HUMAN GENE THERAPY 9:2745–2760 (December 10, 1998)
Mary Ann Liebert, Inc.

Novel Tools for Production and Purification of Recombinant Adenoassociated Virus Vectors

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ABSTRACT

Standard protocols for the generation of adenoassociated virus type 2 (AAV-2)-based vectors for human gene therapy applications require cotransfection of cells with a recombinant AAV (rAAV) vector plasmid and a packaging plasmid that provides the AAV *rep* and *cap* genes. The transfected cells must also be overinfected with a helper virus, e.g., adenovirus (Ad), which delivers multiple helper functions necessary for rAAV production. Therefore, rAAV stocks produced using these protocols are contaminated with helper adenovirus. The generation of a novel packaging/helper plasmid, pDG, containing all AAV and Ad functions required for amplification and packaging of AAV vector plasmids, is described here. Cotransfection of cells with pDG and an AAV vector plasmid was sufficient for production of infectious rAAV, resulting in helper virus-free rAAV stocks. The rAAV titers obtained using pDG as packaging plasmid were up to 10-fold higher than those achieved using conventional protocols for rAAV production. Replacement of the AAV-2 p5 promoter by an MMTV-LTR promoter in pDG led to reduced expression of Rep78/68; however, expression of the VP proteins was significantly increased compared with VP levels from standard packaging plasmids. Immunofluorescence analyses showed that the strong accumulation of VP proteins in pDG-transfected cells resulted in enhanced AAV capsid assembly, which is limiting for efficient rAAV production. Furthermore, using a monoclonal antibody highly specific for AAV-2 capsids (A20), an rAAV affinity purification procedure protocol was established. The application of the tools described here led to a significant improvement in recombinant AAV vector production and purification.

OVERVIEW SUMMARY

Two novel tools allowing a simplified and more efficient generation and purification of adenoassociated virus type 2 (AAV-2)-derived vectors are described here. First, the generation of an AAV/adenovirus hybrid plasmid, pDG, which contains all packaging and helper functions required for production of recombinant AAV (rAAV), is reported. Cotransfection of cells with pDG and an AAV vector plasmid led to production of several hundred infectious rAAV particles per transfected cell. Using pDG as a helper plasmid, generation of rAAV was no longer dependent on overinfection of the cells with adenovirus, resulting in helper virus-free rAAV stocks. Second, an rAAV affinity purification procedure based on the monoclonal antibody A20, which specifically recognizes assembled AAV-2 capsids, is described. Detailed analyses of rAAV production using the new packaging and purification tools in comparison with standard protocols are presented.

INTRODUCTION

VECTORS DERIVED from the adenoassociated virus type 2 (AAV-2) have gained increasing attention as an alternative to the more established retrovirus and adenovirus vector systems. AAV-2 is a helper-dependent human parvovirus that requires coinfection by an adenovirus or herpesvirus for productive infection in cell culture (for review see Berns and Bohensky, 1987). In the absence of a helper virus, AAV-2 is able to integrate stably into the host cell genome (Cheung *et al.*, 1980; Laughlin *et al.*, 1986) at a preferred site within chromo-

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some 19 (Kotin *et al.*, 1990; Samulski *et al.*, 1991). The virus has a physically stable capsid of approximately 25 nm in diameter harboring a 4.7-kb single-stranded DNA genome. The genome encodes several nonstructural proteins (Rep78, Rep68, Rep52, Rep40) and structural proteins (VP1, VP2, VP3) that are expressed from three promoters (p5, p19, p40) at map unit positions 5, 19, 40. These proteins are required *in trans* for replication and encapsidation of the viral genome. Two identical inverted terminal repeats (ITRs) at the genome ends are necessary and sufficient *in cis* for replication, packaging, integration, and rescue of the genome (Samulski *et al.*, 1989).

Recombinant AAV-2 is attractive as a gene transfer vector for several reasons. So far, the wild-type (wt) virus has not been associated with any disease in humans. AAV-2 vectors contain no viral genes and cause only a mild immune response, allowing long-term gene expression of foreign genes in several tissues and also in immunocompetent animals (Conrad *et al.*, 1996; Kessler *et al.*, 1996; Xiao *et al.*, 1996; Fisher *et al.*, 1997; Halbert *et al.*, 1997; Herzog *et al.*, 1997; Snyder *et al.*, 1997). Readministration of viral vectors is possible, however, with varying success (Fisher *et al.*, 1997; Halbert *et al.*, 1997). Transduced genes are able to integrate into the host cell genome, although with a rather low frequency and not at a preferred site in contrast to wtAAV-2 (Walsh *et al.*, 1992; Russell *et al.*, 1994; Kearns *et al.*, 1996; Malik *et al.*, 1997; Ponnazhagan *et al.*, 1997). The expression of one of the large Rep proteins, either Rep78 or Rep68, is required for an enhancement of integration frequency and directing the vector DNA to a preferred integration site (Balague *et al.*, 1997; Surosky *et al.*, 1997). Recombinant AAV-2 is able to transduce dividing and nondividing cells (Kaplitt *et al.*, 1994; Podsakoff *et al.*, 1994; Russell *et al.*, 1994; Alexander *et al.*, 1996; McCown *et al.*, 1996); however, the extent to which S phase influences transduction efficiency is still a matter of controversy (Flotte *et al.*, 1994; Podsakoff *et al.*, 1994; Russell *et al.*, 1994). One limiting step in gene transduction and expression is the conversion of the single-stranded vector DNA, as delivered by the recombinant virus to the infected cell, into double-stranded DNA from which gene expression can occur (Ferrari *et al.*, 1996; Fisher *et al.*, 1996a). This step can be influenced by several genotoxic agents, irradiation, heat treatment, and certain adenovirus genes (Russell *et al.*, 1995; Alexander *et al.*, 1996; Ferrari *et al.*, 1996). Other processes such as nuclear uptake of vector DNA possibly also limit gene transduction (Weitzman *et al.*, 1996). The phosphorylation status of a cellular protein that binds to a sequence element close to the inverted terminal repeats (the D sequence) correlates with the efficiency of transduction of different cell types and mouse tissues by recombinant AAV-2 (rAAV-2) (Qing *et al.*, 1998).

A major limitation for the use of AAV-2 in clinical gene therapy protocols is the difficulty of producing high-titer vector stocks without contamination with helper virus and wtAAV-2. The standard protocol for generation of recombinant AAV-2 involves cotransfection of cells with a vector DNA, i.e., a transgene flanked by the AAV-2 ITRs, and a packaging plasmid expressing the AAV-2 *rep* and *cap* genes (without ITRs) followed by overinfection with adenovirus at a low multiplicity of infection (MOI) (Samulski *et al.*, 1989). Using this method it is possible to generate fairly high titers of recombinant AAV-2 (up to 10^9 transducing units/ml; Li *et al.*, 1997); however, the stocks are contaminated with helper virus and often with rather high amounts of "wild type-like" AAV-2 particles. Contaminations with wtAAV-2 can be avoided using modified helper constructs that express the *rep* and *cap* genes in opposite orientations (Allen *et al.*, 1997). Several improvements of this basic method have been described that include the development of stable packaging cell lines (Clark *et al.*, 1995; Tamayose *et al.*, 1996), optimization of DNA transfection protocols (Mamounas *et al.*, 1995; Fisher *et al.*, 1996b), creation of producer cell lines with integrated vector sequences (Clark *et al.*, 1995; Flotte *et al.*, 1995; Conway *et al.*, 1997), and the use of packaging plasmids with elements allowing amplification of the packaging plasmid during vector production (Chiorini *et al.*, 1995). A high copy number of *rep* and *cap* genes seems to increase vector production efficiency (Fan and Dong, 1997). Also, replacement of AAV promoters by stronger heterologous promoters to increase *rep* gene expression (Flotte *et al.*, 1995) or *cap* gene expression (Vincent *et al.*, 1997) has been reported to yield higher rAAV-2 titers. However, this was contradicted by experiments in which rAAV-2 titers were reduced as a consequence of Rep overexpression (Li *et al.*, 1997). The use of Ad/AAV-2 hybrid vectors and herpes simplex virus type 1 (HSV-1)/AAV-2 hybrid amplicons has been reported (Thrasher *et al.*, 1995; Fisher *et al.*, 1996b; Johnston *et al.*, 1997) whereby the ITRs containing AAV vector sequences were introduced into cells via the adenoviral vector or the HSV-1 amplicon, respectively. Rep and VP proteins were provided in this system either by cotransfection or by codelivery of a *rep/cap* expression plasmid attached to the adenoviral vector capsid. Finally, replication and packaging functions can also be provided by HSV-1 amplicons (Conway *et al.*, 1997; Johnston *et al.*, 1997), yet resulting rAAV-2 titers were comparable to those from cotransfection procedures. It has been claimed that these improvements can yield between 2- and 245-fold higher vector titers as compared with the basic protocol. However, a correct comparison is not possible in most cases owing to the different methods used for rAAV-2 titration. Triple transfection protocols for the production of recombinant AAV-2 in the absence of helper virus have been described, in which the adenovirus-derived helper functions are provided on a separate plasmid (Ferrari *et al.*, 1997; Salvetti *et al.*, 1998; Xiao *et al.*, 1998).

Only a few methods for AAV-2 purification have been described. The standard procedure for vector purification and concentration involves two to three consecutive CsCl density gradient centrifugations (Snyder *et al.*, 1996). This method is elaborate and is usually associated with significant losses of infectious titer. A novel chromatography method involving sulfonated cellulose has been described, allowing concentration and at least partial purification in one step (Tamayose *et al.*, 1996).

Here we describe a simple and efficient method for rAAV-2 production consisting solely of the cotransfection of two plasmids. This method eliminates the helper virus infection step and thereby yields rAAV stocks free of contaminating helper virus. Importantly, rAAV vector stocks prepared using this method are also free of wtAAV-2 contamination. In addition, we report the establishment of a single-step affinity purification for AAV-2 particles (wild type and recombinant) that exploits the properties of a monoclonal antibody highly specific for AAV-2 capsids.

