

Evidence for Encapsidation of Prokaryotic Sequences during Recombinant Adeno-Associated Virus Production and Their *in Vivo* Persistence after Vector Delivery

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Recombinant adeno-associated virus vectors (rAAV) have been successfully used for long-term gene expression in animal models and in patients. However, while the therapeutic potential of rAAV appears promising, safety issues, including contaminants found in vector stocks, must be further evaluated. We previously reported that a *cis*-acting replication element present within the AAV-2 p5 promoter was responsible for the encapsidation of *rep-cap* sequences observed during rAAV production. In that study, we also noticed that plasmid-derived prokaryotic sequences (such as the ampicillin resistance gene) could be found packaged into AAV capsids. In this report, first we confirmed and extended the latter observation by analyzing rAAV stocks produced using different procedures. Second, we demonstrated that these plasmid-derived sequences were transferred and persisted *in vivo* after rAAV injection into different tissues. Third, our data showed that at least some of these packaged plasmid molecules were linked to the AAV ITRs and were present *in vivo* in a form that could be rescued through bacterial transformation. This study highlights the need for more stringent characterization of rAAV stocks and provides useful information on the development of rAAV production methods that are able to circumvent or limit the generation of such undesirable particles.

Key Words: adeno-associated virus, DNA packaging, gene transfer

INTRODUCTION

Adeno-associated virus type 2 (AAV-2) is a defective and nonpathogenic parvovirus with a 4.7-kb single-stranded DNA genome. Efficient replication requires co-infection with adeno- or herpesvirus, which provide helper functions. The AAV-2 genome consists of two open reading frames, encoding regulatory (Rep) and structural (Cap) proteins, flanked by 145-bp inverted terminal repeats (ITRs) [1,2]. The ITRs are the *cis*-acting elements required for virus replication and packaging. Therefore, recombinant AAV-2 vectors (rAAV), in which the *rep-cap* genome is replaced by an expression cassette encoding the transgene, can be efficiently produced when Rep and Cap are provided *in trans* by a *rep-cap*-expressing plasmid [3,4]. Generation of rAAV stocks requires transfection of the vector and of the *rep-cap* constructs into cells such as 293 cells that are either infected with adenovirus or cotransfected with an

adenovirus helper plasmid [5,6]. An alternative method relies on adenovirus or herpesvirus infection of packaging cell lines that stably harbor AAV *rep-cap* genes and/or rAAV vector DNA [7–11].

The characterization of the rAAV stocks indicated that, despite the lack of homologous sequences between the *rep-cap* and the vector constructs, *rep-cap* sequences were present in 0.1 to 1% of the particles [12–14]. We have reported that a 350-bp sequence embedded in the p5 promoter of the AAV-2 *rep* gene contains a *cis*-acting replication element responsible for the Rep-dependent replication and packaging into AAV-2 capsids of a transiently transfected *rep-cap* sequence [15,16]. Surprisingly, in the same study, we found evidence that encapsidated DNA also hybridized to a plasmid backbone probe, indicating that prokaryotic sequences, such as the ampicillin resistance (amp^R) gene, were similarly packaged during generation of

rAAV-2 particles [15]. This observation was indirectly supported by Miller *et al.*, who found integrated pBR322 sequences following *in vitro* infection of HeLa cells with rAAV particles [17].

In the present study, first we confirmed and extended this observation by quantifying the plasmid-containing particles present in rAAV stocks and by analyzing the origin of such sequences. Second, we showed that these sequences were transferred and persisted *in vivo* after rAAV injection in different tissues. Third, we demonstrated that at least part of these plasmid molecules are linked to the AAV ITR and were present *in vivo* in a form that can be rescued through bacterial transformation. In conclusion, this study highlights the need for more stringent characterization of rAAV stocks and also provides useful information on the development of rAAV production methods that are able to circumvent or limit the generation of such undesirable particles.

RESULTS AND DISCUSSION

Universal Packaging of Plasmid Backbone Sequences during rAAV Production by Transient Transfection

We quantified the presence of particles containing plasmid-derived sequences in rAAV-2 stocks obtained using common procedures. In general, the production of rAAV particles relies on the cotransfection of several plasmids (Fig. 1) the rAAV vector (such as pAAVLZ), the *rep-cap* packaging sequences, and the adenovirus helper plasmids (such as pRC and pXX6, respectively). The last two can be combined in one single plasmid (pDG). Alternatively, since transfection is accomplished using 293 cells, the helper functions can also be supplied by an E1-deleted adenovirus (Ad.dl324). Therefore, we evaluated three different configurations in which the vector plasmid

remained unchanged while the packaging and adenovirus helper functions varied (Table 1). We conducted a small-scale production and purified rAAV-2 particles by two consecutive CsCl gradients. Importantly, the virus was recovered from fractions having a density corresponding to mature AAV particles (1.38 to 1.41 g/ml). In addition, we extracted packaged DNA after extensive treatment of the particles with benzonase, under conditions that have been previously shown to lead to a complete degradation of any contaminating DNA present outside the particles [15]. The results indicated that the *amp^R* sequence was found packaged under all three conditions and represented 1 to \approx 6% of the total vector genomes (Table 1). We found similar ratios by dot-blot analysis of the same stocks (data not shown). In contrast, we found *rep-cap* sequences packaged at levels that were much lower, ranging from 0.3 to 1.2% of the vector genomes (data not shown). To test for the possibility that the rAAV vector plasmid was solely responsible for this observation, we similarly analyzed wild-type AAV-2 particles produced by transient transfection using either pXX6 or Ad.dl324 to supply helper functions. Under both conditions, we detected *amp^R*-containing particles at levels similar to those found using a rAAV plasmid (Table 1).

