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Adeno-associated virus vectors can be efficiently produced without helper virus

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The purpose of this work was to develop an efficient method for the production of adeno-associated virus (AAV) vectors in the absence of helper virus. The adenovirus regions that mediate AAV vector replication were identified and assembled into a helper plasmid. These included the VA, E2A and E4 regions. When this helper plasmid was cotransfected into 293 cells, along with plasmids encoding the AAV vector, and rep and cap genes, AAV vector was produced as efficiently as when using adenovirus infection as a source of help. CMV-driven constructs expressing the E4orf6 and the 72-M_r, E2A proteins were able to functionally replace the E4 and E2A regions, respectively. Therefore the minimum set of genes required to produce AAV helper activity equivalent to that provided by adenovirus infection consists of, or is a subset of, the following genes: the E4orf6 gene, the 72-M_r, E2A protein gene, the VA RNA genes and the E1 region. AAV vector preparations made with adenovirus and by the helper virus-free method were essentially indistinguishable with respect to particle density, particle to infectivity ratio, capsimer ratio and efficiency of muscle transduction in vivo. Only AAV vector preparations made by the helper virus-free method were not reactive with anti-adenovirus sera.

Keywords: *AAV vector production; AAV helper genes; adenovirus*

Introduction

Of the DNA delivery vehicles currently being developed for gene therapy, vectors derived from adeno-associated virus (AAV) type-2 vectors are among the most promising. AAV vectors are quite versatile and have been successfully used for short-term applications, such as the delivery of suicide genes to tumors,[1] as well as for applications requiring long-term transgene expression in immunocompetent animals. While AAV vectors are capable of transducing a wide variety of human and animal cell types, they establish long-term transgene expression primarily in tissues composed of slow growing or post-mitotic cells. These include the central nervous system, skeletal muscle, the lung and the liver. For example, therapeutically relevant serum levels of clotting factor IX are reliably established in mice following a single intramuscular injection,[2] or hepatic portal vein instillation,[3] of AAV vectors encoding factor IX. Similar results have been observed with a diverse group of other transgenes including β-galactosidase[4] and erythropoietin (Epo)[5] expressed in muscle, tyrosine hydroxylase[6] and the GABA receptor[7] expressed in the brain, and the cystic fibrosis transmembrane conductance regulator[8] expressed in the lung. In all cases examined so far, the transgene expression remains at a stable level for greater than 4 months and the level of expression is linearly dependent on the dose of vector.

Because of their ability to establish sustained transgene expression, AAV vector-based stratagies are being developed for the treatment of genetic disease. Recently, a novel approach for the treatment for β-thalassemia was tested in thalassemic mouse models. In this study, single doses of an AAV mouse Epo vector, administered intramuscularly, were used to elevate the hematocrits of mice afflicted with moderate or severe β-thalassemia.[9] This treatment resulted in a substantial and sustained reduction in pathologies including iron accumulation in the spleen and heart enlargement. In addition to thalassemia, AAV-based strategies are being developed for the treatment of hemophilia B,[2,3] cystic fibrosis,[8] Parkinson's disease[6] and inherited retinal degeneration.[10] Conceivably, any inherited disease that can be complemented or ameliorated by the constitutive expression of a small therapeutic protein from post-mitotic or slowly growing cells may be treatable by AAV vector-based gene therapy.

AAV-2, the virus from which the vector system was derived, is a replication defective parvovirus that requires co-infection by adenovirus[11] or herpes virus[12] in order to replicate efficiently. Vaccinia virus acts as a partial helper.[13] The individual adenoviral genes that contribute to AAV helper function have been identified mainly by testing adenovirus mutants for their ability to mediate AAV replication. Neither adenovirus DNA replication nor late gene expression are required for repli-

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cation of AAV type-1 or -2 as adenovirus mutants that are conditionally defective for these functions mediate AAV replication under restrictive conditions.[14–16] Data from similarly designed experiments indicate that the E3 region[17] and the gene encoding the E1B 21-M_r protein[18] are also not required for AAV-2 replication. Adenovirus mutants with defects in the E1A,[19] E1B 55-M_r,[18] E2A[20] and E4 open reading frame 6 (E4orf6)[18,21] genes do not support AAV-2 replication and therefore these genes are presumed to participate in AAV replication. Janik *et al*[22,23] reached a similar conclusion by examining the effect of transfected adenoviral restriction fragments on AAV-2 replication, but also added the virus-associated RNA I (VAI) gene to the list of helper genes.[22,23] Additionally, these authors were able to exclude the E2B gene. Currently, it is believed that the E1A, E1B 55-M_r, VAI, E2A and E4orf6 genes are involved in AAV-2 replication.

Despite a substantial amount of research, a complete set of adenovirus helper genes has not been unambiguously defined nor has it been demonstrated that the combination of the E1A, E1B 55-M_r, VAI, E2A and E4orf6 genes is alone capable of producing levels of AAV help equivalent to that produced by adenovirus infection. Studies examining the helper activity of adenovirus mutants can only conclusively define genes that play no role in AAV viral replication (eg those genes that can be inactivated without causing any reduction of the helper activity of the resulting adenovirus mutant). The transfection approach of Janik *et al* is capable of unambiguously identifying a subset of adenovirus genes capable of producing AAV help equivalent to that produced by an adenovirus infection. Although a fairly comprehensive set of adenovirus fragments was tested, their optimum combination of transfected adenovirus genes produced AAV-2 approximately 1000-fold less efficiently than infection by adenovirus. Consequently, it was not possible to define a complete set of adenovirus helper genes from these data.

