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Analysis of Recombinant Adeno-Associated Virus Packaging and Requirements for *rep* and *cap* Gene Products

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Adeno-associated virus (AAV) is a human parvovirus currently being developed as a vector for gene therapy applications. Because the gene transfer vector commonly retains only the AAV terminal repeats, propagation of recombinant AAV (rAAV) requires that the viral replication (Rep) and capsid (Cap) proteins be supplied in *trans*. In an effort to optimize the production of these vectors, a panel of helper plasmids was constructed to determine if expression of the *rep* and/or *cap* genes is a limiting factor for rAAV packaging. Expression of the Rep and Cap proteins was increased by replacing the endogenous AAV promoters, p5 and p40, with the Rous sarcoma virus (RSV) long terminal repeat (LTR) and the cytomegalovirus immediate-early promoter, respectively. Increased synthesis of the Cap proteins resulted in an approximately 10-fold increase in the yield of rAAV, indicating that production of capsid proteins is one limiting factor for rAAV packaging. Expression of the *rep* gene from the RSV LTR not only failed to increase the yield of rAAV but also prevented activation of p40 transcription with adenovirus infection, resulting in a reduced level of capsid protein synthesis.

Use of the human parvovirus adeno-associated virus (AAV) as a vector for gene therapy has attracted much attention owing to several potential advantages over other viral vector systems, e.g., retroviruses and adenovirus (Ad) (for reviews, see references 3, 17, and 31). Unlike these other viruses, AAV is naturally defective, requiring coinfection with a helper virus (e.g., Ad or herpesvirus) to establish a productive infection. No human disease has been found to be associated with AAV infection (4). AAV is capable of infecting both dividing and nondividing cells in vitro and in vivo (16, 29, 47, 49) as well as cells originating from different species and tissue types in vitro (37, 43). However, the efficiency of AAV vector-mediated gene expression may vary with the precise nature of the cell and its mitotic state (20, 49). In addition, when infection occurs in the absence of a helper virus, wild-type (wt) AAV can integrate into the cellular genome (9, 21, 36). This feature of the viral life cycle provides the potential for long-term gene expression with AAV vectors in vivo. A further potential advantage for gene therapy applications is the finding that AAV-mediated integration in vitro is site specific, occurring at a high frequency into a defined region on the long arm of human chromosome 19 (30, 53).

The AAV genome is relatively simple, containing two open reading frames (ORFs) flanked by short inverted terminal repeats. The nonstructural or replication (Rep) and the capsid (Cap) proteins are encoded by the 5′ and 3′ ORFs, respectively. Four related proteins are expressed from the *rep* gene; Rep78 and Rep68 are transcribed from the p5 promoter, while a downstream promoter, p19, directs the expression of Rep52 and Rep40. The larger Rep proteins (Rep78 and Rep68) are directly involved in AAV replication as well as regulation of viral gene expression (for a review, see reference 45). The *cap* gene is transcribed from a third viral promoter, p40. The capsid is composed of three proteins of overlapping sequence; the smallest (VP-3) is the most abundant. Because the inverted terminal repeats are the only AAV sequences required in *cis* for replication, packaging, and integration (52), most AAV vectors dispense with the viral genes encoding the Rep and Cap proteins and contain only the foreign gene inserted between the terminal repeats.

In spite of the attractive aspects of AAV-based vectors, rapid progress in their evaluation for gene therapy has been hampered by the inability to produce recombinant viral stocks at large scale and to high titer. The conventional method for production of recombinant AAV (rAAV) vectors is cotransfection of one plasmid containing the vector and a second helper plasmid encoding the AAV Rep and Cap proteins into 293 cells infected with Ad (12, 29, 37, 52). This method is cumbersome and results in a low yield of rAAV, typically 10^4 to 10^5 infectious units (IU) or transducing units/ml. Strategies to improve this scheme have included increasing transfection efficiency by complexing plasmid DNA to Ad particles via polylysine (40), delivering the vector sequences as part of a recombinant Ad (56), and amplification of helper plasmid copy number by linkage to a simian virus 40 replicon (10).

An alternate approach for the production of rAAV stocks is the development of packaging cell lines which obviate the need for cotransfection of vector and helper plasmids. A variety of packaging cell lines have been generated. These range from cell lines containing only the *rep* gene expressed from an inducible promoter (25, 26, 61) or the *rep* and *cap* genes expressed from endogenous AAV promoters (59), both of which are fairly inefficient, to cell lines containing the vector only (18) or the vector as well as the AAV helper functions (11) which report increased vector yield. The latter system is the most convenient, requiring only Ad infection to produce rAAV.

The aim of the work described here was to determine the limiting component(s) required for rAAV packaging. An understanding of the process at a basic level should benefit all methods of rAAV production. By selectively increasing expression of either the *rep* or *cap* gene (or both), we have shown that Cap protein production is one limiting factor in the production of rAAV. Use of a helper plasmid containing the *cap* gene under the control of the cytomegalovirus immediate-early (CMV IE) promoter increased rAAV yield approximately 10-fold over a similar plasmid in which the *cap* gene was expressed

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from p40. In contrast, overexpression of Rep78 or Rep68 did not lead to an increase in yield of rAAV.

MATERIALS AND METHODS

Cell lines, viruses, and plasmid DNA. The 293 cell line, an Ad type 5-transformed human embryonic kidney cell line (19), was propagated in Dulbecco's modified Eagle's medium-high glucose (DME; Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific), 20 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin (Gibco-BRL/Life Technologies, Gaithersburg, Md.) per ml at 37°C and 5% CO_2. Ad type 5 mutant ts149 (Ad5ts149) (13) used as a helper virus in these studies has reduced ability to replicate viral DNA at the nonpermissive temperature (39°C) due to a temperature-sensitive mutation in the DNA polymerase encoded by Ad early region 2 (55). Ad5ts149 was grown in 293 cells at the permissive temperature (33°C) and purified by CsCl gradient centrifugation.

Plasmid DNA encoding the recombinant AAV vector, pTRlacZ, as well as helper plasmid pIM45 (41) was generously provided by N. Muzyczka (University of Florida). pTRlacZ consists of the *Escherichia coli lacZ* gene (cytoplasmic) under the transcriptional control of the CMV IE promoter, inserted between the terminal repeats of AAV. The plasmid encoding the amber suppressor tRNA, $pSVtsSu^+$ (amber) (5), was obtained from U. L. RajBhandary (Massachusetts Institute of Technology). pNTC3, an AAV genomic clone containing an amber mutation within the *rep* coding region (8), was kindly provided by R. Owens (National Institutes of Health).

Plasmid constructions. Using pIM45 as the starting plasmid, the endogenous AAV promoters p5 and p40 were replaced with the Rous sarcoma virus long terminal repeat (RSV LTR) and CMV IE promoter, respectively. All manipulations were carried out following standard cloning procedures (51). All restriction and DNA-modifying enzymes were obtained from New England Biolabs and used according to the manufacturer's specifications. Plasmid DNAs were purified with kits obtained from Qiagen (Chatsworth, Calif.).