In the above experiments, all the plasmids used for rAAV production carried the *amp^R* gene. To determine further the origin of the packaged plasmid backbone sequences, we then used another vector plasmid, pAAVGFP(K), that contains the *kan^R* in place of the *amp^R* gene in the plasmid backbone (Fig. 1). We cotransfected the pAAVGFP(K) plasmid into 293 cells with the *amp^R*-containing pDG plasmid and analyzed the rAAV-2 stock by quantitative real time PCR (Q-PCR) or dot-blot searching for both the *kan^R* and the *amp^R* genes (Table 1). We found that both sequences were packaged into

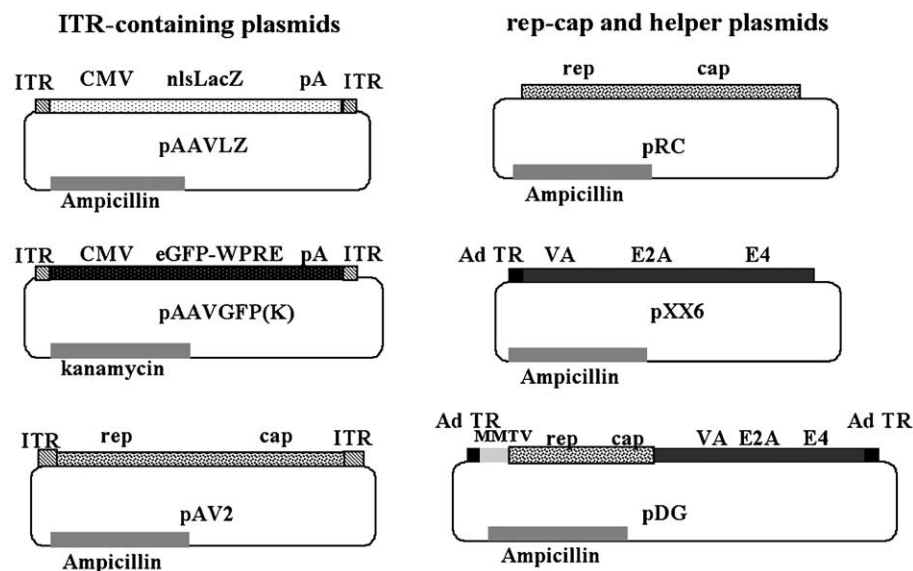


FIG. 1. Structure of the plasmids used for rAAV production. See Materials and Methods for details.

TABLE 1: Quantification by Q-PCR of AAV particles containing vector and plasmid-derived sequences

AAV plasmid	<i>rep-cap</i> plasmid	Helper	AAV (vg/ml)	amp ^R (ag/ml)	kan ^R (kg/ml)
pAAVLZ	pDG	pDG	2.0×10^{10}	1.2×10^9 (6.1%)	ND
pAAVLZ	pRC	pXX6	3.8×10^{10}	1.8×10^9 (4.8%)	ND
pAAVLZ	pRC	Ad.dl324	4.6×10^{10}	5.8×10^8 (1.3%)	ND
pAV2	–	pXX6	2.5×10^{10}	5.5×10^8 (2.2%)	ND
pAV2	–	Ad.dl324	3.7×10^{10}	1.9×10^8 (0.5%)	ND
pAAVGFP(K)	pDG	pDG	3.8×10^{10}	1.8×10^8 (0.5%)	1.5×10^9 (3.9%)

AAV-2 particles produced using the indicated plasmids were purified on two consecutive CsCl gradients and packaged DNA was quantified by Q-PCR using primers recognizing either AAV (rAAV or wt AAV) or plasmid backbone (amp^R gene) sequences. The numbers in parentheses indicate the percentage of amp^R over rAAV or AAV-2 genomes. vg/ml, vector genomes/ml; ag/ml, amp^R genomes/ml; kg/ml, kan^R genomes/ml. ND, not detected.

AAV particles, indicating that plasmid backbone sequences present in the rAAV stocks originated from both the rAAV vector plasmid and the helper plasmid. Nevertheless, the higher ratio of kan^R versus amp^R genomes strongly suggested that the vector plasmid was the major source of contaminating backbone-derived sequences.

This latter result implied indirectly that these plasmid molecules had been able to replicate and to be packaged. The prevalent model to explain AAV replication from a circular plasmid implies that the AAV genome is rescued from the plasmid either before or during replication, thus preventing the concomitant replication of the plasmid backbone [18–21]. Our results suggested that if rescue and preferential replication of the rAAV vector can occur, replication of the entire rAAV plasmid is also taking place at a significant level. Accordingly, replication of backbone sequences was previously observed during *in vitro* replication of AAV-containing plasmids [22,23]. That the pDG plasmid, which has an almost complete deletion of the p5 promoter, was relatively poorly encapsidated, compared to the ITR-containing vector plasmid (Table 1), supports a relationship between replication efficiency and the level of plasmid backbone sequence encapsidation in AAV-2 particles.

Packaging of Prokaryotic DNA Sequences Also Occurs during Production of rAAV Using Stable Producer Cell Lines

The previous results indicated that prokaryotic sequences were packaged when rAAV was produced by transient transfection. However, it could be expected that such undesirable events would not take place during the production of rAAV particles using stable producer cell lines. The stable cell lines used for these experiments were derived from HeLa cells and contained the *rep-cap* gene and a rAAV vector (AAVLZ) integrated in the cell genome [7,10,24]. All of these producer cell clones assembled rAAV particles upon infection with wild-type adenovirus (wild-type Ad5) (Table 2). In addition, we detected amp^R sequences at a ratio varying between 3 and 7% of the total number of vector genomes. Importantly, we found no amp^R sequences by PCR in the wild-type Ad5 stock used in all of these experiments (data not shown).