MATERIALS AND METHODS

Construction of recombinant AAV/Ad plasmids

Plasmid pΔTR, which was used in this study as a standard rAAV-packaging helper plasmid, has been previously described (Weger *et al.*, 1997). Plasmid pMA, which contains the AAV-2 *rep* and *cap* genes with the Rep78/68-encoding gene under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter, was constructed by ligating a *Bam*HI/*Hin*dIII MMTV-LTR fragment from pUC8MMTV (kindly provided by M. von Knebel-Doeberitz, University of Heidelberg) into the *Bam*HI/*Hin*dIII sites of pBluescriptII SK+, yielding pBSM. Next, two sites for the restriction enzymes *Bpu*AI and *Eco*RV were introduced downstream of the MMTV-LTR by annealing and ligating the oligonucleotides 5′-GAAGACGCCCGAGCGCGATATC-3′ and 5′-AGCTGATATCGCGCTCGGGCGTCTTCTGCA-3′ into pBSM between the *Pst*I and *Hin*dIII sites (the annealed oligonucleotides formed a double-stranded molecule with *Pst*I/*Hin*dIII compatible ends), resulting in plasmid pBSMBE. This cloning allowed insertion of the AAV-2 *rep* and *cap* genes from pTAV2-0 (Heilbronn *et al.*, 1990) as *Ava*I/*Hin*dIII and *Hin*dIII/*Sna*BI fragments (AAV-2 bp 263–4491) into pBSMBE cut with *Bpu*AI/*Eco*RV (the *Bpu*AI/*Ava*I and *Eco*RV/*Sna*BI pairs having compatible ends), to yield pMA.

To produce AAV/Ad hybrid plasmids containing the entire Ad-5 genome but having the E1 region replaced by the AAV-2 *rep* and *cap* genes, we followed a protocol based on homologous recombination in *Escherichia coli* as described by Chartier *et al.* (1996). First, a *Xba*I/*Cla*I fragment from pMA containing the MMTV-*rep*/*cap* cassette was cloned into the *Xba*I/*Cla*I sites of the Ad-5 E1-shuttle plasmid pAdRSVβgal (Stratford-Perricaudet *et al.*, 1992), resulting in the plasmid pAdMA. Plasmid pAdRSVβgal contains an RSVlacZ expression flanked by Ad-5 map units 0–1.0 and 9.4–18. In the context of the genuine Ad-5 genome, these sequences flank the E1 region. Likewise, in plasmid pAdMA, the MMTV-*rep*/*cap* cassette is flanked by these Ad-5 sequences. Next, an *Msc*I/*Nsi*I fragment from pAdMA containing the entire MMTV-*rep*/*cap* cassette and a significant amount of the adjacent Ad-5 sequences (Ad-5 bp 288–456 and bp 3384–4477) was isolated. A fivefold molar excess of this fragment was transformed into *E. coli* BJ5183 together with *Cla*I-linearized plasmid pTG3602 (Chartier *et al.*, 1996). Recombination between the overlapping Ad-5 sequences present on the two transformed fragments resulted in exchange of the MMTV-*rep*/*cap* cassette for the E1 region (Fig. 2). Plasmid DNA was isolated and screened for successful recombinations. One of several plasmids that contained the MMTV-*rep*/*cap* cassette was transformed into *E. coli* DH5α for amplification and termed pTGMA.

Plasmid pDG was derived from pTGMA after deleting 18323 bp of Ad-5 sequences not required for rAAV production. First, 1878 bp of the E3 region from pTGMA were removed by homologous recombination. An 8470-bp *Bgl*I fragment from the E3-depleted Ad-5 mutant dl324 (Thimmappaya *et al.*, 1982) containing an E3 deletion (1878-bp *Xba*I fragment) and adjacent sequences was isolated. This fragment was used together with *Spe*I-linearized pTGMA (*Spe*I cuts once near the E3 region) in a recombination experiment as described above. One of the positive clones, i.e., a derivative of pTGMA that lacked the E3 region, was expanded and termed pTGMAΔE3. Second, a large region from the Ad-5 late (capsid) genes (16425 bp) was deleted by cutting pTGMAΔE3 with *Cla*I and *Sgf*I. Since deletion of this fragment also removed the VA (virus-associated) I/II genes, which are necessary for efficient rAAV production, these genes were polymerase chain reaction (PCR)-amplified from pTG3602 using the primers 5′-GCTCCA**ATCGAT**GCATGTCCTTGGGTCCGGCCTGCTG-3′ and 5′-CACCTG**GCGATCG**CCCTGGTTCACCGTCTGCTCGTATGCG-3′. These primers were designed so that the PCR product contained *Cla*I and *Sgf*I (boldface letters) sites at the 5′ and 3′ end, respectively. Conditions for PCR were 2 m*M* $MgCl_2$, 1 m*M* dNTPs, and 30 cycles of 1 min each at 95, 68, and 72°C using Deep Vent DNA polymerase (New England BioLabs, Schwalbach, Germany). The PCR product (1724 bp) was digested with *Cla*I and *Sgf*I and inserted into the ClaI/SgfI sites of pTGMAΔE3, resulting in pDG.

A third plasmid similar to pTGMA, but having the Rep78/68-encoding gene under the transcriptional control of the genuine AAV-2 p5 promoter as opposed to the MMTV-LTR promoter, was also derived from pTG3602. The MMTV-LTR in pAdMA was substituted with the AAV-2 p5 promoter by ligating an *Xba*I/*Sal*I fragment from pΔTR (AAV-2 bp 191–1428) into the *Xba*I/*Sal*I sites of pAdMA. Recombination with pTG3602 was performed as described above, using *Cla*I for linearization of pTG3602. In addition, the E3 region was also deleted from the resulting plasmid as described for pTGMAΔE3, except that *Srf*I instead of *Spe*I was used for linearization, resulting in plasmid pTGAA.

All recombinant AAV/Ad plasmids proved to be stable using standard conditions and methods for growth of transformed bacteria and isolation of plasmid DNA. Since the plasmid yields were reduced when bacterial cultures were started from glycerol stocks, freshly transformed bacteria were always used for the preparation of the AAV/Ad plasmids.

Production and titration of recombinant AAV-2 vectors

Generation of AAV-2 vectors using the standard protocol as described by Samulski *et al.* (1989) involved transfecting 70% confluent 293 cells (in a 6-cm dish, in Dulbecco's Modified Eagles Medium [DMEM] supplied with 10% fetal calf serum [FCS], penicillin, and streptomycin) with 3 μg of the packaging plasmid pΔTR and 3 μg of pTRUF3, an rAAV vector plasmid encoding the green fluorescent protein (GFP) and the neomycin resistance gene (Zolotukhin *et al.*, 1996). Tranfections were carried out using the modified calcium phosphate precipitation protocol according to Chen and Okayama (1987). The cells were infected with Ad-5 at an MOI of 10 for 1 hr after transfection and further incubated for 3 days. For production of AAV vectors using the helper virus-free protocol, the cells were cotransfected with 3 μg of either pTGMA, pTGAA, or pDG and 3 μg of pTRUF3. The amounts of each plasmid were further varied in experiments described in this article. Culture medium was changed after transfection and the cells further incubated for 3 days (or for varying periods of time, as described below). For preparation of rAAV, the cells were lysed via three cycles of freezing and thawing. The cell lysates were centrifuged at 6000 × *g* for 10 min to remove cell debris. If the

standard protocol for rAAV production, which involves overinfection with Ad-5 had been used, the lysates were also incubated at 56°C for 30 min to inactivate the helper adenovirus.

To determine a "replicative titer" of AAV vectors, HeLa cells (5×10^3 cells per well in 96-well plates) were infected with 10-fold serial dilutions of rAAV stocks. In an end volume of 200 μl, the cells were then infected with wild-type Ad-5 and AAV-2 at MOIs of 100 and 10, respectively, to allow amplification of the rAAV vectors. After 3 days, the cells were lysed via two rounds of freezing and thawing and the addition of 120 μl of 1.5 *M* NaOH to each well. Two hundred microliters of each lysate containing the replicated rAAV vector DNA was blotted onto a GeneScreen membrane using a HybriDot manifold (BRL, Eggenstein, Germany). The membranes were ultraviolet (UV) cross-linked, prehybridized in 7% sodium dodecyl sulfate (SDS), 125 m*M* NaH_2PO_4, 125 m*M* Na_2HPO_4, 0.25 *M* NaCl, 1 m*M* EDTA, and 45% formamide for 1 hr, and then hybridized at 42°C with a 731-bp *Not*I fragment from pTRUF3. Nonradioactive DNA probes were generated using the digoxigenin (DIG) labeling kit (Boehringer GmbH, Mannheim, Germany). Filters were washed four times at 42°C in 2× SSC–0.1% SDS (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) for 5 min and twice at 68°C in 0.1 x SSC–0.1% SDS for 20 min and then exposed to Kodak X-Omat films (Eastman Kodak, Rochester, NY).

Analyses of viral DNA replication and protein expression

rAAV replicative DNA forms were detected using the Hirt DNA isolation technique (Hirt, 1967), followed by Southern blot analysis using 0.7% agarose gels and DIG-labeled rAAV vector probes (*Not*I fragment from pTRUF3). Hybridization was performed as described above.