The CMV IE-*cap* cassette was constructed by first amplifying a DNA fragment consisting of AAV genomic sequences between bp 1852 and 4460 (encoding the capsid proteins and including the AAV mRNA polyadenylation site) via PCR (50) using Vent polymerase (New England Biolabs, Beverly, Mass.). This fragment was inserted between the *Bam*HI sites of pCMVβ (Clontech, Palo Alto, Calif.) to generate plasmid pCMV*cap.*

To derive a minimal Rep-encoding sequence, *rep* gene sequences between the *Bam*HI site (bp 1045) and bp 2283 of the AAV genome were PCR amplified and inserted within the pIM45 plasmid digested with *Bam*HI and *Apa*I. The result was a deletion between bp 2283 (just downstream of the Rep termination codon) and the *Apa*I site at bp 4049. This plasmid, pIM*repΔ*, was used to generate a construct in which Rep78 and Rep68 are expressed from the RSV LTR. A 2.4-kb *rep* gene fragment extending from bp 276 (just upstream of the Rep78-Rep68 mRNA initiation codon) to bp 4459 was PCR amplified from pIM*repΔ* and inserted between the *Nhe*I and *Not*I sites of the pRep9 expression vector (Invitrogen, San Diego, Calif.) to create pRSV*rep.*

Because the Rep and Cap protein coding sequences overlap in the region of the AAV intron, there are 431 bp in common between the *rep* and *cap* gene cassettes (between bp 1852 and 2283) of pIM*repΔ* and pCMV*cap*. Prior to insertion of the CMV IE-*cap* fragment into pIM*repΔ* to create p5*repΔ*-CMV*cap*, p40 sequences within pIM*repΔ* were mutated to inactivate the promoter. This was done to prevent the generation of wt AAV as a consequence of recombination between the shared sequences. Mutagenesis was carried out by overlap extension PCR (23). pIM*repΔ* was used as a template for the first PCR using flanking primer 1 (5′-GGATTACCTCGGAGAAGCAGTGGATCC-3′; bp 1024 to 1050 of the AAV genome) and mutagenic primer 1 (5′-GTTTGGGTTCAC TGATGTCTGCGTCACTG-3′; AAV bp 1821 to 1841; mutated nucleotides are underlined). The result is the introduction of three base pair mutations in the region of the p40 TATA box: from TATAAGTGAG to CATCAGTGAA. The G-to-A change ablates a *Ban*II site to enable screening by restriction analysis. pIM*repΔ* was also used as a template for the second PCR using flanking primer 2 (5′-GTGTGGAATCTTTGCCCAGATGGGCCCGGTTTGAGCTTC-3′; AAV bp 2260 to 2283 and 4049 to 4066) and mutagenic primer 2 (5′-CAGTG ACGCAGACATCAGTGAACCCAAACG-3′; AAV bp 1821 to 1841). After gel purification of the above PCR products, a third PCR was performed by annealing the two earlier products and carrying out a final amplification step using only the flanking primers, thereby generating a 1,285-bp DNA fragment. This fragment was digested with *Bam*HI and *Apa*I and cloned into the corresponding sites of the pIMRepΔ backbone. The resulting plasmid was pIM*repΔ*/p40Δ. The helper plasmid p5*repΔ*-CMV*cap* was constructed by inserting an *Sph*I fragment from pCMV*cap* containing the CMV IE promoter and *cap* gene cassette into the unique *Sph*I site of pIM*repΔ*/p40Δ. Similarly, in order to construct p5*repΔ*-p40*cap*, a PCR fragment with *Sph*I ends extending from AAV bp 1715 to 4461 was generated from pIM45 and cloned into the *Sph*I site of pIM*repΔ*/p40Δ.

The p5 promoter regions in plasmids pIM45, p5*repΔ*-CMV*cap*, and p5*repΔ*-p40*cap* were replaced with the RSV LTR promoter by first cleaving pRSV*rep* with *Xba*I. The *Xba*I site was made blunt with DNA polymerase I Klenow fragment, and the DNA was restricted with *Sfi*I to release a fragment containing the RSV promoter and the 5′ end of the *rep* gene. This fragment was then cloned between the *Sma*I and *Sfi*I sites of the parental plasmid.

To introduce an amber mutation into pIMRSV, an *Sfi*I-*Bam*HI fragment containing the mutation (at bp 1033 of the AAV genome) was isolated from plasmid pNTC3 (8) and cloned into the corresponding sites of pIMRSV.

Transient transfections and analysis of rAAV replication and packaging. For small-scale experiments, 293 cells were seeded at a density of 10^6 cells per 6-cm-diameter dish 48 h before transfection. The cells were infected with Ad5ts149 in DME–10% FBS at a multiplicity of infection (MOI) of 20 for 1 h at 37°C prior to transfection. Transfection procedures were carried out using the calcium phosphate ProFection kit (Promega, Madison, Wis.) according to manufacturer's instructions. In general, for rAAV packaging, each dish received a mix of 1.5 μg of vector DNA (i.e., pTRlacZ) and 15 μg of helper DNA. Following incubation at 37°C for 5 h, the infection-transfection was terminated by replacing the medium with fresh DME–10% FBS; the dishes were then transferred to 39°C (the nonpermissive temperature for Ad5ts149).

For analysis of rAAV packaging, cells were harvested at 48 h posttransfection by low-speed centrifugation in a clinical centrifuge. The pellet from each dish was resuspended in 100 μl of phosphate-buffered saline (PBS) and freeze-thawed four times to liberate the rAAV. Ad was heat inactivated by incubating the lysate at 56°C for 30 min. The lysate was subjected to a second low-speed spin to pellet cellular debris, and the supernatant was collected. The rAAV titer was determined on 293 cells (with or without coinfection with Ad5ts149; MOI = 20) by endpoint dilution. Following staining of the cells with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 20 to 24 h, titers were calculated with a computer program based on Karber's method (39).

Replication of vector DNA in transfected cells was assayed by isolating extrachromosomal DNA 48 h posttransfection according to the Hirt fractionation method (24). DNAs were restricted with *Dpn*I (to digest input DNA) prior to agarose gel electrophoresis and Southern analysis. The *lacZ* and wt AAV probes used were both 50-mer oligonucleotides, 5′-ACTGCTGCCAGGCGCTGATGT GCCCGGCTTCTGACCATGCGGTCGCGTTC-3′ and 5′-TCGGAGGAAGC AAGGTGCGCGTGGACCAGAAATGCAAGTCCTCGGCCCAG-3′ (AAV nucleotides 1501 to 1550), respectively. These were labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase according to standard procedures (51). The filter was hybridized and washed as described below for the Northern blot analysis, except that the prehybridization, hybridization, and final wash steps were at 60°C.

Protein extraction and immunoblotting. For analysis of Rep and Cap protein expression from the various helper plasmids, 293 cells were first transfected as described above. Nuclear fractions were prepared 48 h posttransfection according to the procedure described by Mendelson et al. (44). Sample volumes were normalized according to DNA content (by optical density at 260 nm), mixed with 15 to 20 μl of sample buffer (500 mM Tris-HCl [pH 6.8], 10% sodium dodecyl sulfate [SDS], 20 mM EDTA, 10% β-mercaptoethanol, 10% glycerol, and 0.2% bromophenol blue), and boiled for 5 min prior to loading.