Like the parent virus, AAV vectors require adenovirus infection of the producer cells in order to replicate. AAV vectors are typically produced by cotransfecting separate plasmids encoding the AAV vector (composed of a gene of interest bounded by AAV inverted terminal repeats) and the AAV genome (the AAV *rep* and capsid genes without the AAV inverted terminal repeats) into 293 cells followed by infection with adenovirus. During the subsequent 72 h culture period, both recombinant AAV particles and adenovirus are produced. The recombinant AAV particles are then extracted from the cells, purified away from the contaminating adenovirus and cell debris, and then incubated at 56°C to inactivate any residual adenovirus. Even after purification by cesium chloride density centrifugation, the AAV vector preparations are still substantially contaminated with adenoviral structural proteins.

The purpose of this study was to develop a method for the efficient production of AAV vectors in the absence of helper virus. To do this, the subset of adenovirus genes that are required for efficient production of AAV vector in 293 cells was identified and assembled into a helper plasmid. When this adenoviral helper plasmid was cotransfected into 293 cells, along with plasmids encoding the AAV vector and *rep* and *cap* genes, AAV vector production occurred at levels equal to those achieved by the use of adenovirus infection as a source of help. Elimination of adenovirus from the AAV vector production protocol should result in a less complicated large-scale production procedure and a potentially safer preparation of higher purity.

Results

Virus-free production of AAV vectors

Initially, it was not clear whether transfected plasmids encoding adenoviral genes would be capable of providing efficient help for AAV vector production. Janik *et al*[22,23] had demonstrated that AAV-2 could be produced using transfected adenovirus DNA, but when compared with an adenovirus infection, their optimal set of transfected genes produced AAV-2 approximately one 1000-fold less efficiently.[22,23] To resolve this question, pJM17 (a plasmid that contains the entire adenovirus-5 genome) and pW4389lacZ (a plasmid that encodes both an AAV *lacZ* vector and the AAV-2 *rep* and *cap* genes) were cotransfected into 293 cells, and AAV *lacZ* vector production was assessed. AAV *lacZ* vector produced by the standard method employing adenovirus-2 infection was used as a control. The results are shown in Figure 1. When no source of help was provided, little AAV vector production was detected. Surprisingly, cultures cotransfected with pJM17 and cultures infected with adenovirus-2 produced similar amounts of AAV *lacZ* vector. pJM17 does produce extremely low levels of adenovirus when transfected into 293 cells in the presence or absence of AAV vector and helper plasmids (typically 5×10^6 cells transfected with 10 μg of pJM17 by the calcium phosphate method[24] produce a total of 10^4 plaque forming units (p.f.u.)). This is due to an infrequent rearrangement of the circular genome that occurs in a small number of transfected cells. It is unlikely that this small amount of adenovirus mediated the production of a 10 000-fold greater amount of AAV vector.

Data from adenovirus mutants and transfection studies suggest that only the E1A, E1B, VA RNA, E2A and E4 regions are involved in AAV-2 replication. In order to reduce the size of the helper plasmid, and to prevent the production of adenovirus, helper plasmids were assembled that contained only those regions thought to be necessary for AAV help. Since 293 cells stably express the E1A and E1B proteins,[25] only the E2A, E4 and VA RNA regions were used. Two plasmids were built, pVAE2AE4–5 was constructed with adenovirus-5 genes derived from pJM17 and pVAE2AE4–2 was constructed using genes isolated from purified adenovirus-2 DNA. Both of these plasmids contain promoterless copies of the 100K, 33K and pVIII genes because the E2A regions used to construct these plasmids encode the L4 region on the opposite strand. pVAE2AE4–5 also contains the promoterless adenovirus protease gene. These open reading frames are probably not transcribed because they lack promoters. When pVAE2AE4–2 and pVAE2AE4–5 were tested for AAV vector production, they proved to be as efficient as pJM17 and a few-fold more efficient than adenovirus infection (Figure 1).

pVAE2AE4–2 was dissected to determine whether any subsets of the E2A, E4 and VA RNA regions were capable of producing adenoviral help comparable with an adenoviral infection in 293 cells. Plasmids containing each of the regions alone and containing all of the pairwise com-

