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Helper Virus-Free, Optically Controllable, and Two-Plasmid-Based Production of Adeno-associated Virus Vectors of Serotypes 1 to 6

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We present a simple and safe strategy for producing high-titer adeno-associated virus (AAV) vectors derived from six different AAV serotypes (AAV-1 to AAV-6). The method, referred to as "HOT," is helper virus free, optically controllable, and based on transfection of only two plasmids, i.e., an AAV vector construct and one of six novel AAV helper plasmids. The latter were engineered to carry AAV serotype *rep* and *cap* genes together with adenoviral helper functions, as well as unique fluorescent protein expression cassettes, allowing confirmation of successful transfection and identification of the transfected plasmid. Cross-packaging of vector DNA derived from AAV-2, -3, or -6 was up to 10-fold more efficient using our novel plasmids, compared to a conservative adenovirus-dependent method. We also identified a variety of useful antibodies, allowing detection of Rep or VP proteins, or assembled capsids, of all six AAV serotypes. Finally, we describe unique cell tropisms and kinetics of transgene expression for AAV serotype vectors in primary or transformed cells from four different species. In sum, the HOT strategy and the antibodies presented here, together with the reported findings, should facilitate and support the further development of AAV serotype vectors as powerful new tools for human gene therapy.

Key Words: adeno-associated virus, gene therapy, serotypes, vector production

INTRODUCTION

Recombinant vectors derived from adeno-associated virus (AAV) serotype 2 are attractive tools for human gene therapy, as they are nonpathogenic and can establish long-term gene expression in a large variety of tissues [1]. This current opinion is based on almost 20 years of preclinical studies and is supported by promising results from a clinical trial with AAV-2 for the treatment of hemophilia B [2]. However, those studies have also revealed two restrictions of AAV-2 vectors that might limit their use in humans. First, the vectors are rather inefficient at transducing some therapeutically interesting targets, e.g., hepatocytes [3], and second, AAV-2 vector-mediated gene transfer might be hampered by neutralizing antibodies, which are highly prevalent in the human population due to prior exposure to the wild-type virus [4]. One promising effort to try and overcome these restrictions is based on utilizing other serotypes of AAV for vector development [5]. As demonstrated in several recent studies, "pseudotyped" AAV vectors (i.e., capsids of AAV serotypes other than 2 bearing a recombinant AAV-2 genome) exhibit host ranges largely different from AAV-2, as found in tissues such as skeletal muscle [6–8], retina [9], lung [10,11], or CNS [12] or in hematopoietic cells [13]. Moreover, AAV pseudotype particles are capable of escaping from anti-AAV-2 immune responses and, as reported for AAV-6, may even overall be less immunogenic than AAV-2 [14]. Also noteworthy is the recent isolation of two new members of the AAV family, AAV-7 and -8, with the latter holding particular promise for liver transduction [15].

Unfortunately, despite these promising results, methods for AAV serotype vector production as reported so far are laborious and hamper further exploration of the vectors. Typical protocols require cotransfection of cells with an AAV-2 vector plasmid and a helper construct encoding a capsid gene derived from an AAV serotype other than 2, as well as the *rep* gene of either the same serotype or of AAV-2 [5]. In addition, the transfected cells have to be infected with helper adenovirus, which inevitably results in contamination of the AAV vectors with a pathogenic reagent. Alternatively, adenoviral helper functions are delivered by a noninfectious DNA construct to eliminate helper virus contamination, but this requires labor- and cost-intensive preparation of an additional plasmid [e.g., 16]. In one study, AAV-2 vector pseudotyping with AAV-5

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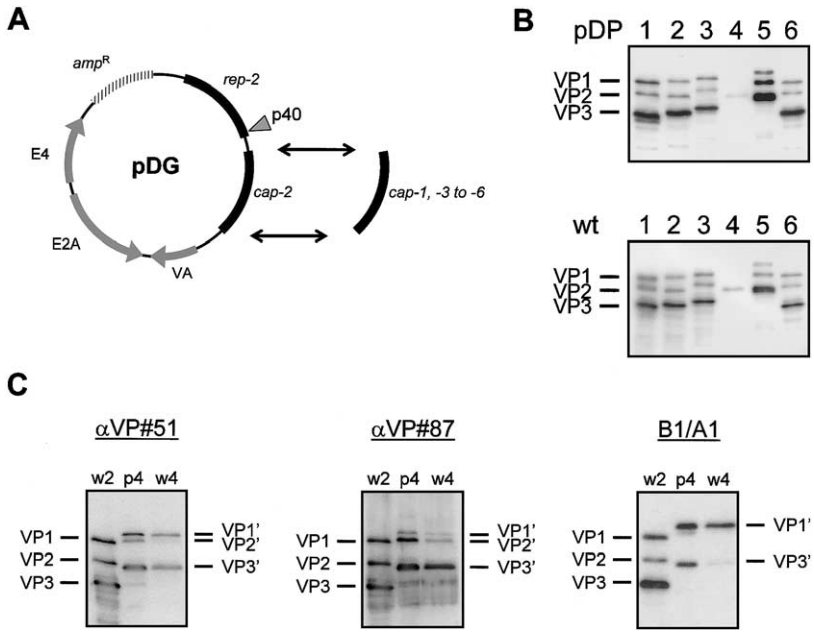

FIG. 1. Generation and characterization of novel AAV serotype helper plasmids. (A) Plasmid generation. Plasmid pDG carries all genes essential for packaging of AAV-2 vectors into capsids of AAV-2 in 293T cells, i.e., AAV-2 *rep* and *cap* genes and VA, E2A, and E4 genes of adenovirus 5 [18]. Five novel helper constructs were derived from pDG by exchanging the AAV-2 *cap* gene with *cap* of AAV serotype 1, 3, 4, 5, or 6 (see Materials and Methods). (B) AAV capsid protein expression. 293T cells were either transfected with the pDP constructs (pDP2 equals pDG; top) or infected with each of the six wild-type (wt) viruses together with helper adenovirus (bottom). Protein extracts from these cells were separated on 12% Tris–HCl gels and Western blotted with the monoclonal antibody B1 [19] for detection of AAV capsid proteins VP1, VP2, and VP3 (marked are the VP proteins of AAV-2). (C) Detection of AAV-4 capsid proteins. 293T cells were either infected with wild-type AAV-2 (w2) or AAV-4 (w4) together with helper adenovirus or transfected with pDP4 (p4). Protein extracts were gel separated and Western blotted with polyclonal anti-AAV-2-capsid protein antisera (αVP#51 and αVP#87) or a mixture of monoclonal antibodies A1 [19] and B1, detecting VP1 or all three VP proteins of AAV-2, respectively. The use of A1 confirmed that the uppermost band observed with the antisera represented AAV-4 VP1 (in contrast to B1, the A1 epitope is fully conserved in AAV-2 and -4). AAV-4 VP proteins presumably corresponding to those of AAV-2 are marked VP1′ to VP3′.

capsids involved even a fourth plasmid, since AAV-2 *rep* and AAV-5 *cap* genes were provided by two separate constructs [17]. Furthermore, none of the current protocols allow for straightforward control of plasmid DNA transfection during vector production runs, albeit this is a crucial parameter determining the final vector yields.