For detection of AAV-2 Rep and VP protein expression after transfection of the various helper plasmids, cells were washed with phosphate-buffered saline (PBS) and then lysed in 2 m*M* EDTA, 100 m*M* Tris-HCl (pH 8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue. Aliquots were subjected to gel electrophoresis using 15% SDS-polyacrylamide gels and Western blotting onto Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The filters were blocked overnight at 4°C or for 60 min at room temperature in PBS containing 12% skimned milk powder and 0.3% Tween 20 (blocking solution) and then incubated for 2 hr at room temperature with the monoclonal antibody 303.9 (diluted 1:5 in blocking solution; Wistuba *et al.*, 1995) or B1 (diluted 1:10; Wistuba *et al.*, 1995) for detection of Rep and VP proteins, respectively. The membranes were washed six times in PBS, 0.3% Tween 20 for 5 min and then incubated with a peroxidase-coupled goat anti-mouse antibody (Dianova, Hamburg, Germany; diluted 1:4000 in blocking solution) for 1 hr at room temperature. After washing, Rep and VP proteins were visualized using an enhanced chemiluminescence detection kit (Amersham, Braunschweig, Germany).

To detect Rep and VP proteins and AAV-2 capsids in double immunofluorescence, small aliquots of the transfected cells were washed with PBS and spotted onto poly-L-lysine-coated slides according to the manufacturer's protocol (Bio-Rad, Munich, Germany). The samples were then fixed via incubation in methanol and acetone (each 5 min at 4°C) and incubated with a polyclonal rabbit serum recognizing VP proteins (Ruffing *et al.*, 1992) diluted 1:50 in the 76.3 monoclonal antibody-containing supernatant (which detects Rep78 and Rep52) or the A20 supernatant (for detection of AAV-2 capsids; Wistuba *et al.*, 1997) for 2 hr at room temperature. The cells were washed five times in PBS for 5 min and then incubated with Texas red isothiolyanate (TRITC)-coupled anti-mouse antibody and fluorescein isothiocyanate (FITC)-coupled anti-rabbit antibody (Dianova) diluted 1:100 in PBS, 0.1% bovine serum albumin (BSA) for 1 hr at room temperature. After washing, the slides were air dried and embedded in a solution containing 0.12% polyvinyl alcohol, 30% glycerol, and 0.01% sodium azide in PBS.

Purification of AAV vectors by immunoaffinity column chromatography

Monoclonal antibody A20 was purified from 400 ml of A20 hybridoma supernatant, using the Dianova quick antibody immunoglobulin purification kit DIAQA2K according to the manufacturer's instructions. After washing the antibody purification columns, 10 fractions each of 1 ml were eluted using 20 m*M* Na_2CO_3, 150 m*M* NaCl, and the protein concentration in each fraction determined. Five fractions containing 10 mg (2 mg/ml) total protein were pooled and dialyzed overnight at 4°C against 500 ml of 0.2 *M* $NaHCO_3$, 0.5 *M* NaCl (coupling buffer). Purified A20 was then coupled to 1 ml of either CnBr-activated Sepharose or *N*-hydroxysuccinimide (NHS)-activated HiTrap-Sepharose (Pharmacia, Freiburg, Germany) according to the manufacturer instructions. Determination of protein content in the various fractions obtained from the coupling protocol revealed that approximately 8–9 mg of purified A20 had successfully been coupled to 1 ml of either Sepharose substrate. Prior to purification of AAV stocks, the columns were equilibrated using PBS. For small-scale evaluation of binding of AAV-2 virions to A20 immobilized on CnBr-activated Sepharose, aliquots of A20-Sepharose (as indicated in Fig. 7) were incubated with 2×10^7 infectious wtAAV-2 units for 3 hr at room temperature. AAV-2 virions were eluted using 0.2 *M* glycine pH 2.7. Flow-through, wash, and elution fractions were precipitated using trichloroacetic acid (TCA, 15% final concentration) and analyzed via Western blotting using the B1 antibody for the detection of VP proteins. For evaluation of conditions for elution of AAV-2 virions from A20 immobilized on either CnBr-activated Sepharose or NHS-activated HiTrap-Sepharose, 5×10^7 or 5×10^8 (as indicated in Results) wtAAV-2 particles were incubated with the two A20-Sepharose columns for 3 hr at room temperature. AAV-2 virions were eluted under various conditions as described in Results (Fig. 7 and Table 2), precipitated with TCA (final concentration 15%), and analyzed via Western blot using antibody B1 for VP detection. Alternatively, the eluted particles were pelleted through a sucrose cushion (Ruffing *et al.*, 1992) and titrated by the replication assay described above. For larger scale purification experiments, rAAV- or wtAAV-containing supernatants were diluted 1:10 in PBS, applied to A20-HiTrap columns, and slowly recirculated at 4°C overnight using a peristaltic pump (Pharmacia). The columns were washed twice with 3 ml of PBS and once with 4 ml of PBS. Elution of AAV-2 particles bound to the columns was achieved using 2.5 *M* $MgCl_2$, 50 m*M* Tris-

HCl (pH 7.0). The columns were washed with 10 ml of PBS and stored at 4°C in PBS containing 0.01% sodium azide for future purifications.

RESULTS

Generation of a hybrid AAV/Ad plasmid

A plasmid containing all of the AAV and adenovirus (Ad) genes required for the production of rAAV was generated using the *in vitro* recombination system described by Chartier *et al.* (1996). The main objective in creating this AAV/Ad hybrid plasmid was to generate a recombinant adenovirus containing the AAV-2 *rep* and *cap* genes [rAd(rep/cap)]. Since constitutive high expression of Rep78/68 mediated by the AAV-2 p5 promoter was expected to prevent replication of an rAd(rep/cap), a set of plasmids was constructed that contained ITR-depleted AAV-2 genomes with the p5 promoter replaced by various inducible promoters. Among these plasmids, plasmid pMA (Fig. 1A) carrying an MMTV-LTR exhibited a low basal level of Rep78/68 expression after transfection, which could be induced to normal levels by treating the transfected cells with dexamethasone (data not shown). In the absence of dexamethasone, expression of Rep78/68 was significantly lower in cells transfected with pMA relative to cells transfected with a plasmid that contained an ITR-depleted AAV-2 genome with the genuine p5 promoter (pΔTR; Fig. 1B). Expression of Rep52/40 was similar following transfection of pΔTR and pMA, whereas VP expression from pMA was slightly reduced. The MMTV-*rep/cap* expression cassette from pMA was subcloned into an adenoviral E1 shuttle plasmid (pAdRSVβgal; Stratford-Perricaudet *et al.*, 1992), yielding plasmid pAdMA (Fig. 2A). The MMTV-*rep/cap* cassette of pAdMA was then substituted for the E1 region of plasmid pTG3602, which contains the entire Ad-5 genome, via homologous recombination as described in Materials and Methods. Thus, plasmid pTGMA was finally obtained (Fig. 2A).

Helper virus-independent generation of recombinant AAV-2

Following the initial goal, namely the generation of an rAd(*rep/cap*), 293 cells were transfected with linearized pTGMA and the cells overlaid with agarose, as recommended by Chartier *et al.* (1996). Although several Ad plaques appearing 6–20 days after transfection could be isolated, generation of a recombinant Ad containing an intact AAV-2 *rep* gene was not successful. Western blot analyses of 293 cells transfected with either pΔTR or pTGMA showed that transfection of pTGMA resulted in rather strong expression of AAV-2 proteins, including Rep78/68 (Fig. 2B), which may explain the difficulty in generating an rAd(*rep/cap*). Importantly, the high expression of Rep and in particular VP proteins from pTGMA occurred without additional overinfection of the transfected cells with helper adenovirus. This led to the idea that pTGMA could be used as a novel type of helper/packaging plasmid allowing helper virus-independent generation of rAAV. In contrast to the standard protocol, which involves cotransfection of an rAAV vector plasmid and a *rep/cap* expression plasmid as well as additional overinfection with a helper virus (usually adenovirus), rAAV production following the new protocol would simply require the cotransfection of a vector and an AAV/Ad helper/packaging plasmid, such as pTGMA (Fig. 3). Indeed, helper virus-independent generation of rAAV particles could be achieved in packaging reactions performed using the pTGMA helper/packaging plasmid as shown below. The most important benefit of this novel packaging procedure, besides its simplicity, was supposed to be that no helper virus should be generated that otherwise must be removed from the rAAV vector preparation.

Deletions in pTGMA further increased the safety and efficiency of rAAV production

Although transfection of 293 cells with pTGMA did not lead to generation of an rAd(*rep/cap*), it sometimes resulted in the

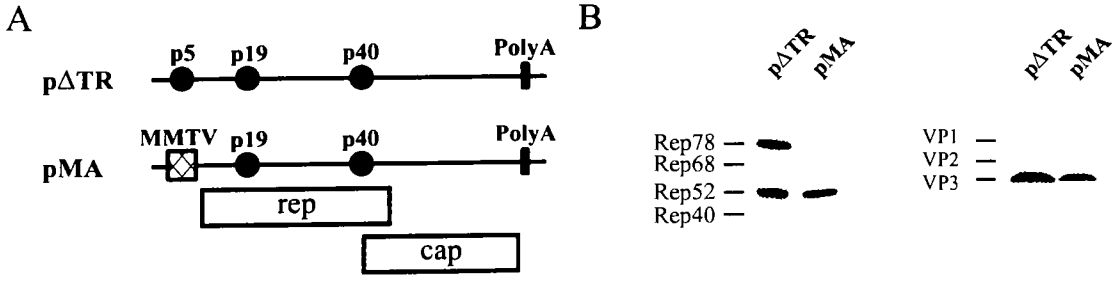

FIG. 1. Structure and characterization of plasmid pMA. (**A**) Plasmid pMA was derived from plasmid pΔTR by exchanging the AAV-2 p5 promoter for an MMTV-LTR promoter fragment as described in Materials and Methods. Generation of plasmid pΔTR has been described previously (Weger *et al.*, 1997). (**B**) Western blot analysis of 293 cells transfected with pΔTR or pMA and overinfected with Ad-5 at a multiplicity of infection (MOI) of 10. AAV-2 proteins were detected using monoclonal anti-Rep (303.9) or anti-VP (B1) antibodies. The positions of the Rep and VP proteins were routinely determined by comparison with the positions of these polypeptides in an extract from Ad-5/AAV-2-coinfected HeLa cells (not shown). Blots were developed using peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection. MMTV, Mouse mammary tumor virus-long terminal repeat promoter; p5, p19, p40, promoters of AAV-2; PolyA, polyadenylation signal of AAV-2. *rep* and *cap* indicate the regions of the AAV-2 genome coding for the replication (Rep) and capsid (VP) proteins, respectively.