Following electrophoresis in 10% polyacrylamide–0.1% SDS gels, proteins were transferred from the gel to Hybond polyvinylidene difluoride membranes (Amersham, Arlington Heights, Ill.). Prior to staining, the filters were blocked for 1 h at room temperature in 5% milk powder dissolved in TBST (10 mM Tris-HCl, [pH 8.0], 150 mM NaCl, and 0.05% Tween 20). The primary antibodies used for Rep and Cap Western blots were both mouse monoclonal antibodies (American Research Products, Belmont, Mass.): anti-AAV Rep protein, 303.9 (used at a dilution of 1:10 in TBST), and anti-VP1, -VP-2, and -VP-3 of AAV, B1 (used at a dilution of 1:5 in TBST), respectively. These were incubated on the filter for 2 h at room temperature with vigorous shaking. Following a wash step in TBST (three times for 15 min each), the filter was incubated in the secondary antibody, goat anti-mouse immunoglobulin G (Fab specific)–peroxidase conjugate (Sigma, St. Louis, Mo.), for 1 h at room temperature. The filter was then washed as before and developed with an enhanced chemiluminescence kit (Amersham).

Isolation of RNA and Northern analysis. Total RNA was isolated from transfected 293 cells by using RNAzol B (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's instructions. Prior to electrophoresis, 10 μg of each RNA was combined with denaturation cocktail (50% dimethyl sulfoxide, 10% formaldehyde, 20 mM MOPS [morpholinepropanesulfonic acid; pH 7.0], 10 mM sodium acetate, 1 mM EDTA) and loading dyes (5% glycerol, 0.1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol) and heated at 65°C for 15 min. Electrophoresis was through a 1% agarose–0.65% formaldehyde gel assembled and run in MOPS running buffer (20 mM MOPS [pH 7.0], 10 mM sodium acetate, 1 mM EDTA). Transfer to a GeneScreen nylon membrane (NEN-DuPont, Boston, Mass.) was carried out by capillary action overnight in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate [51]).

The filters were prehybridized for 4 to 5 h at 65°C and then hybridized with probe overnight at 65°C in hybridization buffer (5× SSC, 0.5% SDS, 5× Denhardt's solution [51], 100 μg of denatured salmon sperm DNA per ml). The probe was a 1.6-kb *Hin*cII fragment of pIM45 (AAV bp 2397 to 3987) labelled with [α-^{32}P]dATP (specific activity, 3,000 Ci/mmol; NEN-DuPont) by using a random primer labelling kit (Stratagene, La Jolla, Calif.). The filter was washed for 5 min at room temperature in 2× SSC–0.5% SDS, 15 min at room temperature in 2× SSC–0.1% SDS, and then for 2 h in 0.1× SSC–0.5% SDS at 65°C and exposed to film.

Large-scale transfection and rAAV purification. Prior to transfection of 293 cells for large-scale growth of rAAV, the cells were seeded in roller bottles such that they would reach 60 to 80% confluence on the day of transfection (final

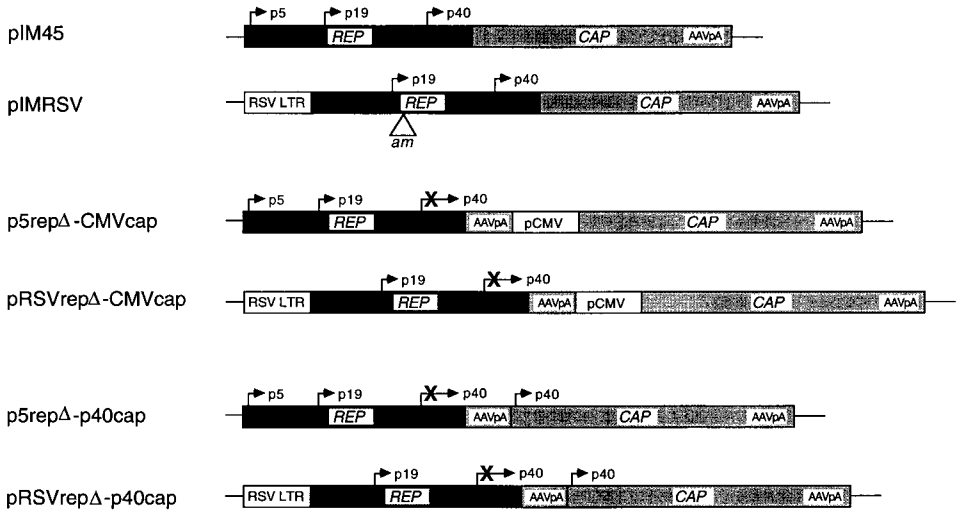

FIG. 1. AAV helper plasmids. Shown is a schematic of the structure of each of the helper plasmids used in this study. The plasmids are represented in linear form with the thin line (only a portion of which is shown) depicting the backbone plasmid DNA. The thick bars represent the Rep and Cap coding regions and their associated control regions. The arrows above the bars depict the positions of the endogenous AAV promoters, p5, p19, and p40. The X indicates that the p40 promoter has been inactivated by mutation. The triangle below pIMRSV depicts the position of the amber mutation (bp 1033 of the AAV genome). For a description of the plasmids and their construction, see Materials and Methods.

density was approximately 10^8 cells/bottle). Transfection was carried out in OptiMem medium (Gibco-BRL/Life Technologies) using lipid #53:DOPE (38), 22 μg of vector DNA, and 218 μg of helper DNA per bottle. Cells were infected with Ad5ts149 at an MOI of 20 at the time of transfection and incubated at 39°C for 48 h prior to harvest.

At the time of harvest, cells were dislodged from the bottles by gentle shaking. The cells were pelleted by centrifugation in a Sorvall RC-3B swinging-bucket rotor (2,500 rpm, 4°C, 15 min) and frozen. For purification of rAAV, the cells were resuspended in PBS containing 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 10% glycerol, and 0.1% Tween. Benzonase (Nycomed Pharma A/S, Copenhagen, Denmark) was added (10 μl/10^8 cells), and the suspension was incubated with shaking for 1 h at room temperature. Trypsin (Gibco-BRL/Life Technologies) was added to a final concentration of 0.25%, and the suspension was incubated again with shaking for 1 h at room temperature. The cell debris was collected by centrifugation (3,000 rpm for 15 min at 4°C in a Sorvall RC-3B rotor), and the lysate was filtered through a 0.45-μm-pore-size filter.

The lysate was then subjected to centrifugation through a CsCl step gradient (4 h, 26,000 rpm, 4°C, SW28 rotor) in which the top and bottom layers were 1.37 and 1.5 g of CsCl per ml, respectively. The top layer was collected (between the CsCl interface and the Ad5ts149 band), adjusted to 1.41 g of CsCl per ml, and centrifuged through a 1.41-g/ml CsCl equilibrium gradient (16 to 20 h, 4°C, 35,000 rpm, NVT.65 rotor). Fractions were collected and assayed on a refractometer; fractions with a density of 1.36 to 1.41 were pooled and dialyzed against PBS–1% sucrose for 6 h at 4°C. Sucrose was added to a final concentration of 5%, and the purified virus was stored in aliquots at −80°C. The average yield of rAAV by this procedure was 12%.

Characterization of purified rAAV stocks. The rAAV titer of the purified rAAV stock was determined in the presence and absence of Ad5ts149 (MOI = 20) by endpoint dilution as described above. The titer of contaminating Ad5ts149 was determined in a similar manner except staining was for hexon by using anti-Ad (hexon)–fluorescein isothiocyanate conjugate (Chemicon, Temecula, Calif.). The level of contaminating wt AAV was assayed by the infectious-center assay as described elsewhere (12).