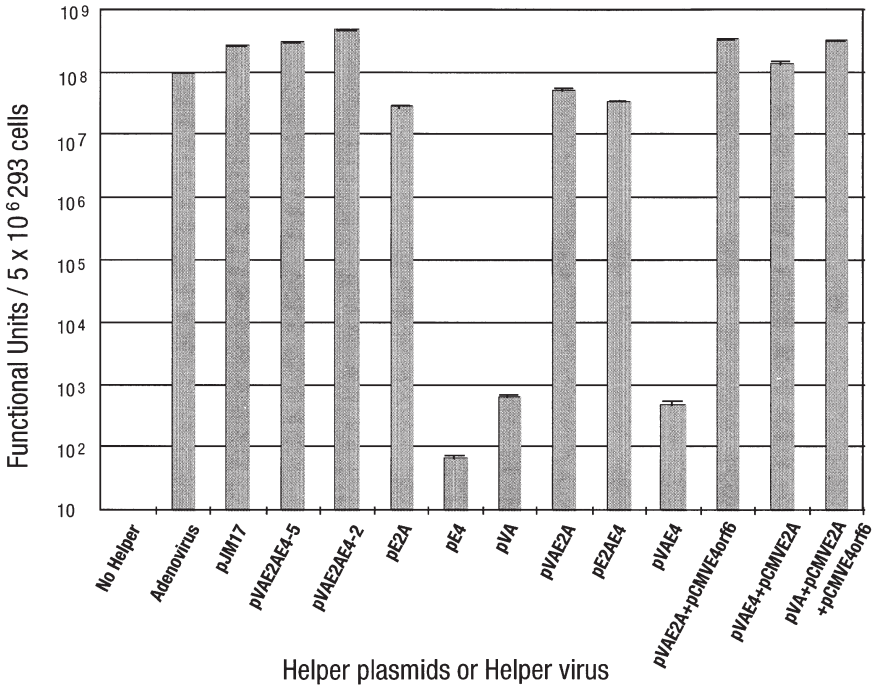

Figure 1 *Comparison of transfected adenoviral gene combinations and adenovirus infection with respect to AAV helper function. AAV lacZ vector was produced by transfecting 10 cm dishes (5×10^6 cells) of 293 cells with pW4389lacZ (a plasmid that encodes both an AAV lacZ vector and the AAV-2 rep and cap genes) and various combinations of cloned adenoviral genes. LacZ vector was also produced using adenovirus-2 infection. Helper function for AAV vector production was assessed by measuring the amount of AAV lacZ vector produced under each condition. The vector was titrated on 293 cells in the presence of adenovirus-2 and stained with X-gal before counting. Each bar represents the average value from triplicate cultures and the error bars represent the standard deviation of the mean.*

binations of the adenovirus-2 E2A, E4 and VA RNA fragments were constructed. This set of six plasmids was then tested for their ability to mediate AAV vector production. As it turned out, each of these three regions contributed to full helper activity and no plasmid containing a subset of these three regions could provide help equivalent to pJM17, pVAE2AE4–2, pVAE2AE4–5 or adenoviral infection. Among the plasmids encoding single regions, the E2A plasmid produced the highest amount of AAV vector but this level was approximately 16-fold less than that produced by pVAE2AE4–2. pVA and pE4 produced approximately 100 000- and 1 000 000-fold less AAV vector than pVAE2AE4–2, respectively (Figure 1). The plasmids containing the pairwise combinations of these three adenoviral genes, pE2AE4, pVAE2A and pVAE4, produced approximately 13-, 9- and 100 000-fold less AAV vector than pVAE2AE4–2, respectively.

The E4 region of adenovirus-2 encodes seven proteins, E4 open reading frames 1, 2, 3, 3/4, 4, 6 and 6/7.[26] Among these, only the E4orf6 34-M_r protein is thought to contribute to AAV-2 production.[18,21] To demonstrate that this is the case, a CMV-driven construct expressing only the E4orf6 34-M_r protein (pCMVE4orf6) was assembled and tested for helper activity. This construct does not express E4orf6/7. In addition, the promoterless open reading frames (100K, 33K and pVIII) in the E2A fragment make it difficult to formally conclude that only the E2A,72-M_r DNA binding protein participates in AAV-2 helper function. To resolve this matter, a CMV-driven construct expressing only the DNA binding protein (pCMVE2A) was constructed and tested for helper function as well. To test these single gene constructs in the AAV vector production assay, pCMVE2A was paired with pVAE4, and pCMVE4orf6 was paired with pVAE2A. The combination of pCMVE2A, pCMVE4orf6 and pVA was also tested. All three of these combinations were essentially equivalent to pVAE2AE4–2 with regard to AAV vector production (Figure 1).

Characterization of helper virus-free AAV vectors

Mid-scale preparations (25 225 cm^2 flasks) of AAV *lacZ* vectors were made with adenovirus-2 or with pVAE2AE4–5 and then purified using a two-step procedure consisting of polyethylene glycol precipitation followed by a single banding on a cesium chloride density gradient. These preparations were then compared with respect to particle to infectivity ratio, particle density, capsimer ratio, adenoviral protein immunoreactivity, and *in vivo* transduction. Table 1 summarizes the specifi-

Table 1 Mid-scale vector production

	Made with adenovirus		*Adenovirus-free method*	
	Crude	*Purified*	*Crude*	*Purified*
Total function units (*lacZ*)	2×10^{11}	8×10^{10}	3×10^{11}	1.4×10^{11}
Total genomes	1×10^{13}	5×10^{12}	4×10^{13}	2×10^{13}
Particle/Functional ratio	50	62.5	133	143
Density (mg/ml)		1.4		1.4

cations of these two preparations. The AAV vector production and purification yields were similar whether measured by functional units (*lacZ* staining) or by quantification of vector genomes. Neither the particle to infectivity ratios nor the particle densities differed significantly.

