

ARTICLE

Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells

Rajendra Kumar-Singh and Jeffrey S. Chamberlain*

Department of Human Genetics, University of Michigan Medical School, 1150 West Medical Center Drive, Med Sci II, Rm 3726, Ann Arbor, MI 48109-0618, USA

Received February 23, 1996; Revised and Accepted April 4, 1996

Adenovirus-mediated gene transfer to muscle is a promising technology for gene therapy of Duchenne muscular dystrophy (DMD). However, currently available recombinant adenovirus vectors have several limitations, including a limited cloning capacity of ~8.5 kb, and the induction of a host immune response that leads to transient gene expression of 3–4 weeks in immunocompetent animals. Gene therapy for DMD could benefit from the development of adenoviral vectors with an increased cloning capacity to accommodate a full-length (~14 kb) dystrophin cDNA. This increased capacity should also accommodate gene regulatory elements to achieve expression of transduced genes in a tissue-specific manner. Additional vector modifications that eliminate adenoviral genes, expression of which is associated with development of a host immune response, might greatly increase long-term expression of virally delivered genes *in vivo*. We have constructed encapsidated adenovirus minichromosomes theoretically capable of delivering up to 35 kb of non-viral exogenous DNA. These minichromosomes are derived from bacterial plasmids containing two fused inverted adenovirus origins of replication embedded in a circular genome, the adenovirus packaging signals, a β -galactosidase reporter gene and a full-length dystrophin cDNA regulated by a muscle-specific enhancer/promoter. The encapsidated minichromosomes are propagated *in vitro* by *trans*-complementation with a replication-defective (E1+E3 deleted) helper virus. We show that the minichromosomes can be propagated to high titer ($>10^8$ /ml) and purified on CsCl gradients due to their buoyancy difference relative to helper virus. These vectors are able to transduce myogenic cell cultures and express dystrophin in myotubes. These results suggest that encapsidated adenovirus minichromosomes may be useful for gene transfer to muscle and other tissues.

INTRODUCTION

Duchenne and Becker muscular dystrophy (DMD/ BMD) are X-linked recessive disorders characterized by progressive muscle weakness, cardiomyopathy and early death. Prevalence estimates for DMD are of the order of 1 in 3500 newborn males with one-third of these cases being sporadic. Patients typically begin using a wheelchair by the age of 8–11 years due to progressive degeneration of limb and axial musculature and usually die in their early twenties as a result of respiratory and/or cardiac failure (1).

DMD and the milder version of the disease, BMD, are due to mutations in the gene encoding dystrophin, a 427 kDa sarcolemma-associated protein that is also expressed as multiple isoforms in non-muscle tissues (2). The *mdx* mouse, an animal model for DMD, has a point mutation in exon 23 of dystrophin eliminating the 427 kDa muscle and brain isoforms (3,4). We have previously shown that expression of full-length dystrophin

in transgenic *mdx* mice eliminates dystrophic symptoms without toxicity, suggesting that gene therapy may be an effective treatment for this debilitating disorder (5). We and others have also demonstrated that truncated dystrophin minigenes can almost completely prevent the appearance of dystrophic symptoms in *mdx* mice; however, this incomplete correction may not be optimal for clinical trials (6,7).

Methods to deliver dystrophin in an efficient manner to muscle tissues are currently being investigated in many laboratories. Human adenovirus (Ad) has many favorable characteristics that make it a promising vector for gene delivery to muscle. These characteristics include a high tropism for muscle tissue, no proven integration into the host genome and convenient mass production yielding high titer stocks (8,9). However, current generation Ad-based gene therapy vectors are of limited use due to their relatively small cloning capacity (~8.5 kb) and their induction of an inflammatory response that leads to transient gene expression