AAV particle titer was quantitated by a procedure modified from that of Samulski et al. (52). The purified rAAV sample was first treated with proteinase K in 0.1% SDS at 37°C for 3 h. Appropriate dilutions as well as standard curve DNAs (for TRlacZ virus, pTRlacZ DNA was used as a standard) were treated with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 10 min at room temperature, and a 1-ml volume was applied to a GeneScreen Plus (Amersham) membrane with a slot blot apparatus (Schleicher and Schuell, Keene, N.H.). After loading, the slot was washed with 300 μl of 0.5 M ammonium acetate, pH 5.2. The filter was dried and hybridized as described above. The probe (a *Pvu*II fragment of pTRlacZ) was labelled with a Prime-It Fluor labelling kit (Stratagene). Following a series of washes as described above (except the final wash at 65°C was for 10 min), the filter was developed with an Illuminator detection kit (Stratagene). Particle concentrations were estimated by comparing the sample signal with that of the standard curve.

RESULTS

Construction of AAV helper plasmids. A series of helper plasmids was constructed to determine if rAAV packaging is limited by expression levels of the *rep* and/or *cap* genes (Fig. 1). Expression of Rep and Cap proteins was increased by replacing the endogenous AAV promoters, p5 and p40, with the RSV LTR and the CMV IE promoter, respectively. The starting helper plasmid, pIM45 (41), contains a sequence encompassing the wt AAV genome but excluding the terminal repeats (nucleotides 145 to 4493). pIMRSV is a modification of pIM45 in which the RSV LTR replaces p5. Because p40 is located within the Rep coding region, the *rep* and *cap* genes were separated to allow replacement of p40 with the CMV IE promoter (as in p5*rep*Δ-CMV*cap*). This strategy generated a vector with a direct repeat of 431 bp of sequence downstream from the p40 and CMV promoter. To prevent generation of wt AAV through recombination, the p40 promoter lying within the *rep* ORF of this construct was inactivated by site-directed mutagenesis (see Materials and Methods). p5*rep*Δ-p40*cap* was constructed to express the *rep* and *cap* genes from endogenous AAV promoters as in pIM45, but to be more directly comparable to p5*rep*Δ-CMV*cap*, the Rep and Cap coding regions were separated. RSV*rep*Δ-CMV*cap* and RSV*rep*Δ-p40*cap* are derivatives of p5*rep*Δ-CMV*cap* and p5*rep*Δ-p40*cap*, respectively, in which p5 is replaced by the RSV LTR.

Rep and Cap gene expression from AAV helper plasmids. The amounts of Rep and Cap proteins expressed from each of the AAV helper plasmids were estimated by Western blot analysis (Fig. 2A). The four Rep proteins produced following transfection into 293 cells in the presence of an Ad5ts149 infection (MOI = 20) comigrate with the corresponding proteins detected after coinfection of 293 cells with wt AAV and Ad5ts149. For each of the helper plasmids, Rep78 and Rep52 are the major proteins produced. Rep68 and Rep40, which are translated from spliced messages, were observed at a lower level. These were also detected as minor proteins in the wt AAV infection.

When the p5 promoter was replaced with the RSV LTR,

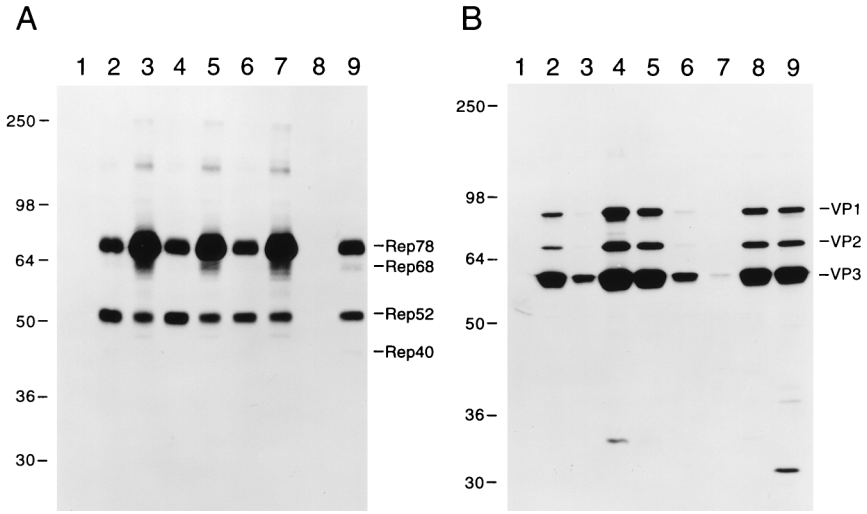

FIG. 2. Rep and Cap gene expression from AAV helper plasmids. Ad-infected 293 cells were transfected with 1.5 μg of vector (pTRlacZ) and 15 μg of the appropriate helper DNA. Nuclear fractions were isolated 48 h posttransfection and analyzed by Western blot using anti-Rep (A) and anti-Cap (B) primary antibodies. Lanes in each panel correspond to samples derived from cells transfected with the following helper DNAs: lane 1, mock; lane 2, pIM45; lane 3, pIMRSV; lane 4, p5*rep*Δ-CMV*cap*; lane 5, pRSV*rep*Δ-CMV*cap*; lane 6, p5*rep*Δ-p40*cap*; lane 7, pRSV*rep*Δ-p40*cap*; lane 8, pIMRSV-*am*; lane 9, wt AAV, MOI = 10. Molecular size standards (in kilodaltons) are depicted on the left; each of the AAV Rep and Cap proteins is identified on the right. As a control for transfection efficiency, this experiment was performed at least twice with different preparations of each plasmid DNA.

an increase in the level of Rep78 was observed. This was the case for all three helpers, pIMRSV, RSV*rep*Δ-CMV*cap*, and RSV*rep*Δ-p40*cap*. There was no change in the amount of Rep52, because in all constructs it was derived from a p19 transcript.

The three capsid proteins VP1, VP2, and VP3 were produced from all helper plasmids in the 1:1:10 ratio observed in a wt AAV infection (Fig. 2B). Synthesis of all three capsid proteins was enhanced when p40 was replaced by the CMV IE promoter (Fig. 2B, lane 2 versus lane 4). However, expression of the *rep* gene from the RSV LTR appeared to have a down-regulatory effect on *cap* expression from p40. Thus, the level of capsid protein was reduced for pIMRSV relative to the parental plasmid containing p5 as the promoter regulating expression of *rep* (pIM45) (compare lanes 3 and 2). A similar but less dramatic effect was observed with capsid protein expression from the CMV IE promoter (lane 5 versus lane 4). In the latter case, a corresponding reduction was also seen in *cap* mRNA by Northern analysis (results not shown), suggesting that overexpression of Rep78 results in transcriptional down-regulation of the CMV IE promoter. Cap protein synthesis was also reduced relative to pIM45 when the *rep* and *cap* genes were expressed from separate transcription units as in p5*rep*Δ-p40*cap*, where the AAV pA signal separates the *rep* and *cap* ORFs.

It should be noted that the total level of AAV proteins produced in the transient transfections was comparable to that observed in a wt AAV infection at an MOI of 10. While Rep78, Rep52, and the capsid proteins appeared at levels similar to that observed in the wt AAV infection when expressed from the AAV promoters (p5, p19, and p40, respectively), expression from the heterologous promoters, the RSV LTR and the CMV IE promoter, increased the amount above that observed in the viral infection. This is especially significant when it is considered that transfection efficiency ranges from 20 to 50% (data not shown) while infection at an MOI of 10 should occur with greater efficiency. This suggests that the concentration of each viral gene product per transfected cell is higher in the transient transfections than in the wt AAV infection.