Aliquots of the crude extracts and the purified fractions of the mid-scale preparations made with adenovirus or by the helper virus-free method were resolved on an 8% acrylamide gel, blotted to nitrocellulose, and probed with either a monoclonal antibody that reacts with all three AAV-2 capsid proteins or a polyclonal antibody raised against adenovirus-2 particles. The crude and purified aliquots used in the Western analysis all contained 1×10^{11} AAV *lac*Z vector genomes. Production of AAV vectors by the helper virus-free method does not seem to change the AAV vector capsimer ratios either in the crude extract or purified fractions (Figure 2). When the crude extract of the AAV vector preparation made with adenovirus was probed with the anti-adenovirus polyclonal serum, a spectrum of adenoviral structural proteins was seen. After the two step purification, this preparation was still heavily contaminated with a single 62-M_r adenoviral protein that seems to have selectively copurified with the AAV vector. This contaminant was identified as the adenovirus-2 fiber protein after a portion of the preparation was digested with the protease Lysine-C and the resulting peptides were separated and sequenced (data not shown). Neither the crude extract nor the purified fractions of the AAV vector preparation made by the helper virus-free method showed any immunoreactivity with the anti-adenovirus-2 polyclonal serum. Several subsequent AAV vector preparations made with the adenovirus-5-based pVAE2AE4–5 have been similarly probed for immunoreactivity against polyclonal sera raised against adenovirus-5 and were entirely nonreactive (data not shown).

To assess the *in vivo* transduction abilities of AAV vectors made by the helper virus-free method, purified AAV vectors encoding the human erythropoietin (hEpo) cDNA were prepared with and without adenovirus, and then used to transduce the hind limb muscles of Balb/c mice. The mice were injected i.m. at three sites in each hind limb with a total of 3×10^{10} AAV hEpo vector particles. On days 14 and 35 after injection, the hematocrits were determined and the serum levels of hEpo were measured using an ELISA specific for human Epo (Figure 3). Gene expression from muscle transduced with AAV vectors rises steadily over a 6 week period before reaching a stable plateau.[5] The mice transduced with AAV vector preparations made with and without adenovirus showed this pattern with serum hEpo levels and hematocrits significantly elevated over control at day 14 and more so at day 35. At day 35, the mice had comparable hematocrits and serum hEpo levels whether they received vector made with adenovirus or by the helper virus-free method. At day 14, the hematocrits of both groups of mice were similar but the mice receiving the helper virus-free preparation had slightly higher concentrations of hEpo in their sera. The significance of this small difference in serum hEpo concentrations at the early time-point is not clear.

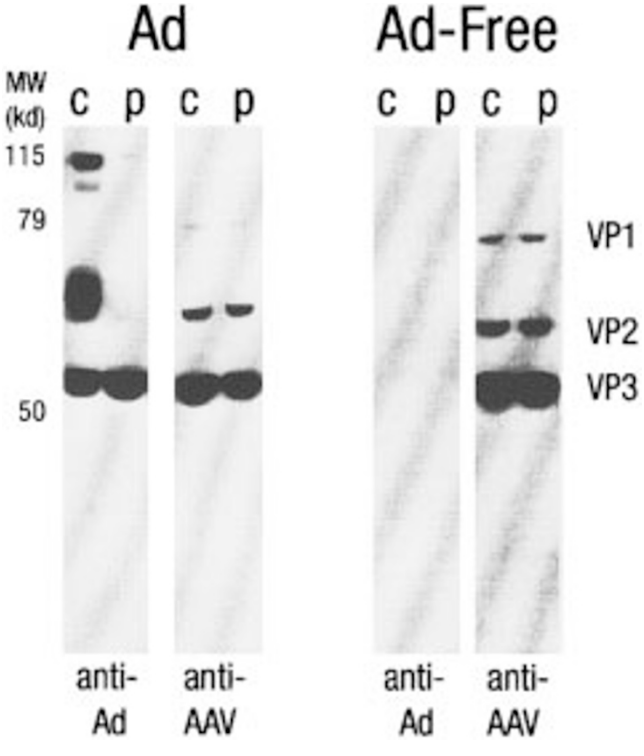

Figure 2 *Immunological characterization of mid-scale preparations of AAV lacZ vector made with adenovirus-2 and by the helper virus-free method. Crude lysates (c) or purified AAV lacZ vector fractions (p) from these two mid-scale preparations were resolved on an 8% polyacrylamide gel, blotted to nitrocellulose, and probed with a monoclonal antibody that recognizes all three AAV-2 capsid proteins (anti-AAV-2) or a polyclonal serum raised against adenovirus-2 particles (anti-Ad). Each lane represents 1×10^{11} AAV lacZ vector genomes.*

Discussion

AAV vectors are conventionally produced using adenovirus as a helper. This procedure usually results in the production of approximately equal numbers of AAV vec-