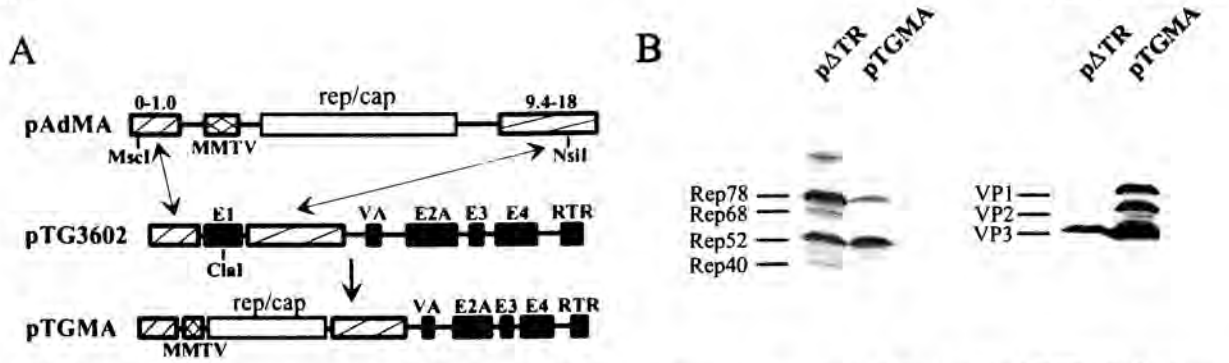

FIG. 2. Generation and characterization of pTGMA. **(A)** Plasmid pAdMA was generated by ligating the MMTV-rep/cap cassette from pMA into an Ad-5 E1 shuttle plasmid. In pAdMA, the MMTV-rep/cap cassette is flanked by Ad-5 map units 0–1.0, which include the Ad-5 left terminal repeat and the Ad packaging sequence, and map units 9.4–18, respectively. These sequences are also present on plasmid pTG3602, which contains the entire Ad-5 genome (Chartier *et al.*, 1996). Homologous recombination between the two fragments in *E. coli* BJ5183 (as indicated by arrows) yielded plasmid pTGMA. Plasmid pTGMA contains all functions of both AAV (rep, cap) and Ad (E2A, E4, VA) essential for packaging of an rAAV vector DNA. **(B)** Western blot analysis of 293 cells transfected with pΔTR or pTGMA. Cells transfected with pΔTR were additionally overinfected with Ad-5 (MOI of 10). For detection of AAV-2 proteins see the caption to Fig. 1. RTR, Right terminal repeat of Ad-5; E1–E4, early regions 1–4 of Ad-5; VA, VA (virus-associated) I/II genes of Ad-5.

production of a detectable level of Ad that had major parts of the *rep/cap* genes removed. Since these adenoviruses would consequently contaminate rAAV stocks produced by cotransfection of pTGMA and an rAAV vector plasmid, essential parts of the Ad-5 capsid genes (bp 3309–9861 and bp 11,585–21,458) of pTGMA were deleted, yielding a truncated plasmid that could no longer produce Ad. A further deletion (1878 bp) spanned the Ad-5 E3 region, which, in addition to the Ad-5 capsid genes, is also not required for rAAV production. The resulting plasmid was termed pDG (Fig. 4). These two deletions reduced the plasmid size from 40,169 (pTGMA) to 21,866 (pDG) bp. In addition, it was of interest to determine whether or not replacement of the MMTV-LTR in pTGMA by the stronger AAV-2 p5 promoter would further increase the efficiency of rAAV production. Therefore, a third AAV/Ad plasmid, pTGAA, was generated that was derived from the E3-deleted pTGMA plasmid and in which the MMTV-LTR promoter was replaced by the p5 promoter (Fig. 4). Small-scale rAAV packaging reactions were then performed comparing pΔTR(+Ad-5) with the three AAV/Ad helper/packaging plasmids. Subsequent analyses included the levels of DNA replication, Rep and VP protein expression, capsid formation, and titers of infectious recombinant AAV particles produced. The titers of rAAV produced using the different helpers were determined by end-point dilution in a replication assay. Cotransfection of pDG and the vector plasmid pTRUF3 (Zolotukhin *et al.*, 1996) yielded the highest rAAV titers of up to 500 infectious units per transfected cell, these being slightly higher than those obtained using the parental pTGMA plasmid (Table 1). On the other hand, transfection of pTRUF3 and either the stan-

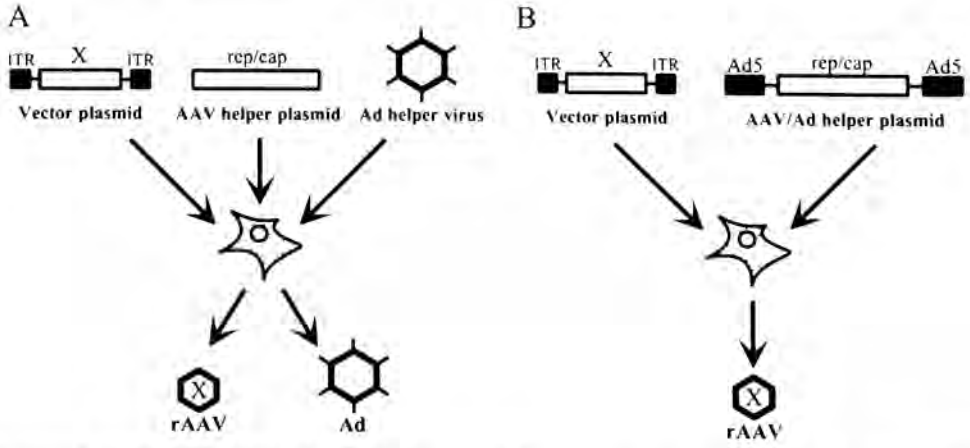

FIG. 3. Packaging of rAAV vector DNA. **(A)** Standard protocol. Recombinant AAV particles are generated by cotransfection of cells, e.g., 293 cells, with an rAAV vector plasmid and a packaging plasmid encoding the AAV-2 Rep and VP proteins. In addition, the cells must be overinfected with a helper virus, e.g., Ad, which provides multiple functions for rAAV generation. As a consequence, the cells produce not only rAAV, but also Ad, which contaminate the rAAV stocks. **(B)** Novel protocol. rAAV particles are generated by cotransfection of 293 cells with an rAAV vector plasmid and the novel AAV/Ad plasmid. Helper virus is not produced. X, Foreign gene inserted into rAAV vector; ITR, inverted terminal repeats of AAV-2; Ad5, general for Ad-5 helper regions present on the AAV/Ad helper plasmid.

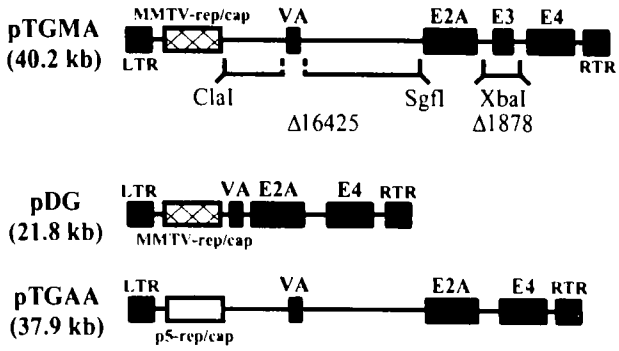

FIG. 4. Generation of plasmids pDG and pTGAA. For generation of pDG, two deletions were introduced in plasmid pTGMA covering 16,425 and 1878 bp, respectively. The first deletion covered major parts of the Ad-5 capsid genes (Δ18149). Plasmid pTGMA was cut with *Cla*I and *Sgf*I, thereby deleting the Ad-5 capsid genes as well as the VA region. The VA region, which encodes essential helper functions of Ad-5 for rAAV production, was then amplified by PCR using primers that contained *Cla*I and *Sgf*I sites and inserted into the pTGMA vector cut with *Cla*I and *Sgf*I. The second deletion (Δ1878) covered the Ad-5 E3 region. This deletion was introduced by homologous recombination. Both deletions together led to a reduction in plasmid size of approximately 18.3 kb and resulted in plasmid pDG. Plasmid pTGAA was derived from pTG3602 by exchanging the MMTV-LTR of pAdMA with the genuine AAV-2 p5 promoter and then performing a homologous recombination as described for pTGMA. In addition, the E3 region was deleted from the resulting plasmid as described above. The final size of pTGAA is 37,891 bp. Note that all three plasmids contain the essential packaging and helper functions of both AAV (*rep*, *cap*) and Ad (E2A, E4, VA) for generation of rAAV.

dard packaging plasmid pΔTR(+Ad-5) or the pTGAA hybrid plasmid led to comparable rAAV titers, which were on average 10-fold lower than those achieved using pTGMA or pDG. Plasmid pTGAA was therefore not included in all further analyses. In a second set of assays, Hirt extracts were prepared and analyzed for rAAV vector DNA replication by Southern blot analyses, using a fragment from the vector plasmid as a probe. The presence of the RF1 and RF2 replicative forms indicated that replication of the rAAV vector DNA had occurred following transfection of either packaging/helper plasmid (Fig. 5A). The signals corresponding to RF1 and RF2 were more intense in the extracts from cells transfected with pΔTR and overinfected with Ad-5. The expression of Rep and VP proteins was analyzed via Western blot and immunofluorescence assays using anti-Rep and anti-VP antibodies, respectively. Western blot analyses showed a stronger accumulation of Rep78 (and Rep68) in cells transfected with pΔTR (+ Ad-5) relative to pTGMA or pDG (Fig. 5B). On the other hand, cells transfected with pTGMA or pDG produced more Rep52 and Rep40 and, perhaps more importantly, larger amounts of VP1, VP2, and VP3 proteins than cells transfected with pΔTR(+Ad-5). Rep and VP expression as well as capsid formation efficiency were also monitored at the single-cell level via indirect immunofluorescence (Fig. 5C, summarized in Table 1). Transfection efficiencies for the three packaging plasmids were similar at about 30% as shown by Rep78/52 staining (Table 1). Yet, the fluorescence intensity corresponding to Rep78/Rep52 expression was highest in pDG-transfected cells, indicating elevated accumulation of those Rep proteins. Likewise, VP expression in pTGMA- or pDG-transfected cells was also markedly stronger than in cells