*To whom correspondence should be addressed

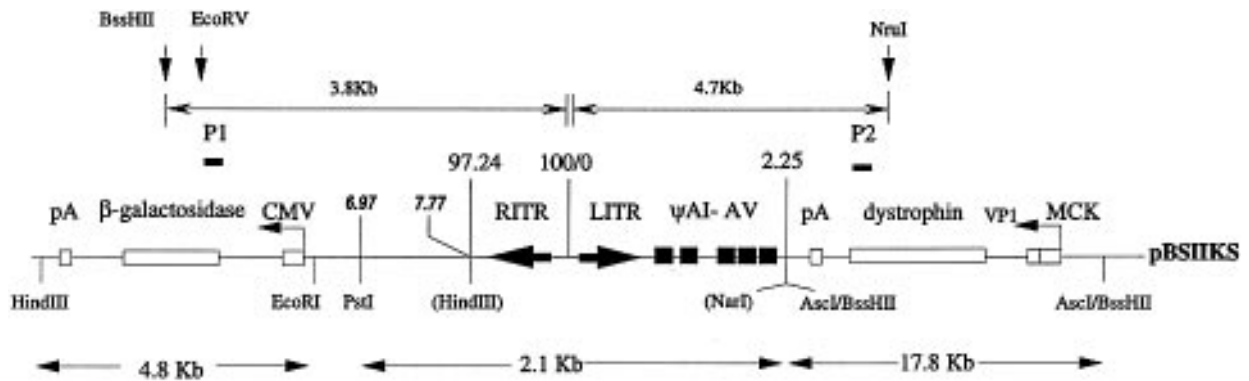


Figure 1. Structure of pAd5 β dys (27.8 kb). The two inverted adenovirus origins of replication are represented by a left and right inverted terminal repeat (LITR/RITR). Replication from these ITRs generates a linear genome whose termini correspond to the 0 and 100 map unit (mu) locations. Orientation of the origin with respect to wild-type adenovirus serotype 5 sequences in mu is indicated above the figure (1 mu = 360 bp). Encapsidation of the mature linear genome is enabled by five (AI–AV) packaging signals (Ψ). The *E. coli* β -galactosidase and *Mus musculus* dystrophin cDNAs are regulated by cytomegalovirus (CMV) and muscle creatine kinase (MCK) enhancer/promoter elements, respectively. Both expression cassettes contain the SV40 polyadenylation (pA) signal. Since the E1A enhancer/promoter overlaps with the packaging signals, pAd5 β dys was engineered such that RNA polymerase transcribing from the E1A enhancer/promoter will encounter the SV40 late polyadenylation signal. Pertinent restriction sites used in constructing pAd5 β dys are indicated below the figure. An adenovirus fragment corresponding to mu 6.97–7.77 was introduced into pAd5 β dys during the cloning procedure (see Materials and Methods). P1 and P2 represent the location of probes used for Southern blot analysis. Restriction sites destroyed during the cloning of the Ad5 origin of replication and packaging signal are indicated in parentheses.

in vivo (10–12). This small cloning capacity restricts the inclusion of gene regulatory elements or large gene cassettes such as dystrophin. Although current versions of Ad vectors have greatly diminished replication capacities *in vivo* due to deletions of the viral genes *E1a* and *E1b*, low level expression of the remaining viral genes is believed to alert the host's immune system, triggering a cytotoxic T-cell-mediated response that attacks Ad-infected cells (13,14). Initial steps to address these problems have demonstrated that temperature-sensitive viruses partially defective in E2A (DNA binding protein) significantly prolong transgene expression by limiting viral replication and gene expression (14,15).

In this report we describe the construction of an encapsidated adenovirus minichromosome (EAM) consisting of an infectious encapsidated linear genome containing Ad origins of replication, packaging signal elements, a β -galactosidase reporter gene cassette and a full-length (14 kb) dystrophin cDNA regulated by a muscle-specific enhancer/promoter. EAMs are generated by co-transfecting 293 cells with supercoiled plasmid DNA (pAd5 β dys) containing an embedded inverted origin of replication (and the remaining above elements) together with linear DNA from E1-deleted virions expressing human placental alkaline phosphatase (hpAP). All proteins necessary for the generation of EAMs are provided *in trans* from the hpAP virions and the two can be separated from each other on equilibrium CsCl gradients. Such EAMs may prove useful for gene transfer to a variety of cell types both *in vitro* and *in vivo*.

RESULTS

Generation and propagation of encapsidated adenovirus minichromosomes

To establish a vector system capable of delivering full-length dystrophin cDNA clones, we combined the minimal region of Ad5 needed for replication and packaging with a conventional plasmid carrying both dystrophin and a β -galactosidase reporter gene. The resultant vector, pAd5 β dys (Fig. 1) contains 2.1 kb of