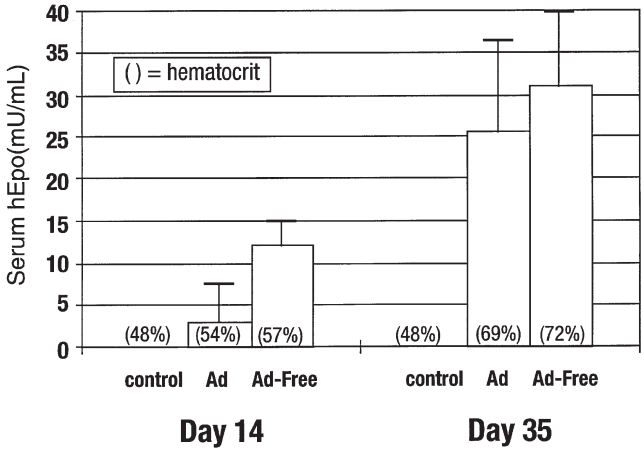

Figure 3 *Comparison of the efficiency of muscle transduction of AAV vector preparations made with adenovirus-2 and by the helper virus-free method. Balb/c mice were injected in the hind limb with 3×10^{10} AAV hEpo vector particles made with adenovirus or by the helper virus-free method. The mice were assayed on days 14 and 35 for hematocrit and serum concentration of human Epo. Each bar represents the average value from three mice and the error bars represent the standard deviation of the mean. The average hematocrit value is given in parentheses.*

tors and adenovirus particles. While purification removes most of the contaminating helper virus, and heat treatment inactivates the remainder, these AAV vector preparations are still substantially contaminated with adenoviral structural proteins, and with the fiber protein in particular. The reason why fiber protein selectively copurifies with AAV vectors on density-based separations is not clear. Fiber has been shown to bind DNA[27] and it may be that a complex composed of fiber protein and sheared cellular DNA has a density similar to that of AAV vectors. In any case, the presence of contaminating helper virus proteins in AAV vector preparations is undesirable because the adenoviral proteins may increase the immunogenicity of the AAV vector preparations and may adversely affect some target cell types.[27] The elimination of potentially pathogenic adenovirus from the production of clinical grade AAV vector preparations would clearly be advantageous.

This article shows that helper virus functions can be mimicked by a transfected plasmid encoding the adenoviral VA, E2A and E4 genes in 293 cells. When plasmids encoding these three regions are cotransfected along with the AAV vector and *rep*/*cap* plasmids, AAV vector production is comparable to that produced by transfection of the entire adenoviral genome (pJM17) or by adenoviral infection. While all three of these regions are required to produce levels of help equivalent to that produced by adenoviral infection, some level of AAV vector production occurs in the absence of each of these regions indicating that none of them is essential to the process.

Constructs expressing only the 34-M_r, E4orf6 protein or the 72-M_r, E2A protein are capable of functionally replacing the E4 and E2A regions, respectively, with no reduction in helper activity. This observation, along with the data of others,[18,21] suggests that these proteins alone may account for the activity of their respective regions. Additionally, the fact that the CMV IE promoter can be substituted for the E2A and E4 regulatory regions suggests that the intricate interplay that occurs between these adenoviral promoters and their gene products is probably irrelevant in the transfection-based helper system. Taken together, these data indicate that AAV-2 helper activity equivalent to that provided by adenovirus infection can be produced by expression of the E4orf6 gene, the 72-M_r, E2A protein gene, the VA RNAs and the E1 region. The minimum set of adenovirus genes capable of producing efficient AAV help may be a subset of this group.

The roles played by the VA RNAs, the 34-M_r, E4orf6 protein and the 72-M_r, E2A protein in AAV-2 replication are understood to varying degrees. The VA RNAs increase mRNA translation by inhibiting the interferon-inducible, RNA-dependent e1F-2 alpha protein kinase.[28–30] The 34-M_r, E4orf6 protein forms a heterodimer with the 55-M_r, E1B protein, and as such, facilitates the export of viral mRNA from the nucleus while inhibiting the translocation of host cell mRNA.[18,31–33] The E4orf6 protein as a monomer also inactivates p53.[34] The E2A region encodes a 72-M_r, DNA binding protein with affinities for single- and double-stranded DNA. AAV production in the absence of the E2A protein results in lower intracellular levels of the single- and double-stranded forms of the AAV genome, the cap proteins, and the spliced forms of the rep proteins.[35–37] The mechanisms by which the E2A protein affects AAV DNA, mRNA and protein metabolism are not currently understood. This protein also acts to inhibit transcription from the adenovirus early gene promoters.

When these three regions were examined for helper function separately, or in pair-wise combinations, it was apparent that the E2A region contributed much more to AAV vector production than did the VA RNAs or E4. When the E2A gene was eliminated from the transfection, the yield of AAV vector was 100 000-fold lower than when using all three genes or adenovirus infection. This result seems to differ from a previous report that an adenovirus mutant, defective for E2A expression, was able to mediate AAV-2 production only 10-fold less efficiently than wild-type adenovirus.[37] These apparently conflicting results may have to do with the differences between AAV-2 and AAV vector production. On the other hand, a key difference between these two experiments is that AAV vector production requires excision of the vector from a plasmid whereas replication of a virus stock introduced into the cell by infection does not. One could imagine that the E2A single-stranded DNA binding protein might facilitate the isomerization of the AAV-2 inverted repeat sequences of the vector plasmid from the fully duplex form, to the 'rabbit ear' form, thereby creating a better substrate for the rep-mediated excision process. The resolution of this question must await further experimentation.