TABLE 1. AAV-2 PROTEIN EXPRESSION AND AAV/Ad PRODUCTION USING VARIOUS HELPER PLASMIDS[a]

	pΔTR	*pTGMA*	*pDG*	*pTGAA*
Rep-expressing cells (% all cells)[b]	10–30	10–30	10–30	10–30
VP-expressing cells (% Rep)[c]	37 ± 3	85 ± 5	87 ± 4	51 ± 6
Capsid-forming cells (% VP)[d]	62 ± 6	>90[e]	>90[e]	70 ± 4
Infectious particles/transfected cell[f]	1–50	50–200	50–500	1–50
Contamination with wtAAV-2[g]	5/12	0/8	0/11	n.d.
Contamination with adenovirus[h]	++	3/19[i]	0/17	n.d.

[a]All numbers given are mean values of at least four independent experiments.

[b]Percentage of cells that expressed Rep proteins after transfection of the various plasmids was estimated via immunofluorescence analyses using the 76.3 monoclonal antibody.

[c]Numbers of Rep-expressing cells also expressing VP proteins were determined via double immunofluorescence using the 76.3 anti-Rep antibody and a polyclonal anti-VP serum.

[d]Percentage of VP-expressing cells also forming AAV-2 capsids was calculated via double immunofluorescence using the A20 antibody for detection of assembled AAV-2 capsids and a polyclonal anti-VP serum.

[e]Precise values could not be determined since the diffuse and speckled patterns of the VP and the capsid stainings prevented counting of exact cell numbers. However, a minimum estimate of VP-expressing cells that formed capsids after transfection of either pTGMA or pDG was 90%.

[f]Amounts of infectious particles were determined via replication analyses as described in Materials and Methods. Infectious particles produced per transfected cell were estimated on the basis of the number of cells that stained positive for Rep expression in immunofluorescence analyses (see footnote b). The total amounts of infectious particles varied slightly depending on the conditions used for rAAV preparation (see Results).

[g]Contamination with wtAAV-2 was detected via immunofluorescence using an anti-Rep antibody (76.3) and via DNA dot-blot titration using a Rep-specific probe (see Materials and Methods).

[h]Contamination with wild-type and recombinant adenovirus was detected via dot-blot titration using 293 cells and an Ad-specific probe. All rAAV stocks produced using pΔTR contained more than 10^5 infectious Ad-5 units/ml owing to the initial overinfection of the cells with Ad-5.

[i]The contaminating Ad found in the pTGMA supernatants were recombinant Ad derived from pTGMA since they contained parts of the MMTV promoter as detected via Southern hybridization. However, the *rep* and *cap* genes were absent (data not shown).

Abbreviation: n.d., Not determined.

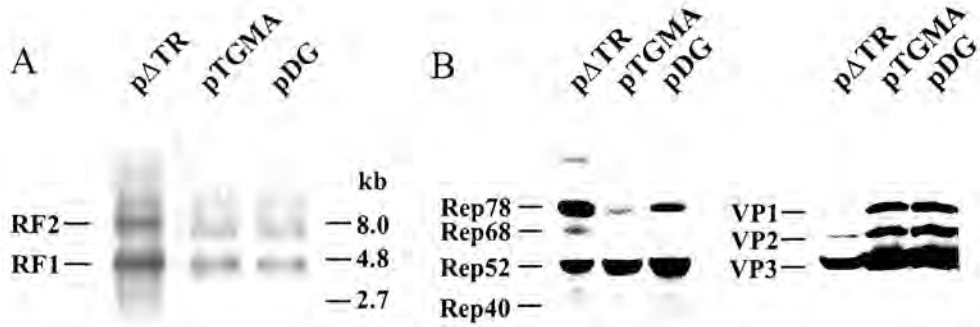

FIG. 5. Analysis of replication, gene expression, and capsid formation using either pΔTR, pTGMA, or pDG as helper plasmids. Recombinant AAV particles were generated by cotransfection of 5×10^5 293 cells with 3 μg of an rAAV vector plasmid (pTRUF3) and 3 μg of either pΔTR, pTGMA, or pDG. Where pΔTR was used to package, the cells were additionally overinfected with Ad-5 (MOI of 10). **(A)** Southern blot analysis. Low molecular weight DNA was isolated 72 hr after transfection, transferred to a nylon membrane, and hybridized with an rAAV vector probe as described in Materials and Methods. All three helper plasmids promoted replication of the rAAV vector as detected by the hybridization of the replicative DNA forms RF1 and RF2, corresponding to monomer and dimer duplex forms, respectively. The sizes (in kilobases) of several fragments from a DNA size standard also run on the gel are indicated on the right-hand side. **(B)** Western blot analysis. Transfected cells were harvested 72 hr after transfection and analyzed for Rep and VP protein expression. Proteins were detected as described in the caption to Fig. 1. **(C)** Double-immunofluorescence analysis of pΔTR(+Ad-5)- or pDG-transfected 293 cells. Expression of Rep and VP proteins as well as capsid assembly was visualized using a monoclonal anti-Rep78/52 antibody (76.3), a polyclonal rabbit anti-VP serum (Ruffing *et al.*, 1992) and a monoclonal anti-capsid antibody (A20), respectively, followed by TRITC-labeled anti-mouse and FITC-labeled anti-rabbit secondary antibodies (Dianova).

transfected with pΔTR(+Ad-5). Furthermore, following transfection of pΔTR(+Ad-5), fewer than 40% of all cells staining positive for Rep proteins were also positive for VP proteins, as detected by double immunofluorescence. On the other hand, more than 80% of Rep-positive cells also showed VP expression after transfection of pTGMA or pDG. Finally, using a monoclonal antibody that selectively recognizes AAV-2 capsids but not nonassembled capsid proteins (A20; Wistuba *et al.*, 1997), it was found that transfection of pTGMA or pDG also resulted in an enhanced AAV capsid assembly rate compared with transfection of pΔTR(+Ad-5). Using pTGMA or pDG as helper plasmids, more than 90% of the cells expressing VP proteins also stained positive for assembled capsids, while in the case of pΔTR(+Ad-5), this ratio was reduced to less than 70%. Moreover, striking differences were observed in the morphology of the immunostainings. While pΔTR-transfected cells showed weak but distinct nuclear localization of VP proteins and assembled capsids, cells transfected with pDG showed a stronger, more diffuse and speckled staining, suggesting a higher level of virus production (Fig. 5C).

rAAV stocks produced using pDG are free of Ad and wild-type AAV-2

To confirm that rAAV particles prepared by cotransfection of pDG and an rAAV vector plasmid were indeed free of contaminating Ad, replication assays of rAAV stocks and immunofluorescence studies on 293 cells transduced with rAAV were performed. Detection of Ad was carried out using an Ad-specific DNA probe (1724-bp *SalI*/*Hin*dIII fragment from pTG3602) and the Ad-specific antibody A30 (Wistuba *et al.*, 1997). 293 cells that had been infected with different dilutions of an Ad-5 virus stock were used as respective positive controls. Although 18% of the rAAV stocks produced using pTGMA were found to contain Ad, all 17 independent rAAV stocks generated using pDG were free of Ad (Table 1). Most importantly, the absence of contaminating wild-type AAV-2 (wtAAV-2) was confirmed in rAAV stocks obtained using pTGMA and pDG via dot-blot as well as in immunofluorescence analyses using an AAV-2-specific DNA probe (*Xba*I fragment from pΔTR) or an anti-Rep antibody (76.3), respectively. In contrast, various rAAV stocks generated using pΔTR(+Ad5) were heavily contaminated with wtAAV-2.

Further optimization of rAAV production using pDG as helper/packaging plasmid

To further improve rAAV production using the helper virus-free system, the effect of transfecting different ratios of packaging and vector plasmid on rAAV titers was examined. First, replication assays showed that a 1:1 molar ratio of pDG and a vector plasmid, e.g., pTRUF3, was sufficient for maximum rAAV production. Increasing this ratio further did not lead to significantly higher rAAV titers. However, using less than equimolar amounts of pDG led to a severe decline in rAAV production (data not shown). Second, the release of rAAV from the cells during the rAAV production period was analyzed. It was found that when pΔTR(+Ad-5) was used to package, more than 40% of the rAAV particles were released into the medium 24 hr after transfection. This increased further after prolonged incubation time due to the Ad-induced cytopathic effects (CPEs). Using the pDG helper/packaging