In the present study, we describe the development of an improved strategy for production of pseudotyped AAV vectors that overcomes the drawbacks of earlier protocols. In particular, we present a set of six novel helper plasmids, which upon simple cotransfection with an AAV vector construct allow helper virus-free and highly efficient vector cross-packaging into capsids of AAV serotypes 1 to 6. In comparison to conventional helper plasmids, we characterize our constructs for AAV protein expression, as well as their ability to support replication and encapsidation of vector templates, based on four different AAV serotypes. Furthermore, we describe the unique feature of the novel plasmids to express reporter fluorescent proteins after transfection, allowing monitoring and optimizing of DNA transfection efficiencies and particle production. We finally report results from infection of a large panel of cells with AAV vectors of serotypes 1 to 6 and demonstrate how the virus capsid not only influences cell tropisms, but also the kinetics of transgene expression.

RESULTS

Generation and Characterization of Novel AAV Serotype Helper Plasmids

We have previously reported the AAV-2 helper plasmid pDG, expressing AAV-2 *rep* and *cap* genes, as well as adenovirus type 5 genes encoding products essential for AAV-2 vector production [18]. As shown, simple cotransfection of cells with pDG and an AAV-2 vector construct is sufficient for helper virus-free generation of high-titer recombinant AAV-2 [18].

In the present study, we further developed this two-plasmid-based protocol for AAV vector pseudotyping with capsids from five other AAV serotypes, AAV-1 and AAV-3 to -6. In general, cross-packaging of AAV vector DNA requires three components (in addition to the vector genome itself): AAV-2 Rep proteins, capsid (VP) proteins from a non-type-2 AAV serotype, and adenoviral helper functions [5]. Since the pDG construct already contains AAV-2 *rep* and adenoviral helper genes, a straightforward approach to evolve this plasmid was to replace AAV-2 *cap* with the *cap* genes of the five alternative AAV serotypes. Therefore, we extracted AAV DNA from cells infected with the different wild-type AAVs and helper adenovirus and then PCR-amplified and cloned the various AAV *cap* genes into the pDG backbone. In the resulting constructs, termed pDP1 and pDP3 to pDP6 (P for pseudotyping), each non-type-2 AAV *cap* gene is under the transcriptional control of the AAV-2 p40 promoter (Fig. 1A).

To confirm correct expression of AAV serotype VP proteins, we transfected cells with the pDP plasmids or pDG and analyzed protein extracts by Western blotting. As positive controls, we included extracts from cells that were infected with the corresponding wild-type AAVs and helper adenovirus. For detection of AAV VP proteins, we used the monoclonal antibody B1 [19], which binds to an 8-amino-acid-long epitope conserved among all AAV serotypes (Fig. 1B). The only exception is AAV-4, whose VP

TABLE 1: Detection of proteins and capsids of AAV serotypes 1 to 6 with mono- or polyclonal anti-AAV-2 antibodies

	Antibody		AAV serotypes					
Name	Antigen	Ref.	1	2	3	4	5	6
76.3	Rep78, Rep52	[19]	−	++	+	−	−	−
303.9	All Rep	[19]	++	++	++	++	++	++
259.5	All Rep	[a]	++	++	++	++	++	++
294.4	All Rep	[34]	++	++	++	++	++	++
226.7	All Rep	[a]	−	++	−	−	−	−
Rep#90	All Rep	[19]	++	++	++	++	++	++
A1	VP1	[33]	+	++	+	+	+	+
A69	VP1, VP2	[19]	+	++	+	−	−	+
B1	All VP	[19]	++	++	++	−	++	++
VP#51	All VP	[33]	++	++	++	++	++	++
A20	Capsids	[19]	−	++	++	−	−	−
C24	Capsids (VP)	[31]	−	++	+	−	+	−
D3	Capsids (VP)	[31]	++	++	++	++	++	++
C37	Capsids (VP)	[31]	−	++	−	−	−	−

Rep#90, Polyclonal rabbit anti-AAV-2 Rep serum; VP#51, polyclonal rabbit anti-AAV-2 VP serum; all other names denote monoclonal anti-AAV-2 antibodies. Cells were infected with each of the six AAV serotypes, as well as helper adenovirus, and then subjected to Western blot as well as immunofluorescence analyses (see Materials and Methods for details), using the listed antibodies for detection of AAV Rep or VP proteins. Assembled AAV capsids were detected by immunofluorescence analyses only. For antibodies C24, D3, and C37, detection of nonassembled VP proteins cannot be ruled out, although these antibodies tested negative in previous Western blot analyses [31]. Categories of signal intensities were very strong (++), strong (+), or absent (−). Ref., reference.

[a]Previously unpublished monoclonal anti-AAV-2 Rep antibodies that were generated in our group as described in Ref. [19].

proteins are only poorly detected due to a 1-amino-acid exchange within the B1 epitope (arginine to histidine). Therefore, AAV-4 VP proteins were visualized using two different polyclonal anti-VP antisera, as well as the monoclonal antibody A1 [19], which binds to VP1 proteins of all AAV serotypes (Fig. 1C). As evident from Figs. 1B and 1C, VP proteins expressed from the novel helper plasmids were indistinguishable from their wild-type counterparts, thus confirming the integrity of our plasmids.

Interestingly, despite the fact that the capsid genes of all AAV serotypes are of almost identical lengths [5], we observed differences in the gel migration pattern of the respective VP proteins. Most strikingly, capsid proteins of AAV-4 and -5 migrated higher than those of the other

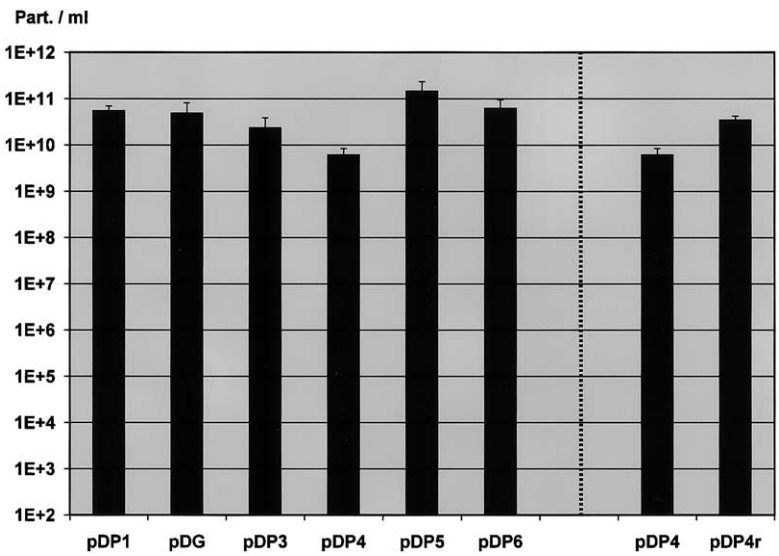

FIG. 2. AAV-2 vector cross-packaging into capsids of AAV-1 to -6. 293T cells were cotransfected with the AAV-2 vector plasmid pTRUFΔ and each of the six pDP helper plasmids. Two days after transfection, particles were harvested from the cells, encapsidated vector DNA was isolated, and particle titers were quantified (see Materials and Methods). Depicted right of the hatched line is a direct comparison of titers obtained with pDP4 or pDP4r as helper plasmid. Shown are mean results of three independent experiments with standard deviations.