AAV vectors can be efficiently produced in the absence of helper virus. With respect to their particle to infectivity ratio, particle density, capsimer ratio and *in vivo* transduction efficiency, AAV vectors made by the helper virus-free method are essentially indistinguishable from vectors made with helper virus. The elimination of adenovirus from AAV vector production will potentially result in preparations more ideally suited for *in vivo* use.

Materials and methods

Plasmids and their construction

pJM17,[38] consists of a circular form of the entire adenovirus-5 genome that contains a copy of pBR322 inserted into the unique *Xba*I site located in the E1A gene. The circularization of pJM17 occurred by ligation of the ends. This plasmid was used as the source of adenovirus-5 genes for the construction of pVAE2AE4–5, pCMVE4orf6 and pCMVE2A. Because neither adenovirus-5 nor pJM17 have been completely sequenced, the sizes of the adenovirus-5 fragments described below are not exact. Since adenovirus-2 has been fully sequenced, and adenovirus types-2 and -5 are thought to be approximately 99% homologous, adenovirus-2 coordinates will be used to describe the adenovirus-5 fragments.

pVAE2AE4–5 encodes the entire E2A and E4 regions, and VA RNA I and II genes. It also contains a promoterless L4 region (100-M_r, 33-M_r, VIII protein genes) and a promoterless adenovirus protease gene. These adenoviral genes and regions were subcloned from the adenovirus-5-based pJM17. pVAE2AE4–5 was made by assembling the VA RNA, E2A and E4 genes into a specifically designed polylinker which was inserted between the *Pvu*II sites of pBSIIs/k–. The assembly plasmid was constructed by ligating a polylinker encoding the restriction sites *Sal*I, *Xba*I, *Eco*RV, *Srf*I, and *Bam*HI (GTCGACAAATCTAGATATCGCCCGGGCGGATCC) to

the 2513 bp *Pvu*II fragment of pBSII s/k–. The adenovirus-5 fragments used were the approximately 1724 bp, *Sal*I–*Hin*dIII fragment encoding the VA RNAs (ad-2 positions 9831–11 555), the approximately 5962 bp *Bam*HI-*Srf*I fragment encoding E2A (Ad-2 positions 21 606–27 568), and an approximately 3663 bp fragment encoding the E4 region (Ad-2 positions 32 172–35 835). Before the E4 region could be assembled into the polylinker, an *Hph*I partial digestion was performed and an *Xba*I site was added to the 3′ end of the fragment at the position corresponding to the *Hph*I cleavage site at position 35 833 of Ad-2. The resulting *Hin*dIII–*Xba*I, E4 encoding fragment, along with the *Sal*I–*Hin*dIII, VA RNA encoding fragment, were ligated to the modified vector plasmid between the *Sal*I and *Xba*I sites in a three part ligation. The *Bam*HI–*Srf*I fragment encoding E2A, was then ligated to the product of this reaction between the *Bam*HI and *Srf*I sites. The genes in pVAE2AE4–5 are arranged such that the 5′ ends of the E2A and E4 promoters abut, causing the regions to transcribe away from each other in opposite directions. The VA RNA genes, which are located at the 3′ end of the E4 gene, transcribe towards the E4 gene. The plasmid is 13 882 bp in length.

pVAE2AE4–2 is composed of the E2A, E4 and VA RNA regions derived from purified adenovirus-2 DNA (Boehringer Mannheim, Indianapolis, IN, USA). This plasmid was assembled as follows: pBSIIs/k+ was modified to replace the 637 bp region encoding the polylinker and alpha complementation cassette with a single *Eco*RV site using oligonucleotide directed mutagenesis and the following oligonucleotide-CCGCTACAGGGCGCGATATCAGCTCACTCAA. A polylinker encoding the restriction sites *Bam*HI, *Kpn*I, *Srf*I, *Xba*I, *Cla*I, *Bst*1107I, *Sal*I, *Pme*I and *Nde*I was then cloned into the EcoRV site (GGATCCGGTACCGCCCGGGCTCTAGAATCGATGTATACGTCGACGTTTAAACCATATG). Adenovirus-2 DNA was digested and restriction fragments encoding the E2A region (a 5335 bp, *Kpn*I–*Srf*I fragment corresponding to positions 22 233–27 568 of the adenovirus-2 genome) and the VA RNAs (a 731 bp, *Eco*RV–*Sac*II fragment corresponding to positions 10 426–11 157 of the adenovirus-2 genome) were isolated. The E2A fragment was installed between the *Sal*I and *Kpn*I sites of the polylinker. An E4 region was first assembled in pBSIIs/k+ by ligating a 13 864 bp, *Bam*HI–*Avr*II fragment corresponding to adenovirus-2 positions 21 606–35 470 (encoding the 5′ end of the gene) and a 462 bp, *Avr*II and *Srf*I, digested PCR fragment corresponding to adenovirus-2 positions 35 371–35 833 (encoding the 3′ end of the gene) between the *Bam*HI and *Sma*I sites of pBSIIs/k+. The oligonucleotides used to produce the PCR fragment were designed to introduce a *Srf*I site at the junction where the E4 promoter and the adenovirus terminal repeat intersect and have the sequences AGAGGCCCGGGCGTTTTAGGGCGGAGTAACTTGC and ACATACCCGCAGGCGTAGAGAC. The intact E4 region was excised by cleavage with *Srf*I and *Spe*I and the 3189 bp fragment corresponding to adenovirus-2 positions 32 644–35 833 was cloned into the E2A intermediate between the *Srf*I and *Xba*I sites. Finally, the VA RNA fragment was inserted into the *Bst*1107 site after T4 polymerase-mediated blunt end modification of the *Sac*II site. The genes in pVAE2AE4–2 are arranged such that the 5′ ends of the E2A and E4 promoters abut, causing the regions to transcribe away from each other in opposite directions. The VA RNA genes, which are located at the 3′ end of the E4 gene, transcribe towards the E4 gene. The plasmid is 11 619 bp in length.