Construction of pIMRSV-*am* and analysis of Cap gene down-regulation. To analyze further the mechanism of down-regulation of capsid protein expression in the helper plasmids containing an RSV LTR-*rep* gene cassette, a derivative of pIMRSV that contained an amber mutation within the *rep* ORF (pIMRSV-*am*) was made. If down-regulation were due to an alteration in *cis* (i.e., replacement of p5 with the RSV LTR), then it should persist in the amber mutant. In contrast, the down-regulatory effect should be relieved in the mutant if it is dependent upon synthesis of full-length Rep protein.

Transient transfections in 293 cells were performed with pIMRSV-*am* as a helper in the presence and absence of Ad infection. Nuclear proteins were isolated and analyzed by Western blot (Fig. 3). With pIM45 as a helper (Fig. 3A), Rep78 appears at a high level in the absence of Ad due to expression of the Ad E1A and E1B genes in 293 cells; hence, there is an apparent lack of induction with the addition of Ad (induction of Rep78 with this construct is observed in HeLa cells [results not shown]). Infection with Ad does result in the appearance of the spliced Rep proteins, Rep68 and Rep40, and a slight increase in the level of Rep52. As expected, expression of Rep78 from the RSV LTR is unresponsive to Ad infection; the same high level appears in the presence and absence of helper virus coinfection. In cells transfected with pIMRSV-*am*, a small amount of full-length Rep78 is observed, indicating that the mutation is somewhat leaky. When cells are cotransfected with pIMRSV-*am* and an amber suppressor tRNA, production of Rep78 is restored to the level observed with pIMRSV. Cotransfection of pIMRSV-*am* with a Rep-expressing plasmid, pRSV*rep*, provides a high level of Rep78 in *trans.*

Capsid protein expression was analyzed in parallel (Fig. 3B). Synthesis of the capsid proteins is significantly enhanced following Ad infection of cells transfected with pIM45. This increase is not observed with pIMRSV (lane 3) but does occur with the pIMRSV-*am* mutant. The pIMRSV phenotype is restored when pIMRSV-*am* is cotransfected with the suppressor tRNA or when Rep protein is supplied in *trans* by cotransfection with pRSV*rep.*

Northern analysis was performed on samples from the same experiment to examine this phenomenon at the RNA level (Fig. 4). With pIM45 (lane 2), the level of the full-length p5 transcript (4.2 kb) was negligible, but the p19 transcript (3.6 kb) was more prevalent and showed an increase with Ad in-

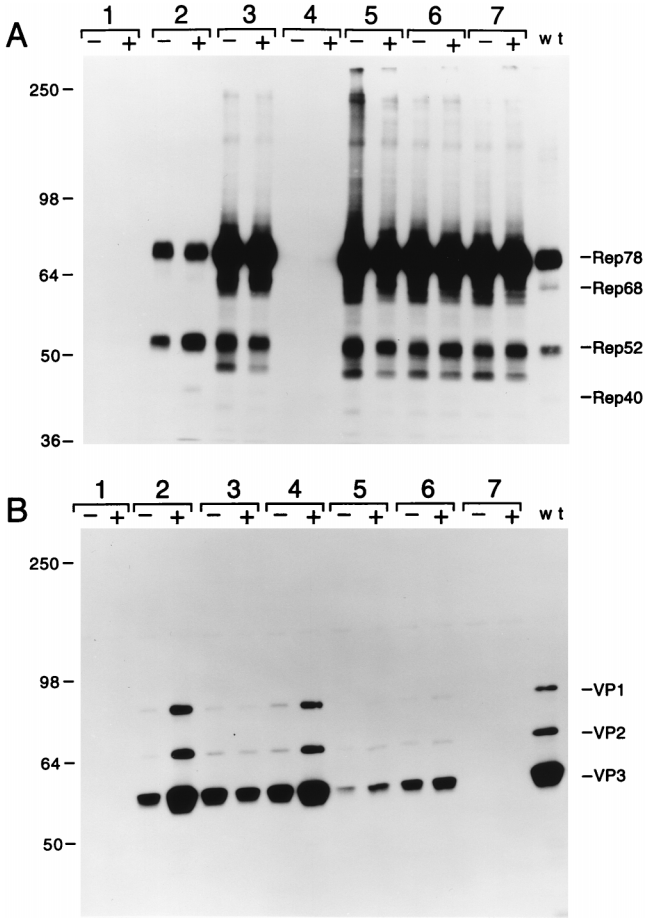

FIG. 3. Analysis of the mechanism of down-regulation of the *cap* genes. Shown in the figure is a Western blot analysis of samples derived from 293 cells transfected with the appropriate DNAs (10 μg total) in the absence (−) or presence (+) of Ad (Ad5ts149, MOI = 20). Where a single helper DNA was transfected, 5 μg was used and the remaining 5 μg was pUC19DNA. Nuclear fractions were prepared 48 h posttransfection. Shown are blots developed with anti-Rep (A) and anti-Cap (B) primary antibodies. Lane 1, mock-transfected cells; lane 2, pIM45; lane 3, pIMRSV; lane 4, pIMRSV-*am*; lane 5, pIMRSV-*am* and suppressor tRNA plasmid; lane 6, pIMRSV-*am* and pRSVRep; lane 7, pRSVRep alone; lane wt, cells infected with wt AAV (MOI = 15) in the presence of Ad (Ad5ts149, MOI = 20). Molecular size markers (in kilodaltons) are shown at the left, and the AAV Rep and Cap proteins are identified at the right. As a control for transfection efficiency, this experiment was performed at least twice with different preparations of each plasmid DNA.

fection. The spliced transcripts from p5 and p19 (3.9 and 3.3 kb, respectively) were not detected. The two unspliced *rep* transcripts (4.2 and 3.6 kb) are also visible in the sample derived from the wt AAV infection (lane wt). Replacement of p5 by the RSV LTR resulted in an increase in the 4.2-kb transcript which contributes to the higher levels of Rep78 produced by this construct. Interestingly, the amount of this transcript was augmented further upon introduction of the amber mutation. When synthesis of Rep is restored by cotransfection of a suppressor tRNA (lane 5) or supplied in *trans* by cotransfection with pRSV*rep* (lane 6), synthesis of the 4.2-kb transcript is again reduced. These results suggest that transcription from the RSV LTR is down-regulated by Rep.

With pIM45, Ad infection results in a significant increase in both the full-length (2.6-kb) and spliced (2.3-kb) p40 mRNA, mirroring the increase in capsid protein synthesis. The prevalence of p40 transcripts over those derived from p5 and p19 is similar to that observed by Labow et al. (35) from wt AAV in Ad-infected KB cells. In general, the ratios of the two p40 mRNAs are shifted in favor of the spliced 2.3-kb transcript with Ad infection. In contrast to pIM45, no increase in the level of either the p19 or p40 transcript is observed with the pIMRSV construct upon Ad infection. Notably, with pIMRSV-*am*, the increase in capsid protein synthesis observed with Ad infection in the Western analysis (Fig. 3B, lane 4) is not reflected by an increase in the level of *cap* mRNA. The level of capsid mRNA observed with pIMRSV-*am* is similar to that of the parental plasmid, pIMRSV. The same lower level is observed in the cotransfection of pIMRSV-*am* and the suppressor tRNA and of pIMRSV-*am* with Rep-expressing plasmid pRSV*rep*. The Northern analysis suggests that the reduced level of capsid protein synthesis observed with pIMRSV is explained at the RNA level by a failure to activate transcription from p40 in response to Ad infection. Furthermore, these results suggest that the increase in capsid protein production following Ad infection with the pIMRSV-*am* mutant is a post-transcriptional effect and that the mutation has relieved a Rep-mediated translational inhibitory effect on capsid synthesis.