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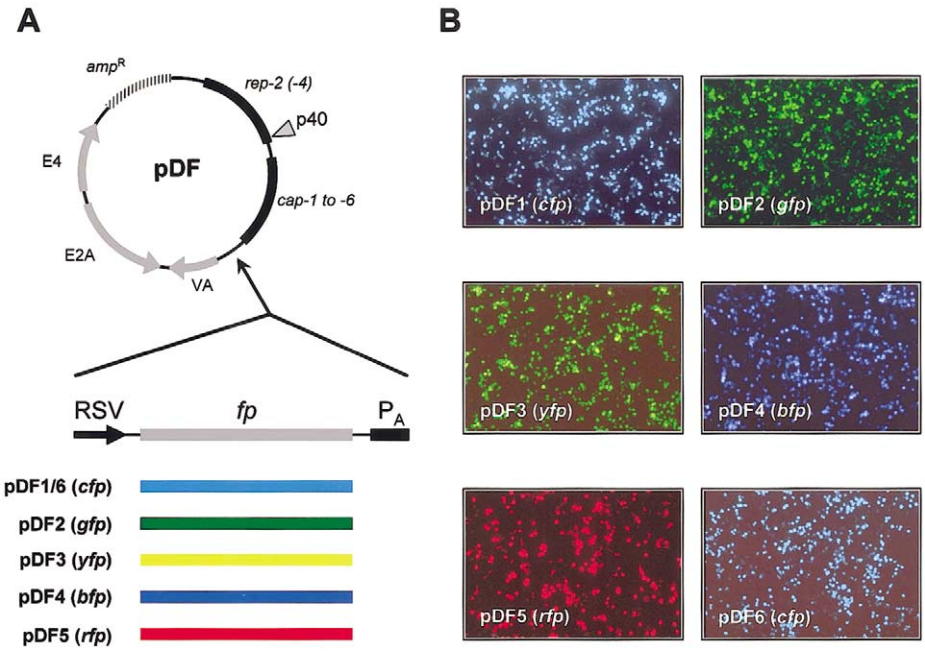

FIG. 3. Generation of AAV helper plasmids expressing a fluorescent marker protein. (A) Plasmid generation. Expression cassettes consisting of a gene (*fp*) encoding a fluorescent protein under the control of the RSV promoter and including an SV40 poly(A) signal (P_A) were inserted into the pDP plasmids and pDG. While pDP1 and pDP6 both received the *cfp* gene, all other plasmids were supplied with a unique *fp* gene. The resulting six novel AAV helper plasmids were labeled pDF1 to pDF6 (F for fluorescent). (B) Expression of fluorescent marker proteins. 293T cells were transfected with the indicated plasmids, and 2 days later expressed fluorescent proteins were visualized using an inverted microscope equipped with fluorescence filters, allowing excitation at the following wavelengths: 470–490 nm for Gfp and Yfp, 545–565 nm for Rfp, and 380–400 nm for Bfp and Cfp. Under these conditions, the difference between Gfp and Yfp was marginal, but discrimination of pDF2 and pDF3 was feasible at a wavelength of 380–400 nm, at which Gfp, but not Yfp, was still visible (data not shown). Bfp, blue; Cfp, cyan; Gfp, green; Rfp, red; Yfp, yellow fluorescent protein.

serotypes, and AAV-4 VP2 ran higher relative to AAV-4 VP1 and VP3, compared to the other AAVs (Figs. 1B and 1C). This suggests that AAV serotype capsid proteins either undergo unique posttranslational modifications or assume SDS-resistant conformations. Also noteworthy, pDP5 yielded the strongest expression of capsid proteins among all the plasmids, although like the *cap* genes in all other constructs, AAV-5 *cap* was controlled by the AAV-2 p40 promoter.

Identification of Antibodies That Detect Non-Type-2 AAV Proteins

The successful detection of AAV serotype proteins with anti-AAV-2 antibodies raised the question whether this would also apply to other antibodies with presumable specificity for AAV-2 present in our group. We therefore infected cells with each of the six wild-type AAVs and helper adenovirus and then analyzed three groups of antibodies for binding to AAV proteins by Western blotting and immunofluorescence. As summarized in Table 1, these groups were antibodies (mono- or polyclonal) initially raised against Rep proteins, VP proteins, or assembled capsids of AAV-2. Importantly, we found that most of the antibodies tested cross-reacted with at least one other AAV serotype, with only 226.7 and C37 proving monospecific for AAV-2 Rep proteins or AAV-2 capsids, respectively. Several monoclonal antibodies were identified to bind Rep proteins of all six AAV serotypes, and likewise, the monoclonal antibody D3 reacted with all capsids, suggesting that the antibodies listed in Table 1 should be useful tools for future work on AAV serotypes.

Helper virus-Free Production of AAV Serotype Vectors

To confirm the functionality of the pDP constructs as helpers for AAV-2 vector pseudotyping, we next cotransfected cells with an AAV-2 vector plasmid (encoding a CMV promoter-driven *gfp* gene) and each of the five pDP plasmids or, as positive control and to provide a basis for comparison, the original pDG. As previously described for AAV-2 vector production [18], recombinant viruses were harvested 48 h after the start of transfection, when a majority of the vector particles was still retained within the cells, as initially reported for AAV-2 [18] and here also found for the five other AAV serotypes (data not shown).

Titration of the virus stocks for amounts of capsids containing AAV-2 vector DNA identified each pDP plasmid as a highly efficient pseudotyping helper (Fig. 2, left of hatched line). In fact, virus particle titers were comparable to those obtained from pDG-mediated packaging of the same vector genome into AAV-2 capsids and varied by no more than fivefold between the different serotypes. Only pDP4 gave significantly lower titers than all other helper plasmids, whereas pDP5 was most efficient. Together with the strong VP protein expression from pDP5 (Fig. 1B), this indicated that capsid protein expression is limiting AAV serotype vector production, substantiating our previous findings with AAV-2 [18,20].

The reasons for the poor performance of pDP4 were not evident, since Western blot analyses ruled out a role for expression of VP proteins from this plasmid (Fig. 1C and data not shown). We therefore speculated that rather

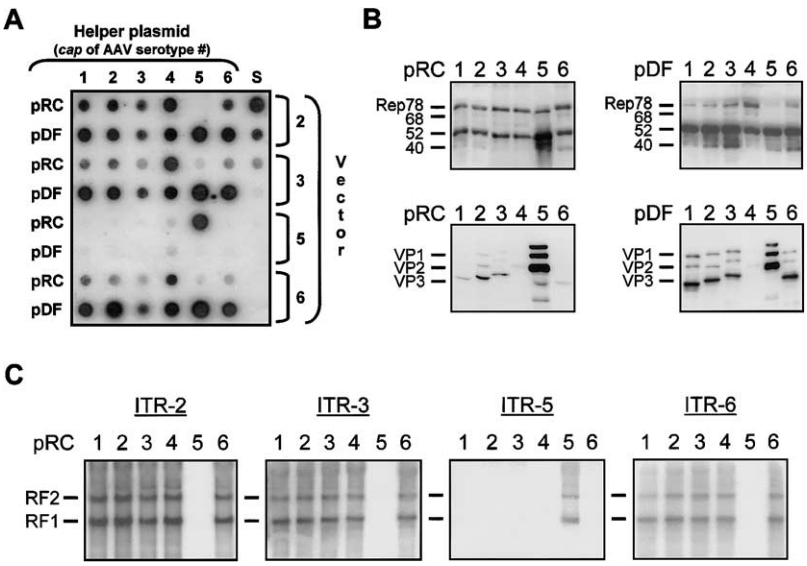

FIG. 4. Cross-packaging of vectors from different AAV serotypes. (A) Dot-blot analysis. 293T cells were cotransfected with vector plasmids carrying a *gfp* expression cassette flanked by ITRs of AAV-2, -3, -5, or -6 (indicated on the right) and the conventional pRC helper plasmids expressing only AAV *rep* and *cap* genes and thus requiring additional adenovirus infection or the novel pDF plasmids. S denotes a fivefold serially diluted vector plasmid standard (starting with 10^9 plasmid copies). Two days after transfection, particles were harvested and processed for dot-blot analysis (see Materials and Methods). (B) Expression of AAV proteins. Shown are Western blot analyses of protein extracts from cells transfected as described for A (using the AAV-2 vector), using the monoclonal antibody 303.9 [19] or B1 for detection of AAV Rep (top) or VP (bottom) proteins. (C) AAV vector DNA replication. Extrachromosomally replicated DNA was isolated from adenovirus-infected cells that had been cotransfected with each of the four different vectors and each of the six pRC helper plasmids and analyzed by Southern blot (see Materials and Methods). The typical AAV replication intermediates RF1 and RF2 are indicated.