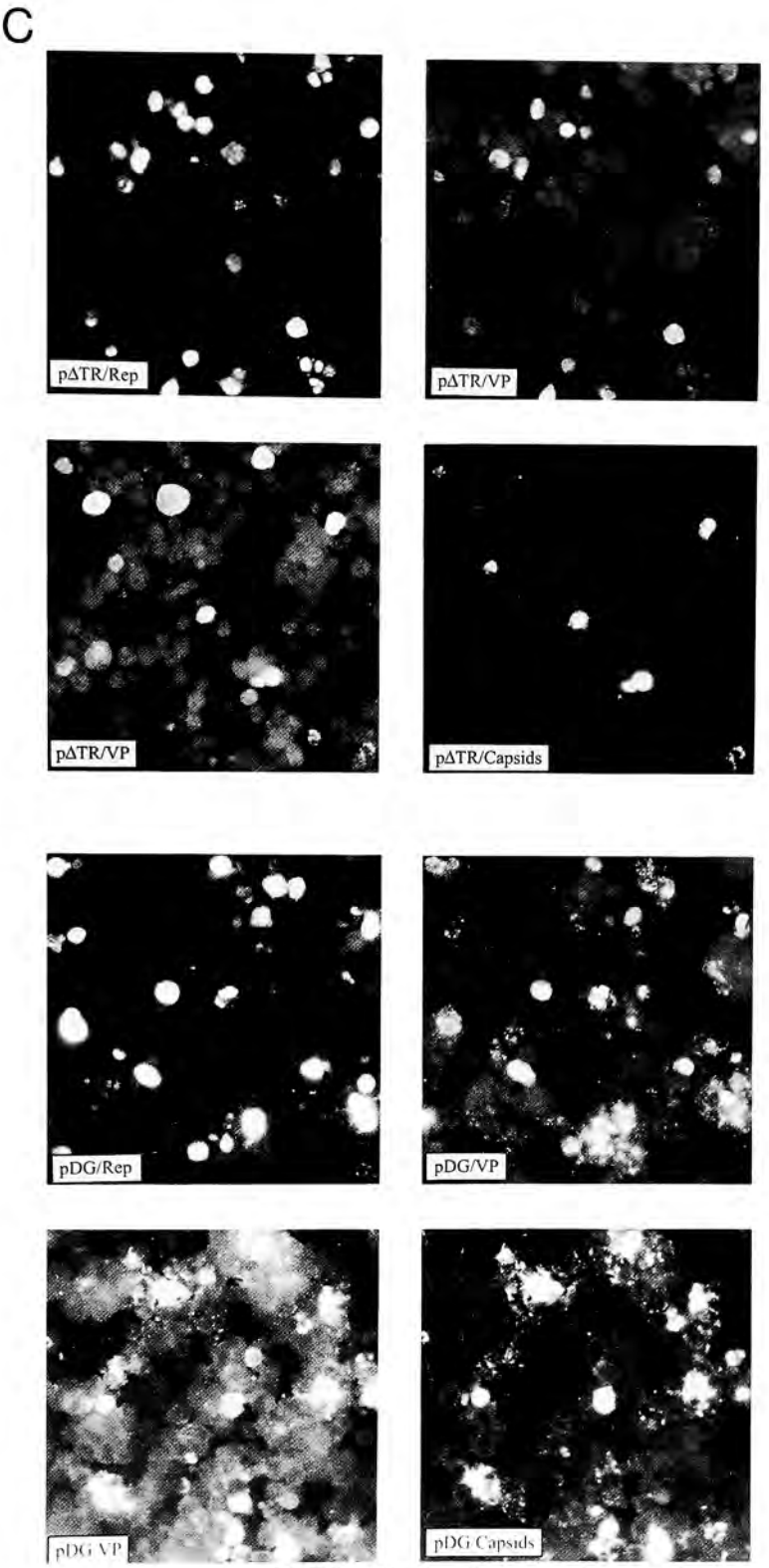

FIG. 5. *Continued.*

plasmid, more than 80% of the rAAV remained inside the cells, which never showed Ad-induced CPE even after 10 days. Furthermore, the dependence of rAAV titers on serum concentration was investigated. Significantly, rAAV titers from media lacking serum were equal to those obtained from complete (10% FCS) serum. Finally, as Ad-induced CPE was never detected following pDG transfection, the capacity of cells for accumulating rAAV over a longer period after trans-

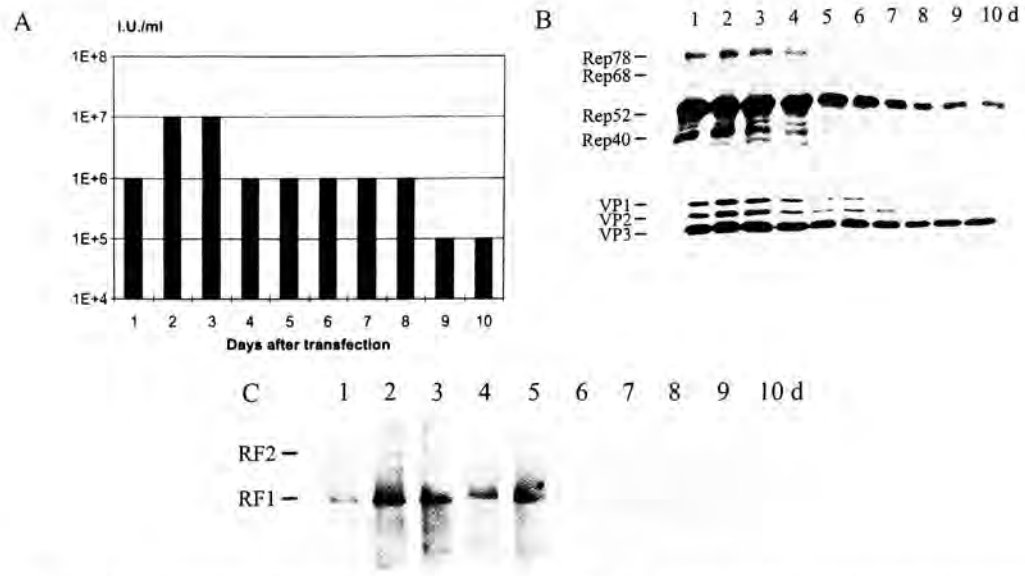

FIG. 6. Time course analysis of rAAV production. 293 cells (5×10^5 cells per 6-cm dish) were transfected with 1.5 μg of vector plasmid pTRUF3 and 4.5 μg of helper plasmid pDG. Cells were harvested daily over a 10-day period after the end of transfection and analyzed for rAAV production, Rep and VP protein expression, and rAAV vector replication. **(A)** Titration of rAAV particles by replication assay. **(B)** Western blot analysis of Rep and VP protein expression. Proteins were detected as described in the caption to Fig. 1. **(C)** Southern blot analysis of Hirt-extracted DNA. Replicative forms of the rAAV vector DNA were detected using a specific rAAV vector probe as described in Materials and Methods.

fection was tested. Small-scale packaging reactions were performed and analyzed for rAAV production each day over a period of 10 days posttransfection. Using replication assays, it was found that maximum rAAV titers were generated after 48 hr (Fig. 6A). This peak was also paralleled by Rep and VP protein expression levels and rAAV vector replication as measured by Western and Southern blot analyses, respectively (Fig. 6B and C).

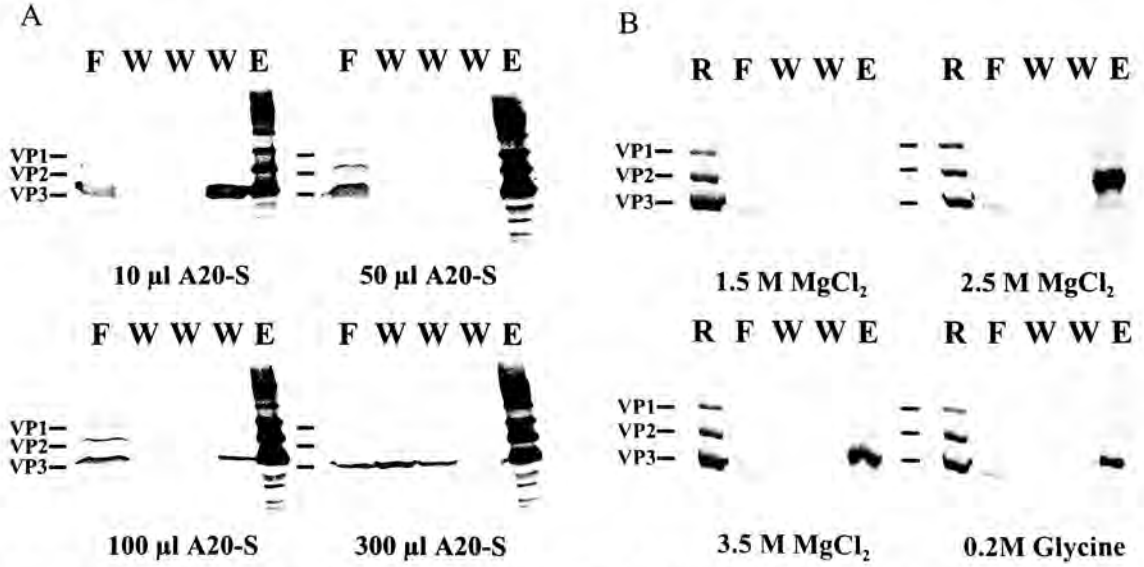

FIG. 7. Evaluation of conditions for immunoaffinity purification of AAV-2 particles. **(A)** Binding of AAV-2 particles to A20 antibody immobilized on CnBr-activated Sepharose was evaluated by incubating 2×10^7 wtAAV-2 infectious units with increasing quantities of CnBr-Sepharose, to which monoclonal antibody A20 was coupled, as indicated. AAV-2 virions were eluted using 0.2 *M* glycine, pH 2.7. **(B)** Evaluation of conditions for elution of AAV-2 particles bound to A20–Sepharose. AAV-2 particles were eluted using different conditions as indicated. The amounts of capsid proteins in the fractions obtained from the purification procedure were determined via Western blot analysis using antibody B1 after precipitation of the proteins with 15% TCA. Western blots were developed using an alkaline phosphatase-coupled secondary antibody. R, Reference AAV-2 virus stock; F, flow-through fraction; W, wash fraction; E, elution fraction.