adenovirus DNA, together with a 14 kb murine dystrophin cDNA under the control of the mouse muscle creatine kinase enhancer/promoter, as well as a β -galactosidase gene regulated by the human cytomegalovirus (CMV) enhancer/promoter (see Materials and Methods). We reasoned that such a plasmid should be packageable into an encapsidated minichromosome when grown in parallel with an E1-deleted virus due to the inclusion of both inverted terminal repeats (ITRs) and the major Ad packaging signals in the plasmid. The ITRs and packaging signals were derived from pFG140, a plasmid that generates E1-defective Ad particles upon transfection of human 293 cells (16). hpAP is an E1-deleted Ad5 containing the human placental alkaline phosphatase gene (17). This virus was chosen to provide the helper functions so that we would be able to monitor the titer of the helper virus throughout serial passages by quantitative alkaline phosphatase assays. Co-transfection of 293 cells with supercoiled pAd5 β dys and linear hpAP DNA produced Ad5 β dys EAMs (the encapsidated version of pAd5 β dys) ~6–12 days post-transfection, as evidenced by the appearance of a cytopathic effect (CPE). Initially, the amount of Ad5 β dys EAMs produced was significantly lower than that of hpAP virions, so the viral suspension was used to re-infect fresh cultures, from which virus was isolated and used for serial infection of additional cultures (Fig. 2). The rate of increase in titer of Ad5 β dys EAMs between transfection and serial passage 6 is ~100 times greater than that for hpAP virions. This result indicates that Ad5 β dys has a replication advantage over the helper virus, probably due to the shorter genome length (a difference of ~8 kb) and hence an increased rate of packaging.

Interestingly, after serial passage 6, there was a rapid decrease in the total titer of hpAP virions, whereas the titer of Ad5 β dys EAMs continued to rise. We hypothesize at least two possible mechanisms for this observation. Firstly, a build up of defective hpAP virions due to infections at high multiplicities may slowly out-compete their full-length counterparts, a phenomenon that has been observed previously upon serial propagation of adenovirus (18–20). Secondly, the emergence of

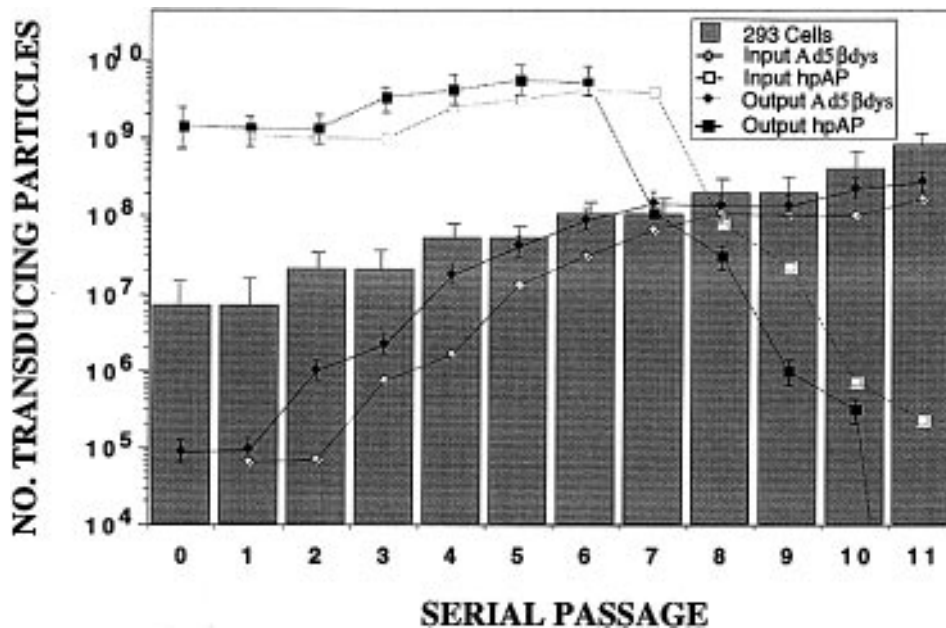


Figure 2. Amplification of Ad5βdys EAMs. The total number of transducing adenovirus particles produced (output) per serial passage on 293 cells, total input virus of either the helper (hpAP) or Ad5βdys, and the total number of cells used in each infection is presented. The total number of input/output transducing particles were determined by infection of 293 cells plated in 6-well microtiter plates. At 24 h post-infection the cells were assayed for alkaline phosphatase or β-galactosidase activity to determine the number of cells transduced with either Ad5βdys or hpAP. The number of transducing particles was estimated by extrapolation of the mean calculated from 31 randomly chosen 2.5 mm² sectors of a 961 mm² plate. The intra-sector differences in total output of each type of virus are presented as the standard deviations, σ , in this figure. For each serial passage, 75% of the total output virus from the previous passage was used for infection.