than the availability of AAV-4 capsids, the process of AAV-2 vector DNA encapsidation was rate limiting. As recently hypothesized by our group, AAV DNA packaging requires interaction of AAV Rep proteins with both the viral genome and the AAV capsid proteins [21,22]. Given that VP proteins of AAV-2 and AAV-4 share only 65% homology, as opposed to 85% for AAV-1, -2, -3, and -6 [5], it was likely that the interaction of AAV-2 Rep and AAV-4 VP proteins expressed from pDP4 was inefficient and thus limiting. To test this, we made a second AAV-4 helper construct, pDP4r, by replacing both AAV-2 *rep* and *cap* in pDG with the corresponding genes of AAV-4. Indeed, pDP4r was approximately fivefold more efficient at cross-packaging AAV-2 vector DNA into AAV-4 capsids than pDP4 (Fig. 2, right of hatched line) and thus equally as efficient as the other pDP helper plasmids and pDG.

Fluorescent Proteins to Monitor Helper Plasmid Transfection

Characteristic for protocols for AAV vector production based on transient plasmid transfection, such as the one presented here and the majority described so far, is that particle titers strongly depend on transfection efficiencies. Monitoring and optimizing this parameter is thus crucial to this type of protocol, but unfortunately not a feature that is implemented in current technology.

For our own approach, we therefore supplied the pDP plasmids and pDG with genes encoding fluorescent proteins (FPs), which once expressed would be easily detectable in living cells. To allow further discrimination of the various constructs after transfection, we provided them with unique *fp* genes, encoding proteins emitting a cyan, green, yellow, blue, or red color upon excitation (Fig. 3A). Among the resulting six new constructs, termed pDF1 to pDF6 (F for fluorescent), the AAV-1 and AAV-6 plasmids both carried the *cfp* gene, which was reasonable since there were only five color variants commercially available and since the *cap* genes of AAV-1 and -6 are highly homologous [7,23].

The examples in Fig. 3B show cells 2 days after transfection with pDF1 to pDF6, emitting bright colors specific for each plasmid. Importantly, addition of the *fp* gene expression cassettes did not impair the functionality of the pDF plasmids as packaging helpers (data not shown).

Cross-packaging of AAV Vectors of Serotypes Other Than 2

Thus far, we had exclusively used a conventional AAV-2 vector plasmid for the pseudotyping experiments, carrying the inverted terminal repeats (ITRs) of AAV-2. Interestingly, vector plasmids derived from other AAV serotypes were also reported recently [23] and appear as attractive objects of study, likely exhibiting unique properties due to the distinct sequences and structures of their ITRs [5].

To test if the pDF helpers would also allow cross-packaging of these non-type-2 AAV vector DNAs, we flanked the *gfp* expression cassette from the AAV-2 vector construct with ITRs from AAV serotype 3, 5, or 6 (AAV-1 or -4 ITRs were not available at the time of our study). The resulting vector genomes were pseudotyped by cotransfection with the pDF helpers or with basic helper constructs (pRC1 to pRC6, see Materials and Methods for details) expressing only *rep* and *cap* genes of each AAV serotype and thus requiring additional adenovirus infection. The pRC helpers were used to evaluate the efficiency of the pDF constructs, as well as to study the impact of AAV serotype Rep proteins on the pseudotyping process. All 48 possible cross-packaging reactions, involving the 4 different AAV vector plasmids (carrying ITRs of AAV-2, -3,

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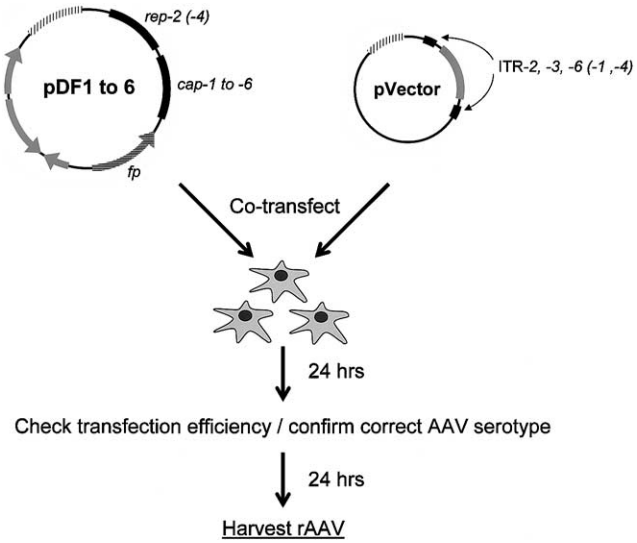

FIG. 5. Helper virus-free, optically controllable and two-plasmid-based (HOT) production of AAV serotype vectors (scheme). To package an AAV vector of serotype 2, 3, or 6 into capsids of any AAV serotype, 293T cells are cotransfected with the vector plasmid and the pDF plasmid expressing the desired *cap* gene. The next day, the fluorescent marker protein expressed from the pDF plasmid can be detected in the living cells, to check plasmid transfection efficiency and to confirm use of the correct helper plasmid and production of the desired AAV serotype. Another day later, the cells containing the recombinant AAV particles are collected and processed. Although not demonstrated, this protocol likely also allows cross-packaging of yet-to-be-made vectors based on AAV-1 and -4 and theoretically can be adapted for any further AAV serotype.

-5, or -6) and the 12 helpers (pRC1 to pRC6, pDF1 to pDF6), were carried out, and yields of genome-containing vector particles were quantified by dot-blot analysis.

A representative example of the results is shown in Fig. 4A, illustrating two main findings. First, the pDF plasmids indeed allowed cross-packaging of the alternative AAV vectors into the six different AAV capsids, the only exception being the AAV-5-based vector. This was, however, not surprising regarding the known inability of AAV-2 Rep proteins, as expressed from all pDF constructs (except pDF4), to replicate AAV-5 vector DNA [24]. For all other vectors, titers varied by no more than 5-fold between the pDF plasmids, with pDF3 being least and pDF5 most efficient, identical to our initial findings with the AAV-2 vector. Second, the pRC plasmids also allowed packaging of the different AAV vector DNAs, but resulting particle titers were up to 10-fold lower compared to the pDF plasmids expressing the same *cap* gene. The only exception was pRC4, which yielded titers equal to those obtained with pDF4. Interestingly, pRC4 also gave highest titers among all pRC constructs, with all AAV vector plasmids except the AAV-5 vector, which was efficiently packaged only by pRC5.

Molecular Analysis of the Cross-packaging Process

To elucidate the molecular basis of the results described above, we analyzed expression of AAV proteins, as well as replication of the vector DNA in cells transfected with the various helper and vector plasmids.

Western blot analyses showed that compared to the pRC plasmids, the corresponding pDF constructs expressed lower amounts of large Rep (Rep78 and Rep68) and significantly higher amounts of VP proteins (Fig. 4B). The only exceptions were the AAV-4- and -5-based pairs of plasmids, which gave similar levels of VP proteins. In the case of pRC4 and pDF4, this paralleled the equal virus titers obtained with both plasmids, while pRC5 and pDF5 could not be compared directly, since pRC5 packaged only the AAV-5 vector. Interestingly, pDF5 not only expressed the highest amounts of VP proteins among all helpers, but also yielded the highest titers with all vectors (except for the AAV-5 vector). Taken together, these findings further correlated VP protein expression from the helper with efficiency of AAV particle production, as we had hypothesized before, here, as well as in an earlier study [18].