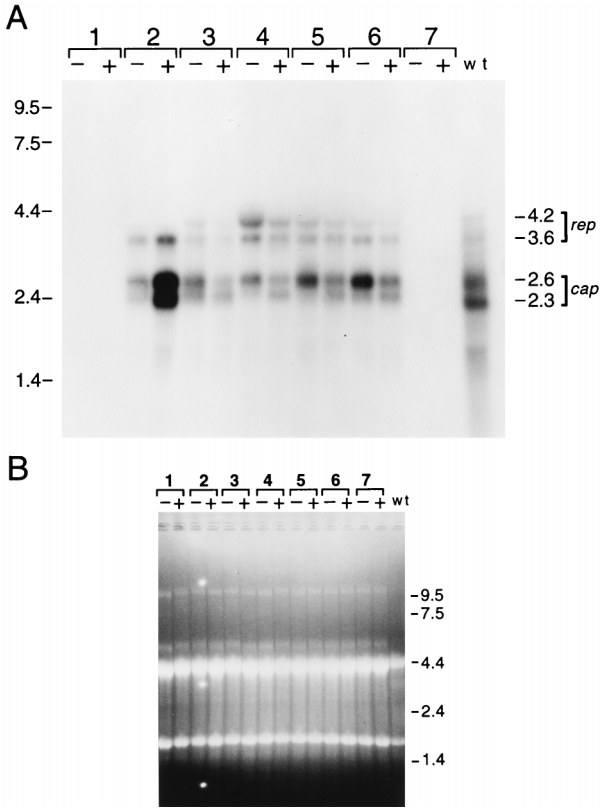

FIG. 4. Analysis of total RNA derived from transfections described in the legend to Fig. 3. RNA was harvested 48 h posttransfection and analyzed by Northern blot as described in Materials and Methods. (A) Northern blot; (B) ethidium bromide-stained gel to demonstrate that equal amounts of RNA were loaded in each lane. Lane 1, mock-transfected cells; lane 2, pIM45; lane 3, pIMRSV; lane 4, pIMRSV-*am*; lane 5, pIMRSV-*am* and suppressor tRNA plasmid; lane 6, pIMRSV-*am* and pRSVRep; lane 7, pRSVRep alone; lane wt, cells infected with wt AAV (MOI = 15) in the presence of Ad (Ad5ts149, MOI = 20). Transfections were carried out in the absence (−) or presence (+) of Ad (Ad5ts149, MOI = 20). RNA size standards (in kilobases) are shown at the left in panel A and at the right in panel B; AAV mRNAs are identified at the right of panel A. As a control for transfection efficiency, this experiment was performed at least twice with different preparations of each plasmid DNA.

TABLE 1. Comparison of Helper Plasmids Containing RSV-*rep* and CMV-*cap*

Sample	Titer (IU/ml)[a]
pIM45	6.0×10^6
pIMRSV	3.2×10^5
p5*rep*Δ-CMV*cap*	5.5×10^7
RSV*rep*Δ-CMV*cap*	1.0×10^7
p5*rep*Δ-p40*cap*	1.3×10^6
RSV*rep*Δ-p40*cap*	1.3×10^5

[a] Shown are the averages from five separate experiments; in each case, the highest and lowest values were omitted and the remaining three values were averaged. These experiments were carried out by transfecting 2×10^6 Ad5ts149-infected 293 cells with vector (pTRlacZ) and the helper plasmids listed above. Lysates were generated by freeze-thaw 48 h following transfection. Titers were determined by endpoint dilution on 293 cells in the presence of Ad5ts149 (MOI = 20).

rAAV packaging: comparison of RSV*rep* and CMV*cap* helper plasmids. The series of helper plasmids were compared with respect to their ability to produce rAAV. Each was transfected into Ad-infected 293 cells in conjunction with the pTRlacZ vector plasmid, and the yield of rAAV in the crude lysate (Table 1) was determined by the titer assay (see Materials and Methods). Increasing capsid protein expression by placing the *cap* gene under the control of the CMV promoter (p5*rep*Δ-CMV*cap*) increased rAAV yield approximately ninefold. In contrast, replacing p5 with the RSV LTR in order to enhance *rep* gene expression resulted in a lower rAAV yield. When the RSV LTR was added to a construct containing p40 (pIMRSV or RSV *rep*Δ-p40*cap*), rAAV titers were decreased 10- to 20-fold, while the RSV*rep*Δ-CMV*cap* helper packaged rAAV 5-fold less efficiently than the comparable construct containing p5. These results correlate with differences in capsid protein expression observed in the Western analysis. As described above, a dramatic decrease in Cap protein production was observed as a result of overproduction of Rep78 with the p40-*cap* constructs, while a more subtle effect was observed on Cap protein expression from the CMV promoter. The results of these experiments comparing the different helper constructs suggest that Cap but probably not Rep protein synthesis is a limiting factor in the production of rAAV.

Table 2 is a summary of the results of large-scale rAAV production using pIM45 and p5*rep*Δ-CMV*cap* as helper DNAs. Notably, the yield of rAAV infectious units per cell increases almost 10-fold when the modified helper is used. This result is also reflected in higher titers (both in infectious units and particles per milliliter) of the purified material. Shown in the table are infectious units per milliliter determined in both the presence and absence of Ad (Ad5ts149). As has been reported by others (14, 15), rAAV titers are approximately 1,000-fold higher in the presence of an Ad infection. The particle/infectious unit ratio of these preparations is 20 to 120 (infectious units with Ad) or 4×10^4 to 7×10^4 (infectious units without Ad). The former value is within the range previously reported (52). While the purification procedure results in a persistent yet variable level of Ad5ts149 contamination (from $<10^3$ to 10^7 IU/ml [results not shown]), the stocks are free of contaminating wt AAV (see below).

Analysis of replication and levels of wt AAV contamination. Hirt analysis was performed on samples from small-scale transient transfections such as those described above to assay replication of the vectors and to assess levels of wt AAV contamination. All of the helper DNAs supported replication of the TRlacZ vector (Fig. 5A); however, in each transfection using a helper plasmid containing an RSV LTR-*rep* cassette, the vector appeared to replicate at a diminished level (the ethidium bromide-stained gel indicated that equal amounts of DNA were loaded in each lane [data not shown]). This result might also help to explain the reduced viral yields obtained with the helpers containing RSV-*rep*. A low level of replication was observed when amber mutant pIMRSV-*am* was used as a helper, confirming, as shown in Fig. 3A, that a small amount of full-length Rep protein is synthesized by this mutant. When the same blot was probed with a fragment from the wt AAV genome, no evidence of replicating wt AAV viral DNA was observed (Fig. 5C). There was, however, hybridization to high-molecular-weight DNA in some lanes. Though this signal appears to comigrate with the dimer replicative form (dRF) in panel A, this is probably not the same species since no monomer replicative form was detected and the distribution of the bands is different (i.e., in some lanes, it appears in panel A but not in panel B). Alternatively, this signal might represent cross-hybridization with Ad DNA or cellular sequences bearing some homology to the wt AAV probe. However, since no band appeared in the negative control (mock) sample (which was also infected with Ad5ts149), an additional explanation might be that the signal is evidence of integration of the helper DNA into the 293 cell genome. Interestingly, this high-molecular-weight band appeared only in cells transfected with helpers carrying the p5-*rep* cassette rather than RSV LTR-*rep*. If this band does indeed reflect integration of the helper plasmid, then this might indicate that sequences within p5 (i.e., the Rep-binding site or RRS) might be required in *cis* for integration in the absence of the terminal repeats.