TABLE 2. EVALUATION OF INFECTIOUS AAV-2 VIRUS RECOVERY FROM A20 IMMUNOAFFINITY COLUMNS UNDER VARIOUS ELUTION CONDITIONS

Column material	*Elution buffer*	*Infectious particle recovery*
CnBr-activated Sepharose	2 *M* $MgCl_2$ in PBS	5×10^5 (0.1%)[a]
	2.5 *M* $MgCl_2$ in PBS	5×10^7 (10%)
	1 *M* KSCN in PBS	5×10^3 (0.001%)
	0.2 *M* glycine, pH 2.7	5×10^5 (0.1%)
NHS-activated HiTrap-Sepharose	2.5 *M* $MgCl_2$ in PBS	$>3 \times 10^8$ (>65%)

[a]In all experiments, starting material was 5×10^8 infectious AAV-2 units. Purification of AAV-2 particles was performed as described in Materials and Methods. The recovery of infectious particles was determined via replication assays.

Application of the monoclonal antibody A20 for immunoaffinity purification of AAV-2 particles

The monoclonal antibody A20, which recognizes assembled AAV-2 capsids but not free capsid proteins, has been described and characterized previously (Wistuba *et al.*, 1997). This property can be exploited to affinity purify and concentrate wild-type as well as recombinant AAV-2 particles from crude virus stocks. A20 antibody was purified and coupled to Sepharose column matrices as described in Materials and Methods. First, the binding of AAV-2 particles to A20 antibody coupled to CnBr (cyanobromide)-activated Sepharose was investigated. Therefore, 200 μl of a crude wtAAV-2 virus stock, corresponding to 2×10^7 infectious units, was purified using increasing amounts of A20–Sepharose. The different fractions obtained from the purification procedures were analyzed via Western blot using the B1 antibody to detect AAV-2 capsid proteins (Fig. 7A). Ten microliters of A20–Sepharose were sufficient to bind the majority of the AAV-2 particles present in the virus stock, which could subsequently be eluted using 0.2 *M* glycine. Thus, the use of increasing amounts of A20–Sepharose of up to 300 μl resulted in only slightly higher recovery of AAV-2 particles in the elution fraction. However, under all conditions tested, a small proportion of AAV-2 capsids remained unbound and was found in the flow-through and wash fractions. Second, the conditions for eluting AAV-2 virions from the A20 affinity matrix were evaluated. Aliquots of 500 μl (5×10^7 infectious units) of a wtAAV-2 stock were incubated with A20 immobilized on CnBr-activated Sepharose and eluted under various conditions. The recovery of AAV-2 virions was determined via Western blot using the B1 antibody for the detection of VP proteins. Of the conditions tested, elution of AAV-2 particles with 2.5 *M* $MgCl_2$ was found to be sufficient to release as many capsids from the column material as when the more stringent elution conditions, including 0.2 *M* glycine, pH 2.7 (Fig. 7B), were used. The eluted and concentrated AAV-2 particles were also subjected to a replication assay to determine the amount of infectious AAV-2 units in the various elution fractions. Surprisingly, the "replicative titers" of those fractions were low and did not correlate with the abundant amounts of VP proteins observed in the respective Western blot analyses. One possible explanation for this finding was that A20 antibody, which was previously shown to neutralize AAV-2 particles, was released from the column matrix under the elution conditions used, as was observed by Western blot analysis (data not shown). Thus, the obvious difference between capsid protein amounts and numbers of infectious units in the elution fractions may result from coelution of AAV-2 virions and neutralizing A20 antibody from the affinity column.

Optimized, highly specific affinity purification and concentration of AAV-2 particles

To overcome the possible reduction of virus infectivity due to coeluting A20 antibody, a different kind of high-capacity coupling gel, namely NHS (*N*-hydroxysuccinimide)-activated HiTrap-Sepharose (Pharmacia), was used for A20 immobilization and subsequent AAV-2 purification. Wild-type AAV-2 (5×10^8 infectious units) was purified using either CnBr-activated Sepharose or NHS-activated HiTrap-Sepharose and eluted under different conditions (Table 2). The use of HiTrap-Sepharose as column material and 2.5 *M* $MgCl_2$ in PBS as elution buffer resulted in the highest recovery of infectious units in the elution fraction. Under these conditions, at least 65% of the infectious particles present in the parental virus stock could routinely be recovered and concentrated depending on the application and elution volumes (Table 2). As an example, Fig. 8 shows the purification and concentration of a crude rAAV stock derived from the vector plasmid pTRUF3 and using pDG as helper plasmid as described above. Eight milliliters of virus stock (containing 2×10^8 infectious rAAV units/ml) were slowly applied to an A20–HiTrap column by recirculating at 4°C overnight. After collecting the flow-through and washing the column with PBS, the rAAV particles were eluted in 2.5 *M* $MgCl_2$ in PBS (Fig. 8A). Virus recovery was determined via replication assays using serial dilutions of the different fractions obtained from the purification procedure. Typically, 70% of the starting material could be recovered (Fig. 8B, solid bars) and fourfold concentrated (Fig. 8B, gray bars). Pelleting of the eluted AAV-2 particles through a sucrose cushion (Ruffing *et al.*, 1992) allowed up to a further 10-fold concentration (not shown). Electron microscopy of the eluted particles revealed an excess of empty to full rAAV particles corresponding to the expected ratios of particle to infectious titers. An additional third type of particle, probably representing packaging intermediates, was also observed. The purity and homogeneity of the eluted AAV-2 particles were confirmed by separation of aliquots obtained during the purification procedure in polyacrylamide gels

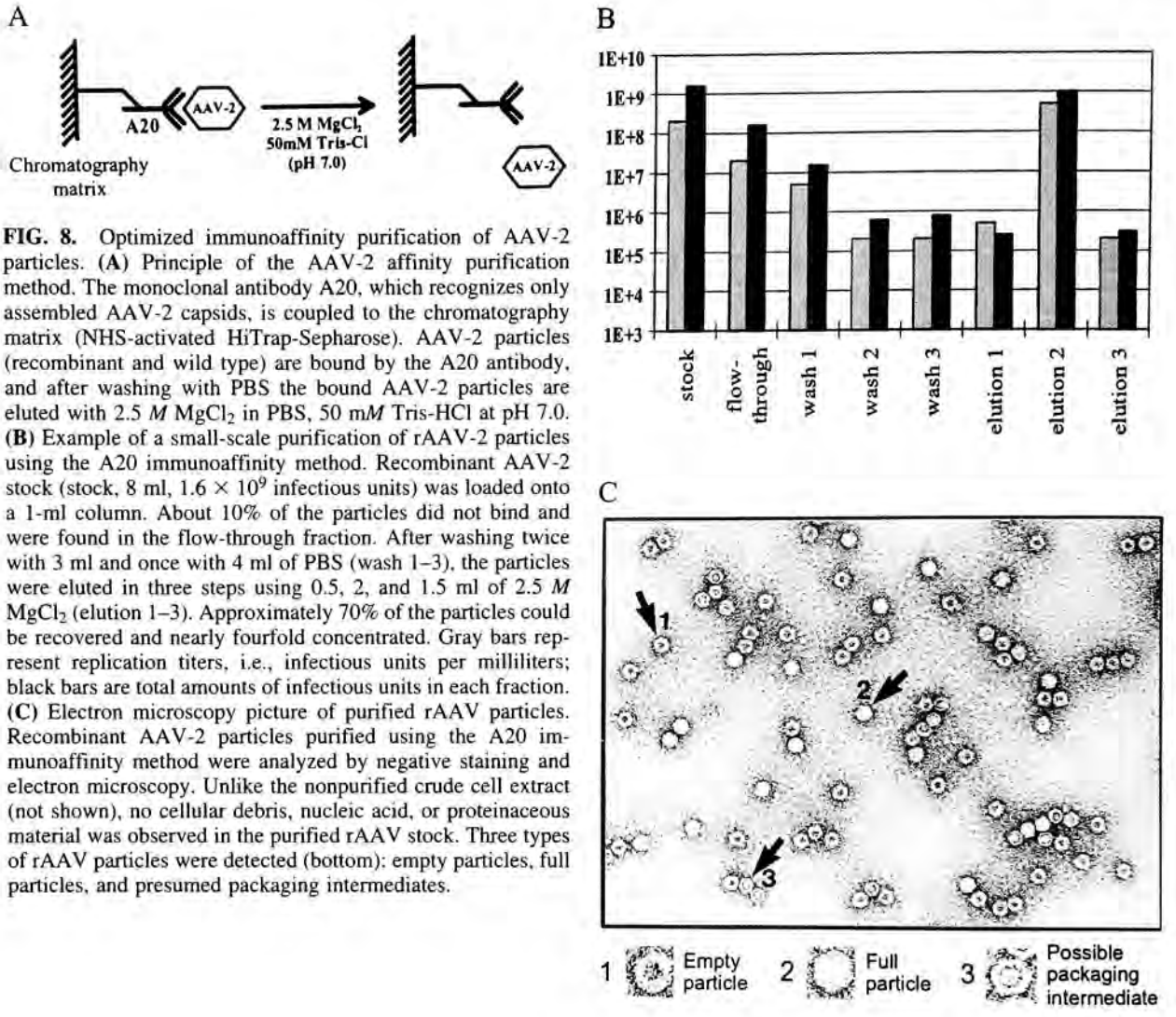

FIG. 8. Optimized immunoaffinity purification of AAV-2 particles. **(A)** Principle of the AAV-2 affinity purification method. The monoclonal antibody A20, which recognizes only assembled AAV-2 capsids, is coupled to the chromatography matrix (NHS-activated HiTrap-Sepharose). AAV-2 particles (recombinant and wild type) are bound by the A20 antibody, and after washing with PBS the bound AAV-2 particles are eluted with 2.5 *M* $MgCl_2$ in PBS, 50 m*M* Tris-HCl at pH 7.0. **(B)** Example of a small-scale purification of rAAV-2 particles using the A20 immunoaffinity method. Recombinant AAV-2 stock (stock, 8 ml, 1.6×10^9 infectious units) was loaded onto a 1-ml column. About 10% of the particles did not bind and were found in the flow-through fraction. After washing twice with 3 ml and once with 4 ml of PBS (wash 1–3), the particles were eluted in three steps using 0.5, 2, and 1.5 ml of 2.5 *M* $MgCl_2$ (elution 1–3). Approximately 70% of the particles could be recovered and nearly fourfold concentrated. Gray bars represent replication titers, i.e., infectious units per milliliters; black bars are total amounts of infectious units in each fraction. **(C)** Electron microscopy picture of purified rAAV particles. Recombinant AAV-2 particles purified using the A20 immunoaffinity method were analyzed by negative staining and electron microscopy. Unlike the nonpurified crude cell extract (not shown), no cellular debris, nucleic acid, or proteinaceous material was observed in the purified rAAV stock. Three types of rAAV particles were detected (bottom): empty particles, full particles, and presumed packaging intermediates.

and subsequent silver staining. Only minor amounts of proteins in addition to the AAV-2 VP proteins were observed, indicating a purity of the eluted and pelleted AAV-2 particles of at least 80% (data not shown).

