and p40 are still present in this construct, down regulation of Rep 78/68 protein by the use of an inefficient translational initiation start signal

(ACG) has resulted in increased AAV capsid protein expression, which results in higher titers of rAAV vectors (10^9). These results strongly argue that highly regulated Rep protein expression is required to generate optimal levels of rAAV vectors. With wt AAV, this delicate balance is most probably achieved by the ability to increase the level of viral templates through DNA replication as Rep mediates the control of the AAV promoters. In summary, we have generated a new AAV helper plasmid that demonstrates a role for regulating Rep proteins to obtain high-level capsid production which directly correlates with an increase in rAAV titers. This plasmid and variations of this strategy should provide additional reagents which will facilitate the ability to produce high-titer rAAV vectors for use in human gene therapy.

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ADDENDUM

While the manuscript was being revised, a report by Vincent et al. (47a), supporting similar conclusions, was published.

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