Lysates harvested in parallel from these transfections were used to infect a second plate of 293 cells in the presence of Ad5ts149, and Hirt samples were prepared. If in fact a small amount of contaminating wt AAV were present, then the virus should have been amplified upon reinfection. Southern analysis and hybridization with a wt AAV probe (Fig. 5D) again showed no evidence of replicating wt AAV viral DNA. When a duplicate blot was probed with a *lacZ* fragment (Fig. 5B), no replicating vector DNA was observed. This latter result is further evidence of the lack of wt AAV, since the presence of wt AAV (i.e., *rep* gene expression) would have allowed vector replication under these conditions.

TABLE 2. Large-scale purification of rAAV (TRlacZ)[a]

Lysate			Purified stock		
Plasmid	Titer (IU/ml with Ad)	IU/cell	Titer (IU/ml with Ad)	Titer (IU/ml without Ad)	Particles/ml
pIM45	2.0×10^8	2.9	4.3×10^7	8.0×10^4	7.4×10^9
p5*rep*ΔCMV*cap*	8.9×10^8	27	5.2×10^8	4.4×10^5	1.9×10^{10}

[a] Shown are the averages of nine preparations for each helper plasmid; these were derived from the transfection of approximately 10^9 cells and purified via CsCl gradient centrifugation (see Materials and Methods).

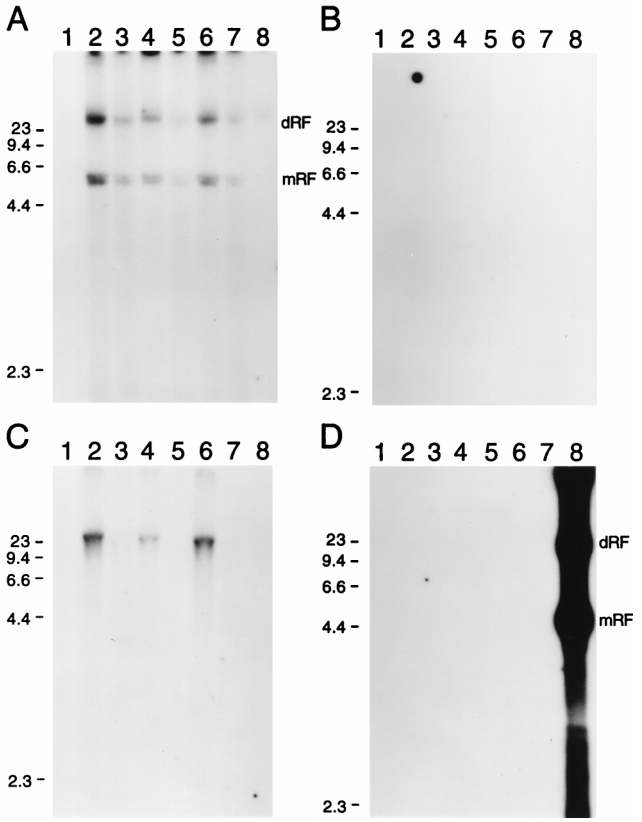

FIG. 5. Analysis of vector replication and levels of wt AAV contamination. Ad (Ad5ts149)-infected 293 cells were transfected with 1.5 μg of vector (pTRlacZ) and 15 μg of the indicated helper DNA. At 48 h posttransfection, duplicate dishes were harvested; the first was used to isolate extrachromosomal DNA by the Hirt method (primary DNAs), and from the second, a viral lysate was prepared. This lysate was subsequently used to infect 293 cells in the presence of Ad5ts149, and a second Hirt preparation (secondary DNAs) was carried out. The replicated viral DNA was then analyzed by Southern blotting; duplicate filters were probed with a *lacZ* gene oligonucleotide to visualize replication of vector DNA and with an oligonucleotide from the AAV genome to detect replication of wt AAV. (A and B) Primary and secondary Hirt DNAs, respectively, probed with the *lacZ* probe; (C and D) primary and secondary Hirt DNAs, respectively, probed with the AAV fragment. For the primary DNAs (A and C), lanes correspond to the following samples: lane 1, mock transfection; lane 2, pIM45; lane 3, pIMRSV, lane 4, p5*rep*Δ-CMV*cap*, lane 5, RSV*rep*Δ-CMV*cap*, lane 6, p5*rep*Δ-p40*cap*, lane 7, RSV*rep*Δ-p40*cap*, lane 8, pIMRSV-*am*. For the secondary DNAs (B and D), the lanes are the same except the sample in lane 8 is derived from cells infected with wt AAV (MOI = 0.001) and Ad (Ad5ts149, MOI = 20). The positions of DNA size standards (in kilobase pairs) are depicted at the left of each panel; hybridizing bands corresponding to the dRF and monomer replicative form (mRF) are identified at the right. The apparent discrepancy in the size of the dRFs (in panels A and D; expected to be ~10 kb) might be explained by the poor resolution capacity of a 8% agarose gel in this size range and/or by gel artifact. The fact that this discrepancy appears in both gels for the wt AAV-infected sample as well as the rAAV (pTRlacZ) DNA supports the identification of the band in panel A as the dRF.

DISCUSSION

Evaluation of AAV-based vectors for gene therapy will require examination of the safety, efficiency, and persistence of AAV-mediated gene transfer in vivo. In order to make such analyses feasible, it will be necessary to generate a sufficient supply of high-titer vector for escalated-dose and repeated-dose animal studies. However, the standard procedure for production of rAAV vectors is cumbersome and inefficient, relying on infection of cells with the helper Ad as well as transfection with two plasmid DNAs: the vector and a helper plasmid commonly containing the *rep* and *cap* genes under the control of endogenous AAV promoters. The typical yield of rAAV by this method (0.1 to 1 particle/cell [52]) is significantly less than that achieved with a wt AAV infection (~10^4 particles/cell [6]). In the latter case, however, large quantities of the AAV replication and capsid proteins are synthesized as the vector genome, which serves as a template for the synthesis of these proteins, is replicated to high copy number within each infected cell. The experiments described here were performed to identify a specific limiting factor(s) for rAAV packaging with the rationale that a basic understanding of this process would lead to improvements in vector production.