The A20 column was also used to purify a wtAAV-2 stock, generated using Ad-5 as helper virus and thus still contaminated with Ad-5 particles. Dot-blot hybridization of the starting material and the eluted particles, using an Ad probe, showed that contaminating Ad-5 could be completely removed from the AAV-2 stock. Moreover, no differences in the efficiency of the purification method were observed when either wild-type or recombinant AAV-2 particles were purified (data not shown).

DISCUSSION

We have described novel and improved protocols for rAAV-2 production and purification. The two methods presented facilitate the still elaborate procedure of rAAV preparation at critical steps. rAAV production is performed in the absence of helper virus by a simple two-plasmid transfection procedure, while purification and concentration of rAAV particles are achieved by one-step affinity chromatography.

A large number of improvements of AAV-2 vector production have already been suggested concerning two major limiting steps, namely the transfer of vector and helper DNAs into the packaging cells and the control of vector replication and *rep/cap* gene expression. However, we attribute the increased efficiency in rAAV production observed here using the pDG helper/packaging plasmid to an improved capsid assembly and a balanced expression of adenoviral and AAV helper functions.

Capsid assembly is dependent on accumulation of high concentrations of capsid (VP) proteins in the host cell (Wistuba *et al.*, 1997). Since it is assumed that packaging of AAV genomes occurs into preformed capsids (Myers *et al.*, 1980), high expression of VP proteins is obviously crucial to provide sufficient amounts of capsids for the DNA encapsidation process. Indeed, it was observed that the low level of capsid protein expression following transfection of an ITR-minus packaging plasmid (pΔTR) resulted in capsid formation in only 30–50% of VP-expressing cells (Weger *et al.*, 1997). In contrast, this ratio was increased to more than 90% after transfection of cells

with an ITR-containing plasmid, correlating with the much stronger VP expression observed. With respect to VP expression and capsid assembly, the pDG packaging plasmid presented in this study clearly resembles an ITR-containing plasmid. As shown by Western blot and immunofluorescence, transfection of cells with pDG led to a strong increase in capsid protein expression relative to a standard helper plasmid (pΔTR) and resulted in capsid assembly in more than 90% of the VP-expressing cells. The pDG plasmid thus counteracts the deficiency in capsid formation of common ITR-minus packaging plasmids, such as pΔTR. However, we cannot fully explain why transfection of pDG, which contains the Rep78/68-encoding gene under the transcriptional control of the MMTV promoter instead of the p5 promoter, leads to stronger VP protein expression than does transfection of plasmids such as pΔTR or pTGAA, which contain the genuine AAV-2 p5 promoter. One could suppose an inverse correlation of the expression of the large Rep proteins with the expression of the capsid proteins. This idea was actually supported by two reports (Li *et al.*, 1997; Vincent *et al.*, 1997) and our own initial attempts to improve rAAV packaging, which included the generation of helper plasmids in which the p5 promoter was exchanged for heterologous stronger promoters, such as the RSV (Rous sarcoma virus) and CMV (cytomegalovirus) promoters. Indeed, overexpression of the large Rep proteins from these constructs led to severe decreases in VP expression and resulted in a loss of capsid assembly (Li *et al.*, 1997; data not shown). Moreover, increased Rep78/68 expression followed by a downregulation of VP expression and capsid assembly, and by reduction in vector titer, was also seen after treating pDG-transfected cells with dexamethasone, a drug that induces the MMTV-LTR promoter (data not shown). However, a reduction in the expression of the large Rep proteins does not necessarily increase capsid protein production, as is evident from a comparison of the plasmids pΔTR and pMA. A possible explanation for the increased VP expression from the pDG plasmid is that it potentially replicates within 293 cells, since it contains the Ad-5 terminal repeats needed for adenoviral replication. Experiments addressing this question further are currently in progress. Replication of the rAAV vector DNA was not limiting under the conditions applied here, since the use of pDG as helper led to essentially higher rAAV titers as compared with pΔTR, although Rep78/68 expression and consequently vector replication were slightly impaired after pDG transfection.

The role of helper virus functions has not been considered much so far in the attempts to improve rAAV production. The complex interactions of Ad helper functions and AAV-2 products, in particular the Rep proteins, are still poorly understood. While Ad at high MOIs strongly stimulates AAV-2 gene expression, including the *cap* gene (Weger *et al.*, 1997), it also limits the time for AAV-2 replication and assembly by inducing cell lysis. If the MOI of Ad is too low, AAV-2 proteins inhibit several adenoviral functions, resulting in decreased AAV-2 *cap* gene expression (Weger *et al.*, 1997) and reduced AAV-2 reproduction. The right amount of helper virus added is thus crucial for high-titer rAAV production. Variations in transfection efficiencies or in the determination of Ad titers or plasmid concentrations can lead to different starting ratios of Ad to AAV gene products, which could result in varying rAAV yields. These problems are all overcome by the pDG packaging plasmid, which provides a constant ratio of AAV-2 and Ad helper functions on one plasmid. An increased vector titer was also observed in reports in which the Ad helper functions were provided in a constant ratio via triple transfection of the AAV-2 vector plasmid and two helper plasmids, one coding for the adenovirus helper functions (Ferrari *et al.*, 1997; Salvetti *et al.*, 1998; Xiao *et al.*, 1998). These protocols also allowed the production of high-titer rAAV preparations independent of Ad infection; however, they required the preparation and transfection of an additional plasmid as compared with the packaging protocol using pDG.

Despite the absence of cytopathic effects using pDG, virus production and recovery declined with time (between 5 and 10 days), probably because the transfected cells died or were metabolically blocked. Only small amounts of recombinant virus were released from the 293 cells (~10–20%), also suggesting that there was no strong cell lysis. Consequently, rAAV particles produced using pDG can be harvested simply by collecting the cell pellets, thus providing a significant advantage over the Ad coinfection procedure, which involves cell lysis and release of more than 50% of the rAAV particles from the cells.

The fact that the packaging system reported here allows the production of rAAV in the absence of both helper virus and serum is most important with respect to safety issues and the use of rAAV as reagents in clinical trials. In addition, rAAV stocks generated using pDG as helper are also free of detectable wild-type AAV-2, which frequently contaminates rAAV stocks produced using standard helper plasmids. Actually, generation of replication-competent wtAAV-2 or "wtAAV-2-like" particles by nonhomologous recombination, as described by Allen *et al.* (1997), was never observed following cotransfection of pDG and a vector plasmid. One possible explanation is that the MMTV-*rep/cap* cassette in pDG, which resulted from the exchange of the AAV-2 p5 promoter for the MMTV promoter, would not be packaged efficiently into AAV-2 capsids owing to its rather large size of 5044 bp (Dong *et al.*, 1996). Another reason could be that replacing the p5 promoter, including its internal *rep*-binding site, by the MMTV promoter strongly reduced the probability for Rep-mediated recombination events that could result in generation of "wtAAV-2-like" genomes.

The AAV-2 purification method presented here involves immunoaffinity chromatography using a column to which a monoclonal antibody selectively recognizing assembled capsids, A20, is coupled. Unlike the other methods for rAAV purification, the A20 chromatography protocol allows for the fast and easy preparation of highly purified and concentrated AAV-2 stocks in a single step. Moreover, the eluates from other columns, e.g., sulfonated cellulose, contain significant contaminations from the tissue culture supernatant, and binding of the virus to these column materials was not achieved with all types of cell extracts (our unpublished observations). Immunoprecipitation of CsCl gradient fractions of wtAAV-2 sedimenting between 1.35 and 1.45 g/cm^3 showed no preference of the A20 antibody for empty particles or one of the full particle types (data not shown). Thus, the relatively large proportion of empty particles seen by electron microscopy in the affinity-purified fractions represents the average of full and empty particles in a standard vector preparation. Furthermore, we could not detect a difference in the binding of recombinant or wild-type AAV-2 particles. As in the initial titration experiment, how-

ever, we observed in all small-scale applications that up to 10% of recombinant particles did not bind to the column. This observation suggests the possibility that a subset of AAV-2 particles does not contain the epitope recognized by the A20 antibody.

In combination, the two new tools described here should significantly facilitate the production of recombinant AAV-2 vectors for preclinical and phase I clinical trials. Production of rAAV in a setting completely free of adenovirus eliminates a number of safety concerns and uncertainties in the interpretation of the biological properties of this gene delivery system.

ACKNOWLEDGMENTS

The authors thank Dr. Jason King for critical reading of the manuscript and for helpful comments. We also thank Dr. Nicholas Muzyczka for providing the AAV-2 vector plasmid pTRUF3 and Jutta Schmidt for photographical work. We are grateful to Dr. Jean-Claude Jauniaux for support in computer-aided simulation of the homologous recombination events that led to the pDG plasmid. D. Grimm was supported by BMBF Grant 01KV9517/6.

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Received for publication April 7, 1998; accepted after revision September 28, 1998.