pVA, pE2A and pE4 were produced by cloning the 743 bp *Sal*I–*Cla*I, 5341 bp *Srf*I–*Bam*HI, and 3185 bp *Srf*I–*Cla*I fragments from pVAE2AE4–2 between the *Cla*I and *Sal*I, *Sma*I and *Bam*HI, and *Sma*I and *Cla*I sites of pBSIIs/k+, respectively.

pE2AE4, pVAE2A and pVAE4 were constructed as follows: pE2AE4 was constructed by cloning the 8526 bp *Cla*I–*Bam*HI fragment (encoding E2A and E4) from pVAE2AE4 between the *Cla*I and *Bam*HI sites of pBSIIs/k+. pVAE2A and pVAE4 were produced by cleaving pVAE2AE4–2 with *Srf*I and *Cla*I, and *Srf*I and *Bam*HI (partial digestion), to produce 8434 bp (VA and E2A encoding) and 6278 bp (VA and E4 encoding) fragments. These fragments were blunt-end modified using Klenow fragment and autoligated.

pCMVE4orf6 expresses only E4orf6 using the CMV immediate–early (IE) promoter. It was created by cloning the approximately 1024 bp, *Bgl*II–*Sma*I fragment encoding the ad-5 E4orf6 gene (ad-2 positions 34 115–33 091) into the expression plasmid p3.3c. The expression cassette of p3.3c is composed of the CMV IE promoter followed by the first intron from the human growth hormone gene, a cloning site for the gene to be expressed, and the SV40 late polyadenylation site. The construct expresses only the E4orf6 gene and does not express E4orf6/7.

pCMVE2A expresses only the 72-M_r, DNA binding protein using the CMV IE promoter. This plasmid was constructed by first subcloning the 2467 bp, E2A encoding, *Msc*I (partial)–*Bam*HI fragment from pE2A between the *Msc*I and *Bam*HI sites of pCITE2A (Novagen, Madison, WI, USA). This sequence corresponds to positions 24 073 (*Msc*I) to 21 606 (*Bam*HI) of the adenovirus 2 sequence. A 1636 bp, E2A encoding, *Nco*I (partial)–*Bsr*GI fragment, was excised from pCITE2AE2A, blunted, and cloned into p3.3c to create pCMVE2A. This fragment corresponds to positions 24 076 (*Msc*I/*Nco*I) to 22 440 (*Bsr*GI) of the adenovirus-2 sequence. This two-step subcloning procedure changes the sequence around the E2A initiator methionine from AGGAA**ATG**G to GCCCC**ATG**G.

pW4389lacZ consists of (in order around the plasmid) an ITR bounded CMV-driven *lacZ* expression cassette, a β-lactamase gene, the AAV-2 *rep* and *cap* genes, and a colE1 origin of replication. The plasmid was constructed as follows: pUC119 was cut with *Afl*III and *Bsp*HI (a partial digestion), blunt end modified with the Klenow enzyme, and ligated to form a circular 1732 bp plasmid containing the bacterial origin and the *amp* gene only (the polylinker and F1 origin were removed). The blunted and ligated *Afl*III and *Bsp*HI form a unique *Nsp*I site. The plasmid was cut with *Nsp*I, blunt end modified with T4 polymerase, and the 20 bp, *Hin*dIII–*Hin*cII fragment (made blunt with the Klenow enzyme) from the pUC119 polylinker was ligated into it. The *Hin*dIII site from the blunted polylinker was regenerated and was positioned adjacent to the bacterial origin of replication. The resulting plasmid was cut at the unique *Sse*8387I site and an *Sse*8387I–*Pvu*II–*Sse*8387I oligonucleotide (GGCAGCTGCCTGCA) was ligated in. The remaining unique *Bsp*HI site was cut, blunted with Klenow enzyme, and the *Asc*I oligonucleotide (GAAGGCGCGCCTTC) was ligated into it, eliminating the *Bsp*HI site. This plasmid was called pWee. In order to create pWlacZ, an

expression cassette that was flanked by AAV-2 ITRs, and that consisted of the CMV IE promoter, the hGH first intron, the adhlacZ gene (Clontech, Palo Alto, CA, USA), and the SV40 early polyadenylation site, was installed in the unique *Pvu*II site of pWee such that the CMV promoter was proximal to the bacterial amp gene of pWee. The construction of this expression cassette has been previously described.[5] pW4389lacZ was created by linkering a DNA fragment that encodes the entire AAV-2 genome excluding the ITR sequences (AAV-2 base pairs 146–4534) with *Asc*I linkers (GAAGGCGCGCCTTC) and ligating the linkered *rep*/*cap* fragment into the unique *Asc*I site of pWlacZ. The AAV-2 gene encoding segments were oriented such that the *rep* gene is proximal to the bacterial origin of replication.