AAV promoters in a standard helper plasmid were substituted with stronger heterologous promoters in order to enhance separately the expression of the Rep and Cap proteins required for rAAV packaging. These experiments showed that rAAV yield was improved approximately 10-fold when *cap* gene expression was increased, implying that the level of capsid protein is one limiting factor for production of rAAV. In contrast, *rep* gene expression is probably not a limiting factor since overexpression of *rep* did not increase rAAV yield. However, it is not possible to make a definitive conclusion on this latter issue, as increases in Rep protein synthesis were always coupled with reductions in capsid protein production. In the case of plasmid pRSV*rep*Δ-CMV*cap*, however, Cap protein production was diminished only slightly relative to that observed with p5*rep*Δ-CMV*cap* (at most twofold, but the level was still higher than that attained with pIM45) while Rep78 expression was enhanced significantly (approximately fivefold). Under these conditions, there was no increase in rAAV yield over that with p5*rep*Δ-CMV*cap*; packaging efficiency was in fact slightly reduced. These conclusions conflict with those made on the basis of a previous study (18) in which use of a construct expressing *rep* from the human immunodeficiency virus LTR (pHIV*rep*/p40*cap*) led to a 10-fold increase in rAAV yield compared to that with a construct in which p5 controlled *rep* expression (pAAV/Ad).

Further evidence supporting the dependence of rAAV yield on the magnitude of capsid protein expression comes from a comparison of the levels of replicated rAAV viral DNA shown in Fig. 5A with the rAAV titers in Table 1. Though the amounts of rAAV viral DNA are similar in cells transfected with pIM45, p5*rep*Δ-CMV*cap*, and p5*rep*Δ-p40*cap*, the rAAV yield is much higher when p5*rep*Δ-CMV*cap* is used as a helper plasmid. This result supports the conclusion that rAAV yield is not necessarily limited by the level of replicated viral DNA (or by Rep function) but is limited by capsid protein production, which is significantly higher with the p5*rep*Δ-CMV*cap* construct than with the other two plasmids.

Another, related factor restricting AAV vector production by the standard protocol is transfection efficiency as the overall level of Rep and Cap protein synthesis is limited both by the number of cells taking up DNA as well as the number of DNA molecules present within each cell. In an attempt to increase transfection efficiency, plasmid DNA has been complexed to replication-competent Ad modified with polylysine, resulting in an increase in rAAV packaging of 40- to 240-fold over the standard calcium phosphate method (40). A number of modifications to the standard rAAV production procedure were made by Chiorini et al. (10); instead of transfecting 293 cells with calcium phosphate, COS cells were electroporated with reported transfection efficiencies of up to 90%. The helper plasmid used for those studies also contained a simian virus 40 replicon, presumably increasing the copy number of the *rep* and *cap* genes within each transfected cell. By this method, a packaging efficiency of over 10^3 rAAV particles/cell was achieved. Alternatively, packaging cell lines have been con-

structed in order to avoid the inefficient transfection step. When vector DNA was introduced into a stable cell line, a fivefold improvement in rAAV yield over cotransfection was reported, resulting in 10^4 particles/cell (18). Clark et al. (11) have constructed a cell line containing both the vector and AAV *rep*/*cap* genes which allows production of rAAV by Ad infection alone. This system yields 30 IU/cell (approximately 400 particles/cell), a value which is comparable to that achieved with the improved helper plasmid described here. Given the experience of others, it is likely that the packaging protocol employed in these studies can be further improved, either by replicating the helper plasmid within the transfected cell or by using the new helper construct to generate a packaging cell line.

The effect that we have observed on Cap protein expression resulting from replacement of p5 with the RSV LTR confirmed the work of others with respect to AAV gene regulation. In addition to their function in replication, the AAV Rep proteins (primarily Rep78 and Rep68 [27, 32]) are known to act as transcriptional regulators. In the absence of an Ad infection, Rep proteins repress transcription from AAV promoters (2, 32, 57, 58), while conversely they activate transcription from these promoters in response to Ad infection (34, 57). McCarty et al. (41) have shown that Rep-mediated activation of the p19 and p40 promoters in the presence of Ad is dependent in *cis* upon sequences located upstream of both p5 and p19. Consistent with this finding was the lack of induction of p40 transcription upon Ad infection when p5 was deleted and replaced with the RSV LTR (as in the pIMRSV plasmid). Similarly, there was also no increase in the level of p19 RNA with this construct. The failure to observe induction was due to removal of sequences required in *cis* since it occurred independently of *rep* gene expression; transcriptional activation of p40 was not restored (Fig. 4) when Rep protein synthesis was prevented by an amber mutation or when Rep protein was supplied in *trans*. Relative to pIM45, pIMRSV lacks only 84 bp upstream of p5 (bp 191 to 275 of the AAV sequence); this deletion is more limited than the one reported by McCarty et al. (bp 191 to 320 [41]) and thus further defines the location of the putative regulatory region required for Rep activation. The region between bp 191 and 275 is known to contain binding sites for the major late transcription factor (USF [7]), YY1 (54), and Rep (33, 42) as well as the p5 TATA box.

Though transcription from the p40 promoter in the pIMRSV-*am* mutant was not activated by Rep in response to Ad infection, Cap protein synthesis was observed to increase. This effect may be attributed to the previously described translational inhibitory activity of Rep. In 293 cells in the absence of an Ad infection, Trempe and Carter (58) observed that the level of p40 mRNA was reduced while chloramphenicol acetyltransferase (CAT) protein expression increased in the absence of Rep compared to a *rep* gene-containing vector. In cells transfected with pIMRSV-*am*, synthesis of the capsid proteins is significantly enhanced with Ad infection. This increase, however, occurs without any alteration in the steady-state level of p40 mRNA, indicating that it is a translational effect. In comparison, capsid protein production also increases in cells transfected with pIM45, but in this case, there is a concomitant increase in the level of both the 2.6- and 2.3-kb p40 mRNAs. The apparent induction in the synthesis of the capsid proteins with pIMRSV-*am* is a *trans* effect of the mutation of the *rep* gene, as it does not occur in any case where the Rep proteins are expressed. Because Rep78 is the major Rep protein produced by pIMRSV, it is presumably the primary mediator of the inhibitory effect; however, a role for Rep68 cannot be ruled out. These results suggest that although Ad infection is capable of significantly increasing the efficiency of translation of p40 mRNA (28, 60), this effect can be counteracted by the Rep proteins. It is not clear whether translational inhibition in the presence of Ad occurs as a normal function of Rep or if it is an artifact of overexpression of the *rep* gene in the pIMRSV construct. Alternatively, inhibition may occur only when the level of p40 mRNA is low and can be overcome when transcription from p40 normally increases with Ad infection. Induction of transcription from p40 was prevented in this case by removal of sequences upstream of p5.

These experiments have also provided further evidence of the ability of the Rep proteins to act as repressors of expression from heterologous promoters. The Rep proteins are known to down-regulate expression of several heterologous genes (1, 27, 35, 46, 48). In the experiments described here, expression of the *cap* gene from the CMV IE promoter as well as the *rep* gene from the RSV LTR were both down-regulated by Rep. Rep has previously been shown reduce the level of expression of the *cat* gene from the CMV IE promoter (22); similar to the results obtained here, this effect was minor (approximately twofold). For both the CMV IE and RSV LTR promoters, inhibition occurred at the RNA level, though since steady-state levels of RNA were assayed by Northern analysis, the effect could be at the level of either transcription or mRNA stability. Down-regulation at the RNA level has also been demonstrated in the case of the human immunodeficiency virus LTR and human papillomavirus type 18 URR promoters and has been attributed primarily to Rep78 and Rep68 (1, 27).

In summary, we have shown that capsid protein but probably not Rep protein synthesis is limiting with respect to rAAV production. With this information, further improvements in the production of recombinant AAV vectors should make AAV a more attractive alternative to other viral vector systems for human gene therapy.

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