The result obtained with the 26Z9 cell clone, in which the *rep-cap* genes were transferred by plasmid transfection, whereas the vector was introduced by infection with purified rAAVLZ particles, suggested that integration of the *rep-cap* plasmid in the original packaging cell clone was sufficient to generate amp^R-containing particles. To confirm this hypothesis, we analyzed the AAV particles produced by a similar packaging cell clone (HeRC32) that was used to generate the two HeAAVLZ clones [7]. The HeRC32 cells contained approximately two to three copies of the entire *rep-cap* plasmid integrated in the cell genome in a head-to-tail configuration (data not shown). As shown in Table 2, infection of HeRC32 cells with wild-type Ad5, in the absence of the vector plasmid, was sufficient to generate a low but detectable level of amp^R-containing particles. These results indicated that amp^R sequences could also be packaged using stable producer cell clones generated by stable transfection of a *rep-cap* plasmid. We have previously described that the integrated *rep-cap* genome in the HeRC32 cell line was subjected to ≈ 100 -fold amplification upon infection of the cells with wild-type Ad5 [25]. This phenomenon was shown to generate large extrachromosomal *rep-cap* molecules. We similarly observed that in HeRC32 cells, the prokaryotic backbone plasmid sequence was also subjected to an intense

TABLE 2: Quantification by Q-PCR of AAV particles containing vector and plasmid-derived sequences produced from stable cell clones

Cell clone	AAV vector ^a	Helper virus	rAAV (vg/ml)	amp ^R (ag/ml)
AAVLZ118	pAAVLZ	wt Ad5	1.9×10^{10}	1.2×10^9 (6.3%)
AAVLZ149	pAAVLZ	wt Ad5	3.2×10^{10}	1.2×10^9 (3.7%)
26Z9	rAAVLZ	wt Ad5	1.0×10^{10}	2.8×10^8 (2.7%)
HeRC32	–	wt Ad5	–	1.08×10^8

AAV particles produced by infecting each stable cell clone with wild-type (wt) Ad5 were purified and quantified as indicated in the footnote to Table 1. The numbers in parentheses indicate the percentage of amp^R over vector genomes.

^a This column indicates whether AAV vector integration into each stable cell clone was obtained by transfection with a rAAV plasmid (pAAVLZ) or infection with a rAAV (rAAVLZ).

amplification upon adenovirus infection (data not shown). As such, these data suggested that the packaging of such plasmid-derived sequences was dependent upon their amplification induced by adenovirus infection.

Globally, these data and those presented in the previous paragraph indicated that such undesirable particles are generated by both production methods. It remains to be determined if nonvector sequences are also packaged using other production methods like those employing baculoviruses or herpesviruses [11,26–28]. In addition, three alternatives can be considered to prevent the formation of such particles. The first is the modification of the prokaryotic plasmid backbone of the vector plasmid by increasing its size and/or by removing the nonspecific Rep-binding site (RBS) sequences that are present in almost all pBR322-derived plasmids [29]. Indeed, the presence of such sequence could favor replication and packaging of prokaryotic backbones. The second alternative would be establishing packaging cell clones with no integrated backbone sequences. Finally, the third and certainly most efficient procedure would consist in the sequential amplification of the rAAV particles from an initial stock produced by plasmid transfection. So far, attempts to amplify the rAAV particles on stable *rep-cap* cell clones using adenovirus as a helper have proved unsuccessful (A. Salvetti, unpublished results) but a recent report indicated that it could be feasible using a recombinant pseudorabies virus containing the *rep* and *cap* genes [27].

The amp^R Sequence Is Found in Genetically Modified Tissues After rAAV-Mediated Gene Transfer

We next asked whether the prokaryotic sequences found packaged into AAV particles could also be detected *in vivo* after rAAV injection. To answer this question, we retrospectively analyzed the tissues from animals used in previous gene transfer studies. This decision was motivated by (i) the availability of several tissues from non-human primates and dogs that would make this analysis particularly pertinent, (ii) the use in those studies of different transgenes and alternative AAV serotypes that are more relevant for clinical applications, and (iii) the availability of tissues from animals injected with rAAV produced either by transient transfection or by stable producer cell clones.

For all these samples, we first measured the presence of amp^R and vector, i.e., transgene, sequences by non-quantitative PCR after extraction of total genomic DNA (Fig. 2, left). We first analyzed the muscles of two macaques that were injected intramuscularly with a rAAV-2 vector encoding the macaque erythropoietin cDNA (AAVEpo) under the control of a tet-regulated promoter [30]. The analysis of total DNA extracted from muscle biopsies, 1 and 5 months after rAAV injection, indicated that vector and amp^R sequences could be easily detected by PCR in both animals, whereas they were

undetectable in the tissue from a mock-injected animal (Fig. 2A). We next analyzed the brain tissue from a macaque and a dog that were injected intracerebrally with a suspension of rAAV-5 particles. In these experiments the rAAV-5 preparation was obtained by cotransfection of a rAAV-5 vector with the AAV-5 *rep-cap*-expressing construct pAAV5-2 and using plasmid pXX6 to supply the helper functions [6,31]. The vector encoding the human α -L-iduronidase (AAVIDUA) was injected into the putamen of a normal macaque and a mucopolysaccharidosis type I-affected dog (8×10^{10} vector genomes in 80 μ l) (J. M. Heard and P. Moullier, unpublished data). Either 5 months (macaque) or 1 month (dog) after rAAV injection, we extracted total DNA from coronal slices surrounding the injection track and analyzed it by PCR to detect transgene and amp^R sequences. In both animals, IDUA and amp^R sequences could be amplified in the slices that were closer to the injection site, whereas more distant sites were negative (Figs. 2C and 2E).

Finally, we looked for the presence of the amp^R sequence *in vivo* after injection of rAAV particles produced using the stable cell clones. For these analyses, we used total DNA extracted from the brain of three mice deficient for the adrenoleukodystrophy protein (ALDP) that had received a preparation of a rAAV-2 encoding the human ALDP (AAVALD), which was injected into the corpus callosum. The vector used in these studies was produced using a stable AAVALD cell line infected with wild-type Ad5 [10]. As shown in Fig. 2G, we found ALD and amp^R sequences in the brains of the three mice analyzed, 6 weeks after rAAV injection. This finding confirmed that, as observed for rAAV stocks produced by transfection, the amp^R sequence packaged by the stable producer cell lines persisted *in vivo* following rAAV injection.