Replication of the four different vector DNAs in the presence of Rep proteins from all six AAV serotypes was analyzed by Southern blot (Fig. 4C). Typical AAV replication intermediates were detected to similar extents in cells transfected with any combination of vector and helper plasmids, except for the AAV-5-based constructs. In fact, pRC5 supported replication only of the AAV-5-based vector, and inversely, this vector was not replicated by any of the non-type-5 helpers. Similar results were obtained when the pDF plasmids, expressing AAV-2 or -4 Rep proteins, were used as helpers to replicate the different vectors (data not shown). Thus, the block of virus production with the AAV-5 vector and any of the non-type-5 Rep-expressing helpers, and vice versa, was already at the level of vector DNA replication. This confirms and extends a previous report on the incompatibility of AAV serotypes 2 and 5 [24] and indicates that a strict requirement for future attempts to cross-package AAV-5 vectors is the inclusion of AAV-5 *rep* in the helper plasmid.

In summary, we showed that the pDF plasmids allowed the pseudotyping of vectors based on three different AAV serotypes with capsids of AAV-1 to -6, at efficiencies equal to or in most cases exceeding those of conventional helper constructs previously used by others [e.g., 23]. A schematic protocol for our novel approach, using the pDF constructs for the efficient HOT, i.e., helper virus-free, optically controllable, and two-plasmid-based production, of AAV serotype vector particles, is shown in Fig. 5.

AAV Serotype Vector Particle-Mediated Transduction of Cultured Cells

Our final goals were to confirm that particles produced with the pDF helper plasmids are functional as transgene delivery vehicles and, moreover, to extend the existing

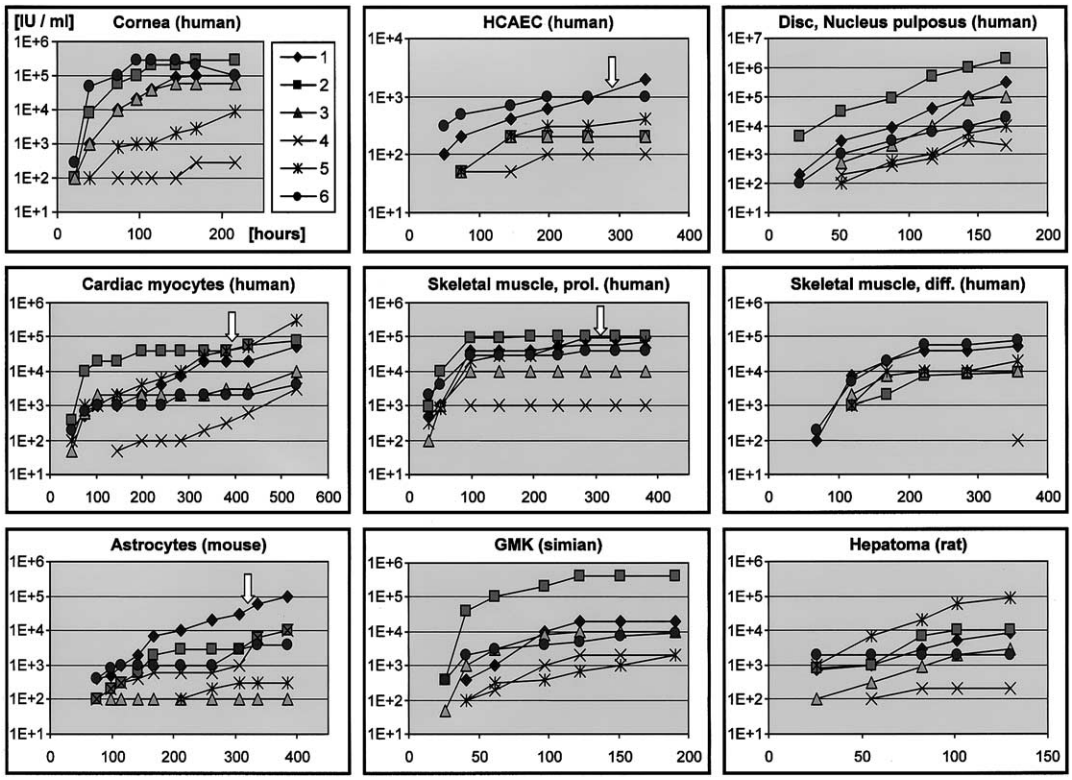

FIG. 6. Transduction profiles of AAV vectors of serotypes 1 to 6. Cells were infected with recombinant *gfp*-expressing AAV-1 to -6 vectors as described in Table 2. Plotted are titers of infectious particles (IU/ml) against time after infection, with the arrows in some graphs denoting a renewal of culture medium.

knowledge of the transduction properties of AAV serotype vectors. We therefore used the constructs for large-scale pseudotyping of a *gfp*-expressing AAV-2 vector DNA with all six capsids and subsequently infected a broad panel of cultured cells (Table 2) with the purified particles. The cells comprised either primary or transformed cells derived from four different species (humans, mice, rats, and monkeys) and included several human cell types for which infectibility with all six AAV serotypes has not been analyzed before, e.g., human endothelial cells and cardiac myocytes.

A central finding was that while most cells were transduced by at least four different serotypes, the particle-to-infectivity ratios frequently ranged over three or four orders of magnitude between the six AAV serotypes on a given cell type, which emphasized the largely different cell tropisms of AAV-1 to -6. Moreover, although the majority of cell types (15/29) was most efficiently infected by AAV-2, there were interesting exceptions, such as three types of human primary endothelial cells being preferentially transduced by AAV-1, suggesting a high prevalence of the currently unknown AAV-1 receptor. Similar findings were obtained for the AAV-5 and -6 pseudotypes (Table 2). Also noteworthy is the shift found for human primary skeletal muscle cells: in the undifferentiated state they were best infected with AAV-1 and -2 whereas AAV-6 was superior after differentiation, followed by AAV-1 and -5, which extends previous studies [6,17].

To analyze the transduction kinetics of the different AAV serotypes in more detail, we plotted titers of Gfp-expressing units against time points at which cells were counted. Representative examples of the resulting graphs are shown in Fig. 6. Interestingly, while for all vectors numbers of Gfp-expressing cells steadily increased to eventually reach a plateau, AAV-6 often exhibited a unique transduction profile, e.g., in human cornea or HCAEC cells. In these cells, AAV-6 yielded the strongest Gfp expression among all serotypes at early time points, suggesting that the AAV-6 pseudotype particles had been taken up and/or intracellularly processed fastest and most efficiently.