AAV vector production

293 Cells (5×10^6 cells plated on 10 cm dishes) were transfected with pW4389lacZ in the presence or absence of various combinations of the adenoviral helper plasmids listed above. The transfections were done by the calcium phosphate method of Wigler[24] using a total of 30 μg of DNA for a period of 6 h. For each transfection, 10 μg of pW4389lacZ was used in combination with 10 μg of each of the adenoviral helper plasmids. If the total amount of required plasmid DNA amounted to less than 30 μg, pBluescript II s/k+ was added to bring the total amount of transfected DNA to 30 μg. After the transfection period, the media were changed, adenovirus-2 (MOI = 10) was added if infection was called for, and the cultures were incubated at 37°C for 18 h. At this time, the serum containing medium was replaced with serum-free medium and the cultures were incubated for another 54 h. The cells were then collected, the media removed by centrifugation (1000 *g* for 10 min), and a lysate was produced using three freeze–thaw cycles (alternating between dry ice-ethanol and 37°C baths). The lysates were made free of debris by centrifugation (10 000 *g* for 10 min). All adenoviral helper transfections were done in triplicate. The transfection efficiency was approximately 70%. This transfection efficiency is consistently obtained irrespective of cell passage or flask size provided that the cells are at least three passages from frozen storage and they are never allowed to reach confluency.

AAV *lac*Z vector production was assessed by titration of the freeze–thaw extracts or purified vector preparations using 293 cells in the presence of an adenovirus-2 superinfection (MOI = 50). The transduced cultures were incubated for 24 h at 37°C before fixation and X-gal staining. The stained cells were counted under light microscopy.

In the case of purified AAV vector preparations, vector titer was also determined by quantitative dot blot hybridization of DNase-treated stocks. In the subsequent text and figures, the AAV vector particle number refers to the number of AAV vector genomes in the sample as determined by the quantitative dot blot assay.

The AAV hEpo vector preparations were prepared as previously described[5] using adenovirus or pVAE2AE4-5, as described above.

AAV vector purification

AAV vector producing 293 cells were dislodged from the culture dishes by gentle pipetting and collected by centrifugation at 1000 *g* for 5 min. The cells were resuspended in 0.1 m Tris-HCl, 0.15 m NaCl pH 8.0 (1×10^7 cells/ml), subjected to three cycles of freeze–thaw lysis, and the tissue debris was removed by centrifugation at 10 000 *g* for 15 min. The supernatant was subjected to a second 15 min centrifugation at 10 000 *g* to remove any remaining turbidity. The cleared supernatant was made 25 mm in $CaCl_2$, and left to precipitate for 1 h at 0°C. The resulting precipitate was removed by centrifugation (10 000 *g*, 15 min) and discarded. The supernatant was then made 0.62 m in NaCl and 8% in PEG(8000), mixed, and left to precipitate for 3 h at 0°C. The AAV vector containing precipitate was collected by centrifugation (3000 *g*, 30 min), and resuspended in a small volume of 50 mm NaHEPES, 0.15 m NaCl, 25 mm EDTA pH 8.0. After removal of the insoluble material by centrifugation (10 000 *g*, 15 min, 4°C), solid CsCl was added to produce a density of 1.4 g/ml, and the sample was centrifuged at 114 000 *g* for 24 h using a SW40 Ti rotor (30 000 r.p.m.) (Beckman, Palo Alto, CA, USA). The gradient was fractionated and the fractions were assayed for AAV vector content by DNA dot blot assay. The pooled AAV vector containing fractions were made 10% in PEG(8000) and the precipitated AAV vector was collected by centrifugation (10 000 *g*, 15 min, 4°C). The purified vector was resuspended in 50 mm HEPES, 150 mm NaCl pH 7.4 and stored at –80°C. Preparations made with adenovirus were incubated at 56°C for 1 h to inactivate any residual adenovirus. This treatment generally results in a 50% decrease in functional titer or *lacZ* transducing units.

Western blot analysis

Protein samples were resolved on 8% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, and probed using an anti-AAV-2 capsid monoclonal antibody (Progen, Belmont, MA, USA) or polyclonal rabbit sera raised against adenovirus-2. Immunoreactive protein bands were identified using the ECL detection system (Amersham, Arlington Heights, IL, USA).

Transduction of mouse muscle with an AAV hEpo vector

Six- to eight-week-old female Balb/c mice (Simonsen Laboratories, Gilroy, CA, USA) were each injected with a total of 3×10^{10} particles of AAV human Epo or AAV *lacZ*. The vector was diluted to a volume of 200 μl with buffered saline and was distributed by i.m. injection equally to six injection sites, three sites in each hind limb. Hematocrits and serum levels of human Epo were assayed bi-weekly. Human Epo was measured using an ELISA specific for human Epo (R & D Systems, Minneapolis, MN, USA).

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