To substantiate these results further we measured the level of plasmid copies, i.e., amp^R, and transgene copies in these samples by Q-PCR and, when possible, compared these values with those measured in the original rAAV stock. The results of these analyses are shown in Fig. 2 (right) and summarized in Table 3. Importantly, we verified that the sensitivity of the Q-PCR was the same using either the amp^R primers or those hybridizing to the transgenes used and extended, in the linear and quantitative part of the assay, from 1 to 10^6 copies of plasmid. Also the sensitivity of this assay was not affected whether DNA was extracted from rAAV particles or from animal tissues (data not shown). The analyses conducted on the original rAAV stocks confirmed that, as previously demonstrated (Table 1), the level of amp^R genomes ranged from 1 to 5% of the total number of vector genomes (Table 3). Surprisingly the percentage of amp^R copies measured *in vivo* was consistently higher than that measured in the original rAAV stock, even though a great variability was observed among the samples. We do not

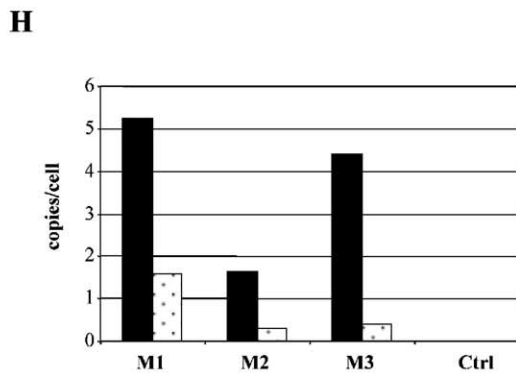
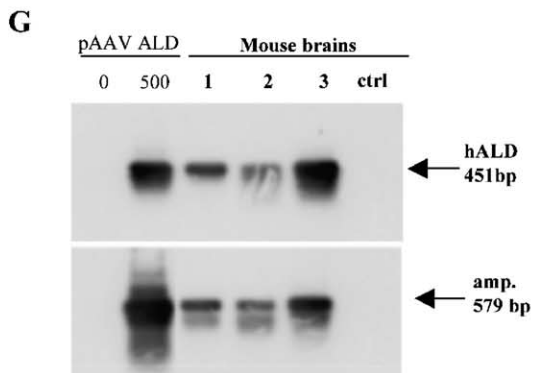
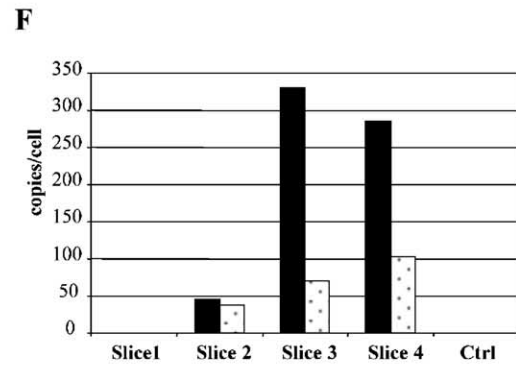
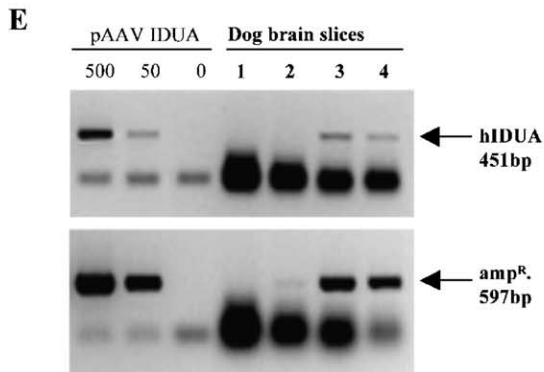
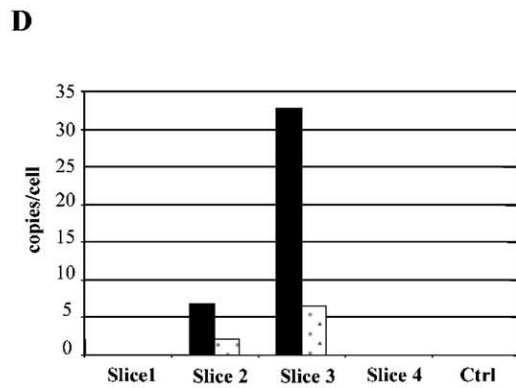
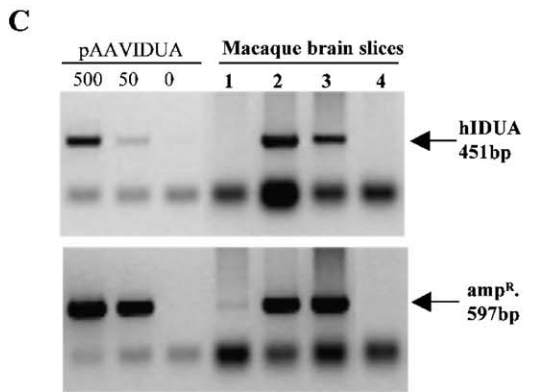
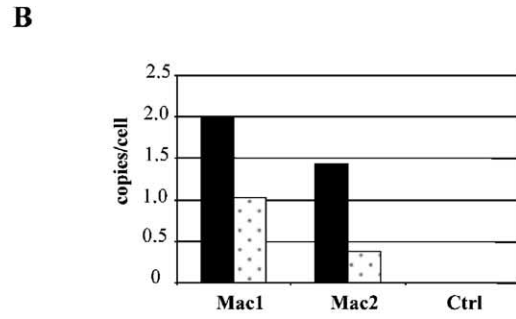
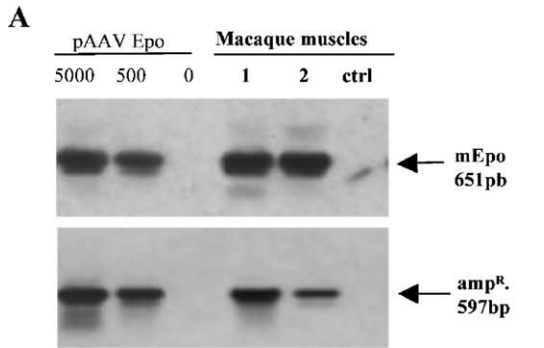