DISCUSSION

In this report, we have presented a novel protocol for production of gene transfer vector particles derived from AAV serotypes 1 to 6. These vector types are increasingly attracting attention as alternatives or supplements to particles built exclusively from components of AAV-2 (i.e.,

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TABLE 2: Ratios of DNA-containing to infectious particles

Cells				AAV serotypes					
Name	Tissue	Origin	Status	1	2	3	4	5	6
911	Eye	Human	t	**1×10^5**	**1×10^5**	2×10^5	2×10^6	4×10^6	1×10^6
A172	Brain	Human	t	**1×10^5**	5×10^5	3×10^6	4×10^8		1×10^7
Adipocytes	Fat	Human	p	7×10^7	**1×10^6**	2×10^8	5×10^7	3×10^7	1×10^7
Alex	Liver	Human	t	1×10^7	**7×10^6**		2×10^8	2×10^8	2×10^7
Astrocytes	Brain	Mouse	p	**2×10^5**	2×10^6	2×10^8	2×10^6	7×10^7	5×10^6
C6	Brain	Rat	t	**5×10^6**	1×10^7			1×10^7	7×10^6
Cardiac myocytes	Heart	Human	p	4×10^5	3×10^5	2×10^6	7×10^6	**7×10^4**	5×10^6
Chondrocytes	Joint	Human	p	3×10^7	**7×10^5**			4×10^8	7×10^6
Cornea	Eye	Human	p	2×10^5	**7×10^4**	3×10^5	7×10^7	2×10^6	**7×10^4**
Disc (nuc. pulp.)	Spine	Human	p	7×10^4	**1×10^4**	2×10^5	7×10^6	2×10^6	1×10^6
Fibroblasts	Foreskin	Human	p	5×10^7	**7×10^6**	1×10^8		3×10^7	1×10^7
GMK	Kidney	Monkey	t	1×10^6	**5×10^4**	2×10^6	1×10^7	1×10^7	2×10^6
HCAEC	Endothelium	Human	p	**1×10^7**	1×10^8	1×10^8	2×10^8	5×10^7	2×10^7
HeLa	Cervix	Human	t	4×10^5	2×10^5	3×10^6		**1×10^5**	2×10^6
Hepatoma	Liver	Rat	t	3×10^6	2×10^6	7×10^6	1×10^8	**2×10^5**	1×10^7
Hep3B	Liver	Human	t	7×10^6	**2×10^6**	1×10^7	2×10^8	7×10^7	7×10^6
HepG2	Liver	Human	t	3×10^7	**1×10^6**	2×10^6	2×10^8	4×10^7	1×10^7
HUAEC	Endothelium	Human	p	**4×10^8**					
Huh-7	Liver	Human	t	2×10^5	**7×10^4**	3×10^5	7×10^7	4×10^6	5×10^5
HUVEC	Endothelium	Human	p	**4×10^8**					**4×10^8**
Osteocytes	Bone	Human	p		**1×10^8**				**4×10^8**
RG2	Brain	Rat	t	2×10^6	**1×10^6**	3×10^7	2×10^7	4×10^6	2×10^6
RPMI	Nose	Human	t	2×10^5	**2×10^4**	7×10^4	2×10^8	5×10^6	2×10^6
Skeletal muscle	Muscle	Human	p, diff.	4×10^5	2×10^6	2×10^6	2×10^8	1×10^6	**3×10^5**
Skeletal muscle	Muscle	Human	p, prol.	**2×10^5**	**2×10^5**	2×10^6	2×10^7	3×10^5	4×10^5
Smooth muscle	Muscle	Human	p	3×10^5	**2×10^5**	2×10^6	2×10^7	1×10^6	1×10^6
Stem cells	Mesenchyme	Human	p	2×10^7	**7×10^6**			5×10^7	4×10^7
TC-1	Lung	Mouse	t	**1×10^6**	1×10^7		3×10^7	3×10^7	2×10^7
Tong	Liver	Human	t	1×10^7	**3×10^6**	2×10^7	4×10^8	2×10^7	2×10^7

1×10^4 cells per well were seeded in 96-well plates and the next day infected with purified recombinant AAV (*gfp*) of serotypes 1 to 6, at doses of 2×10^8 DNA-containing particles for the first well and then with 10-fold dilutions of virus down to 2×10^0 particles, which corresponded to multiplicities of infection of 10^4 to 10^{-4}. Using an inverted fluorescence microscope, Gfp-expressing cells were then counted at regular intervals after the infection to determine a titer of infectious units, until Gfp expression reached a maximum or the cells died due to age. At this point, ratios of DNA-containing particles to infectious units were determined by dividing the two numbers (the initial titer of DNA-containing particles was 2×10^{10}/ml for each serotype). The AAV serotype that showed the lowest ratio for a particular cell type, i.e., was most efficient at transducing these cells, is highlighted by boldface. diff., differentiated; nuc. pulp., nucleus pulposus; p, primary; prol., proliferating; t, transformed.

capsid proteins and ITRs), due to their unique host ranges and immunological profiles [5].

At the center of our protocol is a set of six plasmids, pDF1 to pDF6, delivering all helper functions required for packaging of recombinant AAV genomes into capsids of AAV-1 to -6. These plasmids were derived from the previously described AAV-2 helper construct pDG [18], by cloning of AAV serotype-specific *cap* (and *rep*) genes, together with fluorescent protein expression cassettes, into the pDG backbone. Consequently, the pDF constructs share three major properties with pDG: first, AAV particle generation is accomplished by simple cotransfection of cells with an AAV vector plasmid and one of the pDF helpers. This is in contrast to all previously reported protocols, in which adenoviral and AAV

helper genes were provided from separate plasmids, thus requiring up to four DNA constructs for AAV vector production [e.g., 16,17]. The reduction in number of helper plasmids to only 1 is a significant advance, as it saves time, work, and costs, factors that are particularly crucial when scale-up of the protocol is intended. Moreover, it guarantees that AAV and adenoviral functions are always provided in a fixed and optimal ratio, thus eliminating the risk of variability in vector yields associated with the use of multiple helper plasmids [18].

Second, our method avoids infectious helper adenovirus, which is neither required nor generated as an unwanted by-product, since the pDF plasmids contain only adenoviral genes essential to AAV vector production. Helper virus-free production of AAV serotype vectors is highly desirable, as contamination with pathogenic adenovirus poses an obvious safety risk, despite current technology allowing purification of helper virus from AAV stocks [25,26]. Therefore, others have established adenovirus-independent production of AAV serotype vectors before [e.g., 16], by replacing helper virus infection with transfection of an adenoviral helper plasmid. This, however, caused an unfavorable increase in the total number of plasmid DNAs needed, as opposed to our approach in which adenoviral genes are included in the AAV helper plasmid.

Third, in small- or large-scale preparations, the pDF plasmids routinely yielded titers of DNA-containing particles between 10^{10} and 10^{12} per milliliter, with less than fivefold variation between the serotypes within a production run. Importantly, those titers were equal to yields obtained from the parental pDG plasmid, which we and others previously characterized as an efficient helper for AAV-2 vector production [18,26], proving an equally high efficiency for the novel pDF plasmids.

A fourth benefit unique to the pDF constructs is that they allow direct and simple monitoring of plasmid transfection during vector production runs, by expressing AAV serotype-specific fluorescent proteins that are detectable the day after transfection. The feasibility of simultaneously controlling transfection efficiencies and use of the correct helper construct should facilitate the maintenance of a high efficiency of AAV serotype vector production.

A central part of this study was the comparison of the pDF constructs with conventional adenovirus-dependent helpers to pseudotype vector DNAs derived from alternative AAV serotypes for their efficiency. This appeared interesting regarding the major role played by AAV ITRs during the viral life cycle, e.g., for encapsidation or cellular persistence of the viral genome [27], and considering that AAV serotype ITRs share as little as 60% homology (AAV-2 and -5, see Ref. [5] for overview), together suggesting unique properties for serotype-specific vector genomes. Surprisingly, pseudotyping of non-type-2 vectors has been described only once so far [11], while in a few other cases, vectors based on AAV-3, -5, or -6 were packaged into capsids of the same serotype [e.g., 16,23].