TABLE 3: Summary of the percentage of amp^R versus vector copies found *in vivo* and in the corresponding rAAV stock

rAAV	rAAV prep amp ^R / vector copies	Animal/time postinjection	Injected tissue	<i>In vivo</i> amp ^R /vector copies
AAVEpo 2/2	NA	Macaque/5 months	Muscle	50%
AAVEpo 2/1	1.3%	Macaque/1 month	Muscle	28%
AAVIDUA 5/5	1.4%	Macaque/5 months	Brain	S2, 31%; S3, 19%
AAVIDUA 5/5	1.4%	Dog/1 month	Brain	S2, 80%; S3, 21%; S4, 36%
AAVIDUA 5/5#	1.4%	Dog/3 months	Brain	S1, 5.2%
AAVALD 2/2*	4.1%	Mice/1.5 months	Brain	29%
AAVALD 2/2*	4.1%	Mice/1.5 months	Brain	19%
AAVALD 2/2*	4.1%	Mice/1.5 months	Brain	9%

The numbers following the name of the vector refer to the serotype of vector and the capsid. The amount of amp^R and vector copies was quantified *in vitro* and *in vivo* by Q-PCR as previously described and reported as the percentage of amp^R copies over the total number of vector copies. The analyses on dog and macaque brains were conducted on individual coronal slices (S). The asterisks designate the rAAV stocks produced using stable producer cell lines [10]. All these samples are also presented in Fig. 2, except for the one marked with #. NA, sample not available.

yet have an explanation for this result but it clearly indicates that these plasmid sequences were at least as stable as the transgene.

Detection of ITR–Plasmid Junctions Packaged into AAV Capsids and *in Vivo*

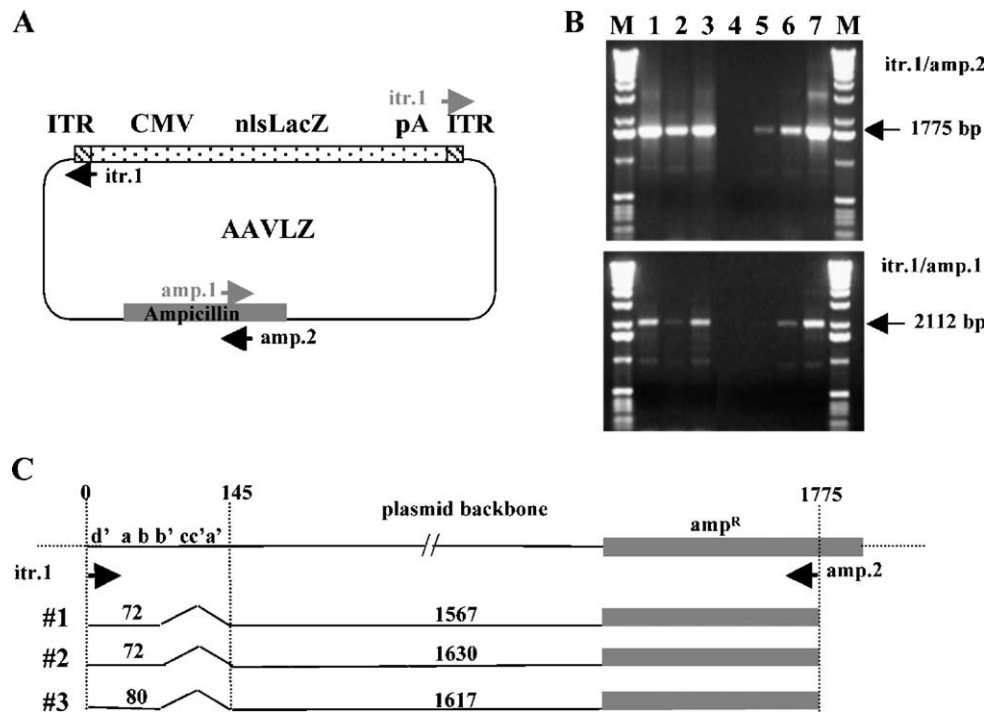
Previous models for rAAV replication from circular plasmids have proposed that the rescue of the rAAV genome occurs by the preferential replication of the sequences flanked by the viral ITRs, i.e., the rAAV vector [18–21]. In this study, the presence of prokaryotic sequences in rAAV stocks implied indirectly that they had been able to replicate, presumably because of the presence of the ITRs on the same plasmid. Consequently, we first asked whether the packaged molecules containing the amp^R sequence also contained the AAV ITRs. For this, we extracted DNA from three different rAAVLZ stocks and amplified it using a first primer in the ITR D region and a second one located in the amp^R gene (Fig. 3A). The analysis of the PCR products indicated that they had a size corresponding to the expected ITR–amp^R fragments (Fig. 3B). The sequencing of these PCR products confirmed the presence of plasmid sequences linked to the AAV ITR (Fig. 3C). However, in all the sequenced products it was not possible to detect the complete ITR and a gap was consistently observed between the B/B' region and the beginning of the plasmid backbone sequences. The same analysis conducted on seven other rAAV stocks pro-

duced with vectors containing different transgenes confirmed the presence of ITR–plasmid sequences with similar extensive deletions in the ITRs (Supplemental Fig. 1). The presence of this deletion in all the clones analyzed strongly suggested that it reflected the real structure of these packaged sequences even though it cannot be excluded that it was introduced during PCR amplification and/or sequencing.