Two main conclusions were drawn from our own experiments: first, the pDF plasmids indeed allowed pseudotyping of vectors derived from AAV-3 or -6 with capsids from all six serotypes, yielding particle titers comparable to those from packaging of an AAV-2 vector with the same pDF helper. Thus, Rep proteins of AAV-2 or -4, as expressed from the pDF plasmids, equally efficiently supported excision, replication, and encapsidation of the different AAV vector DNAs. This finding was substantiated and extended by the use of adenovirus-dependent AAV serotype *rep*- and *cap*-expressing helper plasmids. With the exception of pRC5 (see below), any of these constructs supported cross-packaging of the AAV-2, -3, or -6 vector, albeit at slightly varying efficiencies. In particular pRC4, expressing AAV-4 Rep and VP proteins, routinely yielded the highest titers among all conventional helpers. The fact that vector DNA replication was similar with pRC4 and the other pRC plasmids suggests that AAV-4 Rep proteins are more efficient at encapsidating replicated AAV DNA.

It was not surprising that pRC5 exclusively supported cross-packaging of the AAV-5 vector, due to the previous demonstration of incompatibility of AAV-2 and -5 Rep proteins and ITRs [24]. Our findings extend this former study, by showing that AAV-5 Rep proteins also do not support replication of other non-type-5 vector DNAs and that moreover, AAV-5-based vectors are not efficiently replicated by any Rep proteins other than those of AAV-5. Only pRC4 and pDF4, both expressing AAV-4 Rep, supported pseudotyping of the AAV-5 vector, albeit poorly, indicating a closer functional relationship of AAV-4 and -5. Taken together, our results imply that for pseudotyping of AAV-5-derived vectors, helpers must express AAV-5 Rep *in cis* with VP proteins of another serotype. It will be interesting to determine whether AAV-5 Rep proteins can indeed interact with non-type-5 VP proteins, as would be required for vector DNA encapsidation, since AAV-5 is only distantly related to the other AAV serotypes [28].

A second central result was that independent of the vector plasmid (except for the AAV-5 vector), particle yields from the pRC helpers were up to 10-fold lower than those from the corresponding pDF plasmids. This was concomitant with our previous results with pDG, which was also 10-fold more efficient than a helper virus-dependent AAV-2 *rep*- and *cap*-expressing plasmid [18]. In the former study, we could correlate pDG's high efficiency with strong expression of VP proteins [18], and the same correlation was confirmed here for the five other AAV serotypes. This suggests that in general, AAV VP protein expression and subsequent capsid formation are crucial and limiting parameters for AAV vector production.

The final part of our study yielded further insight into the transduction properties of AAV serotypes: first, we found that almost 50% of the 29 cell types analyzed were

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equally or more efficiently infected with different non-type-2 AAV particles, compared to genuine AAV-2 particles. This confirmed that AAV serotypes differ largely in their host range and thus provides a reasonable basis for vector development. It was moreover particularly interesting to observe great differences in transduction efficiencies for the AAV-1 and -6 pseudotypes, as the two wild-type genomes are highly homologous [7,23], suggesting that AAV-1 and -6 are in fact the same serotype [16]. While our experiments cannot resolve this issue, we showed that the 6-amino-acid difference in the AAV-1 and -6 VP proteins [7,23] results in unique host ranges. Importantly, our finding that both viruses efficiently infect endothelial, corneal, and differentiated muscle cells indicates an abundance of their receptors on these cells. This should prove useful in the eventual characterization of these molecules, which in contrast to the AAV-2, -4, and -5 receptors [29,30] are currently unknown.

Second, we detected different transduction kinetics from AAV serotype vectors, a phenomenon that so far has been noted only sporadically [e.g., 9]. Here, in particular AAV-6 frequently yielded the most rapid onset of gene expression, in contrast to AAV-1, which further highlights a diversity between these two closely related AAV types. It remains an interesting challenge for future studies to uncover the mechanisms underlying the unique transduction kinetics of the different AAV serotypes. Considering that all our particles carried an identical gene expression cassette, we can rule out a role for late steps in transduction, such as transcription or translation. More likely, the different kinetics relate to early steps, such as particle binding to cellular receptors, transport through vesicles to the nucleus, or proteasome-mediated capsid degradation.

In conclusion, with unique transduction kinetics, in addition to distinct host ranges and immunological profiles, vectors derived from AAV serotypes appear as highly promising tools for human gene transfer. The novel efficient technology reported here, allowing vector generation through simple cotransfection, in the absence of pathogenic helper virus and under controllable conditions, should facilitate their further preclinical and eventual clinical evaluation. Importantly, this technology can easily be adapted to any further members of the AAV family, such as the recently described two new AAV serotypes 7 and 8 [15]. Moreover, sequence data reported for these two viruses show that the epitopes for the anti-VP antibodies A1, A69, and B1 are fully conserved, supporting our notion that the antibodies described here are useful tools for future studies on AAV serotypes.

MATERIALS AND METHODS

Cells and viruses. Cells were obtained from the cell bank of or groups at the German Cancer Research Center (911, A172, astrocytes, C6, fibroblasts, GMK, HeLa, all liver cells, RG2, RPMI, and TC-1), from external clinical collaborators (adipocytes, cardiac myocytes, chondrocytes, cornea, disc, osteocytes, and mesenchymal stem cells), or from a commercial source (Promocell, Heidelberg, Germany; all endothelial, proliferating skeletal, and smooth muscle cells). Differentiated human skeletal muscle cells were derived from proliferating cells through treatment with differentiation medium as recommended by the manufacturer (Promocell).

Wild-type AAV virus stocks were present in the group (serotype 2) or kindly provided by U. Bantel-Schaal (serotypes 1, 3, 4, and 5). An AAV-6 virus stock was generated by transfecting adenovirus-infected 293T cells with plasmid pAAV6Bgl [23]. All viruses were amplified and titrated as previously described for wild-type AAV-2 [31].

Plasmid constructions. AAV vector plasmids carrying a CMV promoter-driven *gfp* gene flanked by ITRs of AAV serotype 2, 3, 5, or 6 were generated based on the AAV-2 vector construct pTRUF3 [32]. Therefore, a thymidine kinase promoter-driven neomycin-resistance gene present in pTRUF3 was first removed by digestion with *Sal*I and religation, resulting in the AAV-2 vector construct pTRUFΔ. The CMV-*gfp* cassette was then excised with *Bgl*II and cloned into plasmid pA3LAPSN [23], p7D05 (kind gift from J. Chiorini and R. Kotin), or pAAV6Bgl [23] (all opened with *Bgl*II), to become flanked by ITRs of AAV serotype 3, 5, or 6, respectively.

The adenovirus-dependent AAV helper plasmid pRC2 carrying AAV-2 *rep* and *cap* genes is identical to the previously reported plasmid pΔTR [18]. Similar constructs expressing genes of AAV-3 or -6 (pRepCap3 and pRepCap6, here referred to as pRC3 and pRC6) were also previously published and obtained as kind gifts from D. Russell [23]. To assemble the three helper plasmids expressing *rep* and *cap* genes of AAV-1, -4, or -5, we first PCR-amplified these genes, using as template AAV genomes that were extracted from 239T cells infected with the respective wild-type AAVs and helper adenovirus. The *rep* and *cap* genes were amplified as individual fragments, sharing a central overlapping sequence that contained sites for restriction enzymes, which allowed subsequent ligating of the two genes back together to yield full-length AAV genomes (lacking the ITRs). In addition, the PCR primers binding upstream of *rep* or downstream of *cap* contained sites for restriction enzymes (underlined, see below) to facilitate subcloning of the joined PCR products into *Spe*I–*Cla*I-linearized pBluescript II SK (Stratagene, La Jolla, CA). Primers for *rep* genes were AAV-1, sense 5′-GCTAGTCTAGACGTCAGCGCTGACGTAAATTACGTC-3′ (*Xba*I site underlined), antisense 5′-GCTCCAGGTTTCAAGTCCCACCACTC-3′; AAV-4, sense 5′-CACTGACGTCAATGTGACGTCCTAGG-3′ (*Bln*I), antisense 5′-CGTGACCTCCTTGACCTGGATGTTG-3′; and AAV-5, sense 5′-GTCCATCTAGAGCGCGTATGAGTTCTCGCGAGAC-3′ (*Xba*I), antisense 5′-GTGGGCCCGCTTCAAGGCCCAAAAAC-3′. Primers used to amplify *cap* genes were AAV-1, sense 5′-CCAGGTATGGCTGCCGATGGTTATC-3′, antisense 5′-GTCCAATCGATGCGAAGCGCAACCAAGCAG-3′ (*Cla*I); AAV-4, sense 5′-CCAGATATGACTGACGGTTACCTTCC-3′, antisense 5-GTCCAATCGATGCAGTTGTAAACCGCGAAGCGCAAG-3′ (*Cla*I); and AAV-5, sense 5′-GGAAAACTTGTCAGATTTTGG-3′, antisense 5′-GTCCAATCGATGCCACAAGAGGCAGTATTTTACTGAC-3′ (*Cla*I). PCRs were performed using Deep Vent DNA polymerase (NEB, Frankfurt, Germany), and reaction conditions were 5 min at 95°C, 2 min at 69°C, 3 min at 72°C, followed by 25 cycles of 2 min each at 95, 69, and 72°C and finally 10 min at 72°C.

All adenovirus-independent helper plasmids (pDP1 to 6 and pDF1 to 6) were derived from the previously reported AAV-2 helper construct pDG [18]. Therefore, the AAV-2 *cap* gene was deleted from pDG by digestion with *Swa*I and *Cla*I and replaced with the *cap* genes of the alternative AAV serotypes, which had been amplified as PCR products from wild-type viral DNA. Primers for amplification of AAV-1, -4, or -5 *cap* genes are described above, while the following primers were used to amplify AAV-3 or -6 *cap:* AAV-3, sense 5′-CCAGGTATGGCTGCTGACGGTTATC-3′, antisense identical to AAV-4 *cap* antisense (see above); and AAV-6, sense 5′-CTGGATGACTGTGTTTCTGAGC-3′, antisense 5′-GTCCAATCGATGCGAAGCGCAACTAAGCAG-3′ (*Cla*I). Plasmid pDP4r containing both AAV-4 *rep* and *cap* genes was assembled by isolating these genes from pRC4 with *Xba*I and *Cla*I and ligating this fragment onto *Xba*I–*Nde*I and *Nde*I–*Cla*I fragments from plasmid pDG. The integrity of all AAV *cap* genes in the pRC, pDP, and pDF plasmids was confirmed by DNA sequence analyses (Biotech Core, Inc., Palo Alto, CA).

Cassettes expressing fluorescent protein genes under the control of the

RSV promoter were assembled by first cloning an *Xba*I–*Pst*I fragment, comprising the RSV promoter together with an SV40 poly(A) site, from plasmid pRep8 (Invitrogen, Karlsruhe, Germany) into plasmid pSL1180 (Stratagene). Genes encoding the green (*gfp*), cyan (*cfp*), blue (*bfp*), yellow (*yfp*), or red (DsRed2/*rfp*) fluorescent protein were then inserted into the resulting construct as *Kpn*I–*Not*I fragments. All *fp* genes were isolated from commercially available plasmids (Clontech, Palo Alto, CA). Finally, the various RSV-*fp* expression cassettes were cloned as *Cla*I fragments into the pDP plasmids opened with *Cla*I, with *fp* genes assigned to pDP constructs as described under Results, yielding the pDF helper constructs (pDF4 was derived from pDP4r).

AAV vector production and titration. AAV vector production was carried out in 293T cells and following a previously described calcium phosphate transfection protocol [18]. For analytical small-scale productions, 4×10^5 cells were seeded in 6-cm cell culture dishes and the next day transfected with a total of 6 μg plasmid DNA. For preparative large-scale productions, 4×10^6 cells were seeded in each of 10 15-cm culture dishes and the next day transfected with 90 μg DNA. AAV vector and helper plasmids were transfected in equimolar amounts, i.e., pDG or the pDP or pDF plasmids were mixed with AAV vector plasmids in a 3:1 ratio (accounting for the threefold smaller size of the vector constructs), while pRC helpers and vector plasmids were mixed 1:1. pRC-transfected cells were additionally infected with wild-type adenovirus at a multiplicity of infection of 10, and adenovirus was later inactivated at 56°C for 1 h. Cells were incubated for 48 h after transfection (and infection) and then lysed by two consecutive rounds of freeze–thawing, releasing the virus particles into the medium (small-scale preparations) or PBS (1 ml per 15-cm plate, large-scale preparations). Vector particles from large preparations were purified and concentrated by iodixanol density centrifugation essentially as described [26].

Titration of DNA-containing particles was carried out following a previously reported dot-blot protocol [18,20]. Briefly, particles (in medium from small-scale or 40% iodixanol from large-scale preparations) were incubated for 2 h with 5 units of DNase I to digest nonencapsidated vector DNA, before viral capsids were digested for 2 h with 100 μg proteinase K (all reagents from Roche, Mannheim, Germany). Recombinant AAV genomes thus released were then purified and concentrated by phenol–chloroform extraction, followed by ethanol precipitation. Serial 10-fold dilutions of vector genomes were transferred to nylon membranes by dot blot, along with serial dilutions of AAV vector plasmid as standard. Following hybridization with a radioactively labeled DNA probe binding the samples and the standard, numbers of encapsidated genomes were calculated and then considered equivalent to numbers of DNA-containing viruses in the preparation, assuming that each particle contained one genome.

Immunodetection of AAV proteins. AAV proteins from transfected or infected cells were detected by either Western blot analyses of crude cell extracts or immunofluorescence analyses of fixed cells. Samples for either analysis were prepared and processed essentially as described [18,19]. For Western blot analyses, monoclonal anti-AAV antibodies were diluted 1:10 in 6% skim milk, and polyclonal anti-AAV-antisera were diluted 1:500. For immunofluorescence analyses, monoclonal antibodies were used undiluted, and polyclonal antisera were diluted 1:50 in PBS supplied with 0.1% BSA. Secondary antibodies (Dianova, Hamburg, Germany) against mouse (binding to primary monoclonal antibodies) or rabbit (for primary polyclonal antisera) IgG were coupled with horseradish peroxidase to allow ECL detection (NEB, Frankfurt, Germany) after Western blotting or with rhodamine to label positive cells for immunofluorescence analyses.

DNA replication analyses. Extrachromosomally replicated DNA was extracted from transfected cells following a modified Hirt procedure [18]. Briefly, the cells were lysed by 3 h incubation in lysis buffer (10 mM Tris, 10 mM EDTA, 0.6% SDS, 250 μg proteinase K/ml), and high-molecular-weight DNA was precipitated by addition of sodium chloride to a final concentration of 1 M and 4°C incubation overnight. After 1 h centrifugation at 16,500*g*, viral replicated DNA present in the supernatant was purified by phenol–chloroform extraction and precipitated with isopropanol. Following *Dpn*I digestion to remove transfected input plasmid DNA, replicative AAV DNA was separated on 0.7% agarose gels and transferred to nylon membranes following a standard Southern blot transfer protocol. Immobilized AAV vector DNA was finally detected by hybridization with a radioactively labeled fragment of the CMV promoter binding to all four different AAV vector constructs.

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