Second, we asked if ITR–plasmid junctions were also found *in vivo* in a form that could be rescued through bacterial transformation. For this, *Escherichia coli* cells (DH5 α) were transformed with 100 to 500 ng of DNA extracted from the rAAV-injected tissue and then selected on ampicillin-containing plates. The samples used in this assay were those derived from most of the animals presented in Fig. 2 and Table 3 ($n = 5$). We obtained a single amp^R colony reproducibly ($n = 4$) only with DNA from the AAVIDUA 5/5-injected dog brain that contained the highest number of vector amp^R sequences as detected by Q-PCR (Fig. 2F). Importantly, we obtained no colonies using DNA from uninjected animals. The restriction patterns of these four colonies suggested that they were identical and contained extensive deletions between the two viral ITRs in the transgene portion (data not shown). We determined the complete sequence of one rescued plasmid and confirmed that it derived from the parental pAAVIDUA construct. This rearranged plasmid contained the complete plasmid backbone of pAAVIDUA and had an extensive deletion in the transgene expression cassette

FIG. 2. Detection and quantification of transgene and amp^R sequences *in vivo* after rAAV injection. Total DNA was extracted from the following tissues: (A and B) Skeletal muscles from macaques 5 months and 1 month, respectively, after intramuscular injection of rAAV-2 encoding the mEpo. The same vector was packaged in either AAV-2 (rAAVEpo 2/2 for Mac1) or AAV-1 capsids (rAAVEpo 2/1 for Mac2) [30]. (C and D) Brain coronal slices of a macaque, 5 months after injection of rAAV-5 vector encoding the IDUA and packaged into AAV-5 capsids (rAAVIDUA 5/5). (E and F) Brain coronal slices of a dog, 1 month after injection of rAAVIDUA 5/5. (G and H) Brain slices of ALD^{-/-} mice, 1.5 months after injection into the corpus callosum of rAAV-2 particles encoding the ALDP (rAAVALD 2/2) [10]. In this last case the rAAVALD stock was produced using a stable cell clone [10]. The presence of transgene and amp^R sequences was evaluated by PCR (A, C, E, and G), followed by Southern blot analysis using appropriate probes (A and G). The same samples were analyzed by Q-PCR (B, D, F, and H): black columns, number of transgene copies per cell; dotted columns, number of amp^R copies per cell.

FIG. 3. Detection of packaged ITR–plasmid backbone junctions in rAAV stocks. (A) Position of the itr.1 and amp.1/amp.2 primers on the AAVLZ plasmid used for the preparation of the rAAV stocks. (B) Analysis of the PCR products on an agarose gel. Packaged DNA was extracted from approximately 1×10^9 AAVLZ particles produced by transient transfection of 293 cells. Helper functions were supplied by plasmids pRC and pXX6 (lane 1), pRC and Ad.dl324 (lane 2), or pDG (lane 3) (see Table 1). Approximately 1/10 of the recovered DNA was used for each PCR. As a control, we used pAAVLZ plasmid DNA (0.5, 5, and 50 ng in lanes 5, 6, and 7, respectively) or unrelated plasmid (lane 4). The expected size of the amplified fragment is 1.775 kb using itr.1/amp.2 and 2.112 kb using itr.1/amp.1. (C) Sequencing of the PCR products obtained with primers itr.1/amp.2. (#1, #2, and #3 refer to the same samples as in lanes 1, 2, and 3 of B). The linear structure of the AAVLZ plasmid amplified using these primers is shown on the top. It includes the left ITR (D'ABB'CC'A') and the plasmid backbone to the end of the amp^R gene. The numbers above refer to the nucleotide position, assuming that the sequence starts at the beginning of the D region. The lines below represent the sequences of three independent PCR products obtained with itr.1/amp.2 primers. The numbers above each line indicate the lengths of the sequenced regions.



and in both AAV-5 ITRs (Fig. 4A). In both rearranged ITRs, the conserved sequence was in the A region and covered the RBS (Fig. 4B). Only the right ITR also conserved, in addition to A, a partial C and a complete D region including the terminal resolution site.

In conclusion, these results indicated that DNA molecules containing ITR–plasmid junctions could be isolated both from the rAAV stock and *in vivo* from a rAAV-transduced tissue. In both situations, an extensive deletion of the AAV ITR was observed with the preferential retention of the A region containing the RBS.

The main question raised by these studies is: Does the presence of these particles in the rAAV stocks actually represent a safety issue? On the one hand it could be argued that the findings reported in this study do not implicate any problem in terms of safety given that several long-term studies conducted in small and large animals have not revealed any problem associated with rAAV gene transfer. Also, the finding that, *in vivo*, most of the rAAV sequences are found in an episomal form is a strong

argument supporting the safety of these vectors [32–34]. On the other hand, our *in vivo* data indicated that plasmid sequences and in particular the antibiotic resistance gene are stable and present in the tissue in a potentially rescuable form. The finding that plasmid sequences could be rescued from only one sample, containing the highest amount of vector and plasmid DNA, certainly indicates that this event is not taking place at a high frequency. Nevertheless, this observation may become particularly relevant if one considers that much higher levels of transduction can now be achieved using new AAV serotypes [35]. The use of alternative antibiotic resistance genes, as already recommended by the Food and Drug Administration in the United States (www.fda.gov/cber/gdlns/somgene.pdf) and by the European Agency for the Evaluation of Medicinal Products (CPMB/BWP/3088/99), can certainly reduce the potential risk associated with the transfer of such sequences *in vivo*. Nevertheless, the results from this study emphasize the need to quantify these undesirable particles in the rAAV stocks used for clinical

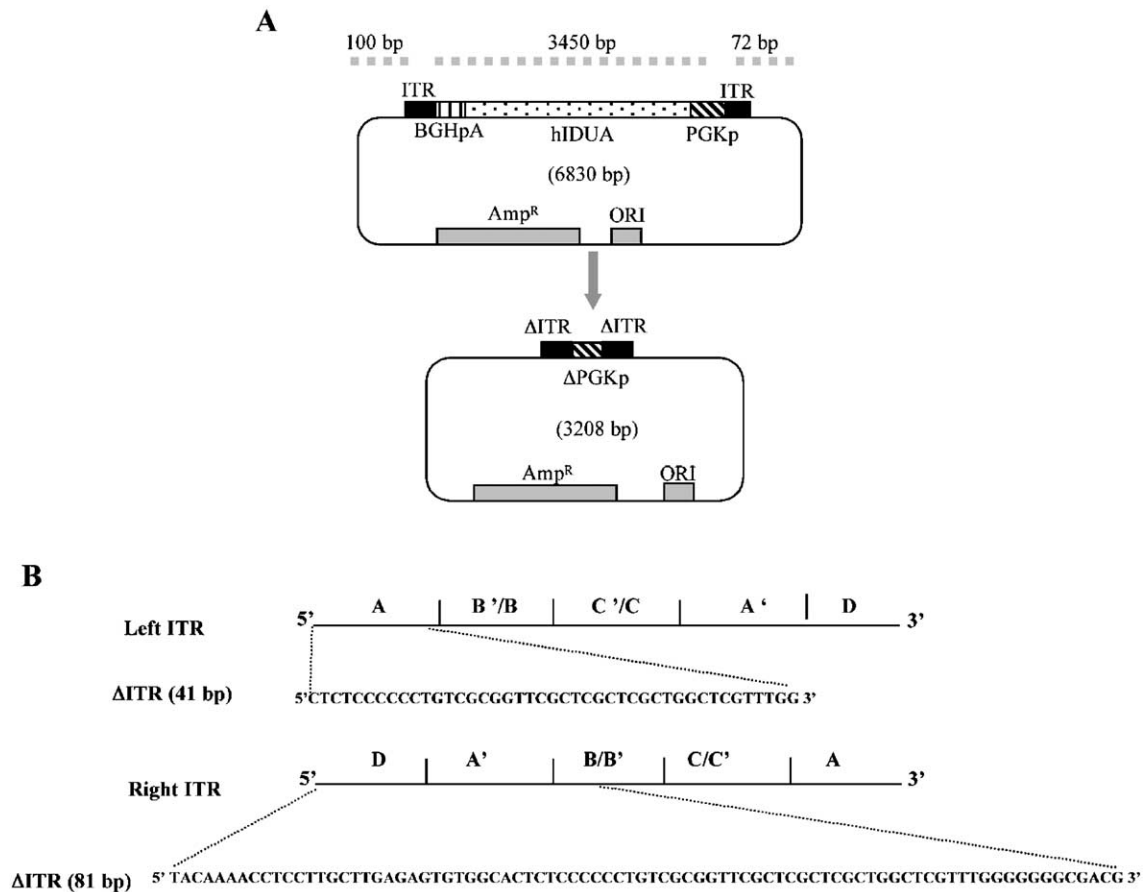


FIG. 4. Rescue and analysis of an amp^R plasmid from the rAAVIDUA 5/5-injected dog brain. *E. coli* cells (DH5 α) were transformed with 100 to 500 ng of total DNA extracted from the coronal slice 3 of the dog brain injected with the rAAVIDUA 5/5 vector (Figs. 2E and 2F) and selected on ampicillin-containing plates. One amp^R colony obtained using this DNA sample was sequenced. (A) The top shows the structure of the parental pAAV-5IDUA plasmid that was used to make the rAAV stock. The bottom shows the structure of the rescued plasmid. The deletions observed in this plasmid are indicated by a gray dotted line above the parental pAAV-5 IDUA construct. BGHpA, bovine growth hormone polyadenylation signal; hIDUA, human α -L-iduronidase cDNA; PGKp, phosphoglycerate kinase-1-promoter; Amp^R, ampicillin resistance gene; ORI, prokaryotic origin of replication; ITR, AAV-5 inverted terminal repeats. (B) Structure of the wild-type and of the rearranged AAV-5 ITRs.

applications and to monitor the presence of these sequences *in vivo*.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Viruses

HeLa and 293 cells were obtained from the ATCC. The packaging cell line HeRC32 was clonally derived from HeLa cells and contained two copies of the ITR-deleted *rep-cap* genome of AAV-2 [7]. The HeAAVLZ118 and HeAAVLZ149 producer cell clones were obtained after cotransfection of HeRC32 cells with both a rAAV vector plasmid encoding the nuclear-targeted β -galactosidase (AAVLZ) and the pSV-Hygro plasmid conferring resistance to hygromycin [10]. The 26Z9 cell line (kindly provided by Richard Snyder, Powell Gene Therapy Center, University of Florida, Gainesville) was obtained by infecting a packaging cell line with rAAVLZ particles [24]. All cell lines were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin/streptomycin (Gibco BRL, 5000 U/ml).

Most plasmids used in this study have been described previously (Fig. 1). The pRC plasmid contains the ITR-deleted AAV-2 *rep-cap* genome (nt

190 to 4484 of wild-type AAV) inserted into the psp72 plasmid (Promega) [13]. Plasmid pDG had both the AAV *rep-cap* genes, in which the p5 promoter region of the *rep* gene was partially replaced with the MMTV promoter, and the adenovirus E2a, E4, and VA1 sequences [5]. Plasmid pXX6 contained a mini-adenovirus genome including the E2a, E4, and VA1 sequences [6]. Plasmid pAV2 had the intact wild-type AAV-2 genome cloned into pBR322 [36]. The AAV plasmid vectors pAAVLZ and pAAVGFP(K) were derived from plasmid psub201 by deleting the *rep-cap* region and replacing it with an expression cassette encoding either LZ or the green fluorescent protein (GFP), both under the control of the cytomegalovirus (CMV) immediate early promoter. All plasmids had the bacterial ampicillin resistance gene except for the pAAVGFP(K), which instead had the kanamycin resistance gene. Plasmid pAAV5-2, used for the production of rAAV-5 particles, contained the AAV-5 ITR-deleted *rep-cap* genome [31].

Wild-type adenovirus type 5 and the E1-deleted Ad.dl324 were produced and titrated on 293 cells according to standard procedures [37]. Adenovirus stocks were routinely checked by PCR for the absence of the *rep* sequence [13].

Vector Production and Titration

The rAAV particles were produced by transient transfection of 293 cells and purified as previously described [13]. An additional method for the

generation of rAAV particles involved using the rAAV producer cell lines HeRC32LZ118, HeRC32LZ149, and 26Z9 infected with wild-type Ad5 at an m.o.i. of 50. The rAAV particles were extracted from cell lysates 48 h after transfection (or infection) and purified on a double CsCl gradient as previously described [13]. Recombinant AAV-containing fractions were titrated by Q-PCR and dot blot to measure the number of particles containing vector and amp^R genomes per milliliter (g/ml).

Detection of rAAV and Plasmid Sequences by PCR and Q-PCR

PCR on DNA extracted from purified rAAV particles. Analysis by PCR was performed on pure or 1/10- and 1/100-diluted samples using a Perkin-Elmer thermocycler (Gene Amp PCR System 9600) as previously described [15]. The PCRs were performed using the following primers sets: 5'-ATTATCGATGAGCGTGGTGG-3'/5'-TGTCTGCTTCATCAGCAGG-3' for lacZ, 5'-AAGTTCATCTGCACCACCG-3'/5'-TGTCTGCTGGTAGTGGTCG3' for gfp, 5'-CATCCATAGTTGCTGACTCC-3'/5'-AAGTCTGCTATGTGGCGC-3' for amp^R, 5'-ATATTCGGCAAGCAGGCATC-3'/5'-TGCTCG-ACGTTGTCACCTGAA-3' for kan^R. To detect ITR-plasmid backbone junctions we used the following primers: itr.1, 5'-GGAACCCCTAGTGATGGAG-3', located in the AAV ITR D region, and either amp.1, 5'-GGT-ATTATCCCGTATTGAC-3', or amp.2, 5'-GTCAATACGGGATAATACC-3', both located at the same position on the amp^R gene. The ITR-amp^R PCR products were sequenced directly using the BigDye Terminator Kit (Applied Biosystems).

Analysis by Q-PCR was performed using SYBR Green quantitative PCR. The C_t values were obtained from the amplification of serial dilutions of plasmid DNA containing the lacZ, gfp, kan^R, or amp^R genes and were, respectively, 32.3 to 5.4 × 10⁵ copies, 41.7 to 6.9 × 10⁵ copies, 41.7 to 6.9 × 10⁵ copies, and 32.3 to 5.4 × 10⁵ copies. AAV vector, amp^R, and kan^R sequences were simultaneously amplified from serial dilutions of DNA extracted from the viral particles. Copy numbers were calculated using the regression curve equations established with each corresponding plasmids. Also, negative controls were provided by plasmids containing either an unrelated transgene or a different antibiotic resistance gene. Primers used were 5'-GTGGTGGTTATGCCGATC-3'/5'-AACCACCGCACGATAGAG-3' for lacZ, 5'-TGCAGTCTTCAGCCGC-3'/5'-ACGTAGCCTTCGGGCATG-3' for gfp, 5'-CCTGCCGAGAAAGTATCC-3'/5'-ATGTTTCGCTTGGTGGTC-3' for kan^R, 5'-TGAAGCCATACCAACGAC-3'/5'-AACTTATCCGCTCCATC-3' for amp^R. Amplification parameters were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, using a Model 7700 sequence detector (Perkin-Elmer Applied Biosystems).

PCR on DNA extracted from animal tissues. Total DNA from genetically modified tissues was extracted using an 8 M urea/1% SDS/10 mM EDTA/300 mM NaCl buffer containing 500 µg/ml proteinase K (Boehringer Mannheim) and further processed as described [30]. The following primers were used: 5'-GAGTAGGCGTACGGTGGGAGG-3'/5'-GTGTCAGCAGT-GATGGTTCGGAG-3' for the CMV-erythropoietin cassette (CMV-Epo), 5'-CATCCATAGTTGCTGACTCC-3'/5'-AAGTCTGCTATGTGGCGC-3' for amp^R, 5'-GCTACCTTCGTCAACAGTGC-3'/5'-CATAGAAGGCGATCTCCTCC-3' for human adrenoleukodystrophy (ALD), 5'-CTTCGAGACGTTGGAATGAGC-3'/5'-GATGACCTTACCACCATGG-3' for human α-L-iduronidase (IDUA).

For Q-PCR, C_t values were obtained from the amplification of serial dilutions of plasmid DNA containing the hIDUA, the hALD, the macaque erythropoietin (mEpo), the macaque chorionic gonadotropin (CG), the mouse CCR5, the canine β-glucuronidase (GUSB), or the amp^R cDNAs and were, respectively, 38 to 6 × 10⁵ copies, 42.6 to 7.1 × 10⁵ copies, 32.3 to 5.3 × 10⁵ copies, 50.1 to 8.5 × 10⁵ copies, 43.6 to 6.9 × 10⁵ copies, 33.9 to 5.4 × 10⁵ copies, and 32.3 to 5.4 × 10⁵ copies. The AAV vector and amp^R were amplified from serial dilutions of total DNA extracted from the tissues. The mouse CCR5, the GUSB, the mEpo, and the macaque CG sequences were used as internal 2N genome standards for murine, canine, and macaque tissues, respectively. Primers used were 5'-ACTTGACCTTCTCAGGGAGAAC-3'/5'-CACCTGCTTGTCTCAAGTCA-3' for IDUA, 5'-TTCATCCAGGAGGGCGTACT-3'/5'-CAAAGGAAGGGCTACTCGGA-3' for ALD, 5'-GCTCCACTCCGAACCATCAC-3'/5'-TCATCTGTCCCTCTCCTGC-3' for Epo, 5'-GCAGCACTTCTGACTGTGGG-3'/5-

CGAGATGGACTTGGAGGGC-3' for CG, 5'-ACACCCTGTTTCGCTGTAGGAA-3'/5'-TGTAGGGAGTCCAGAAGAGAAAGTAGAC-3' for CCR5, 5'-ACGCTGATTGCTCACACCAA-3'/5'-CCCCAGGTCTGCTTCATAGTTG-3' for GUSB, 5'-TGAAGCCATACCAACGAC-3'/5'-AACTTATCCGCTCCATC-3' for amp^R. Amplification parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s and 60°C for 1 min and were conducted using a Model 7700 sequence detector (Perkin-Elmer Applied Biosystems).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2005.06.003.

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