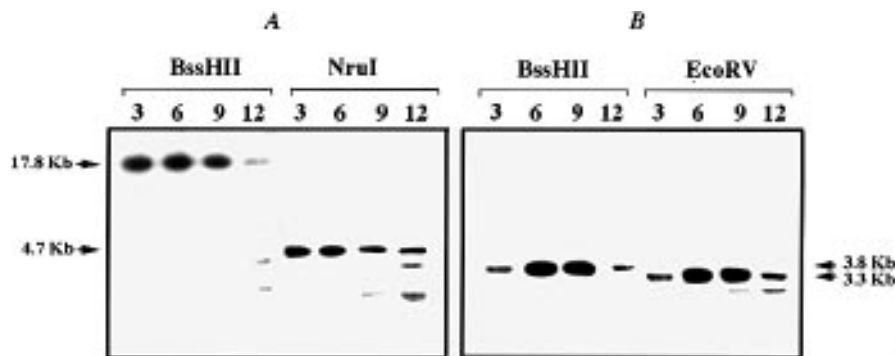


Figure 3. Southern blot analysis of viral DNA from lysates 3, 6, 9 and 12, digested with the restriction enzymes *BssHII*, *NruI* and *EcoRV*, indicating the presence of a full-length dystrophin cDNA in all lysates. Fragments from the C-terminus of *Mus musculus* dystrophin cDNA (A) or the N-terminus of *E. coli* β-galactosidase (B) were labeled with [³²P]dCTP and used as probes (42). The position of these probes and the predicted fragments for each digest is indicated in Figure 1. Note that one end of each fragment (except the 17.8 kb *BssHII* dystrophin fragment) detected is derived from the end of the linearized Ad5βdys genome (see Fig. 1). Low levels of shorter products, presumably derived from defective virions, become detectable only at high serial passage number.

replication-competent virions due to recombination events between E1 sequences in cellular DNA and the hpAP genome could lead to a build up of virus particles defective in expressing alkaline phosphatase (21). Southern analysis of DNA prepared from serial lysates 3, 6, 9 and 12 indicated that full-length dystrophin sequences were present in each of these lysates (Fig. 3A). In addition, the correct size restriction fragments were detected using both dystrophin and β-galactosidase probes against lysate DNA digested with several enzymes (Fig. 3). However, at the later passages (9 and 12) there appeared to be an emergence of truncated Ad5βdys sequences, suggesting that deletions and/or rearrangements may be occurring at later

passages. Hence, we chose to perform most of our experiments with Ad5βdys EAMs derived from the earlier passages. We have also examined the possibility of the emergence of replication-competent (E1-containing) viruses by infection of HeLa cells by purified and crude serial lysates, none of which produced any detectable CPE (data not shown).

Purification of encapsidated adenovirus minichromosomes on CsCl gradients

We have examined the possibility of separating Ad5βdys EAMs from hpAP virions based on their buoyancy difference (due

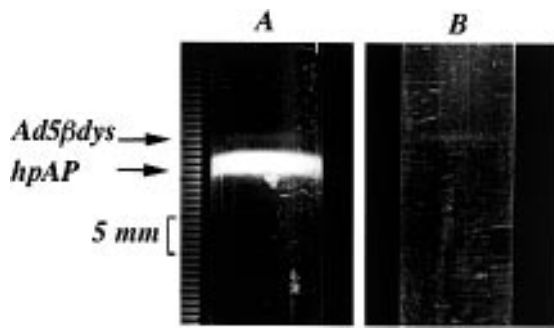


Figure 4. Physical separation of Ad5 β dys from hpAP virions at the third (A) and final (B) stages of CsCl purification (initial $\rho = 1.334$) in a SW41 tube (see Materials and Methods). Aliquots of Ad5 β dys EAMs from the final stage were drawn through the top of the centrifugation tube and assayed for β -galactosidase and alkaline phosphatase expression. Results of this assay are presented in Figure 5.

presumably to their different genome lengths) on CsCl gradients. Repeated fractionation of the viral lysate (see Materials and Methods) allows small differences in buoyancy to be resolved. Results of the physical separation between Ad5 β dys EAMs and hpAP virions are shown in Figure 4. Purification of the band fractions containing predominantly Ad5 β dys EAMs from 20% of the lysate from passage 6 and analyses of contamination by hpAP virions in the various fractions by infection of 293 cells is shown in Figure 5. The maximum ratio of transducing Ad5 β dys EAMs to hpAP virions reproducibly achieved in this study was 24.8—a contamination with helper virus corresponding to ~4% of the final viral isolate.

Dystrophin and β -galactosidase expression by encapsidated adenovirus minichromosomes in muscle cells

To determine if the Ad5 β dys EAMs were able to express β -galactosidase and dystrophin in muscle cells, we infected mouse *mdx* myogenic cultures with CsCl-purified EAMs. Western blot analysis of EAM-infected *mdx* myoblasts and myotubes (3 days post-fusion) indicated that EAMs were able to infect both of these cell types (Fig. 6). Expression of β -galactosidase was detected in both the infected *mdx* myoblasts and myotubes, indicating that the CMV promoter was active at both these early stages of differentiation in muscle cells. However, only infected *mdx* myotubes produced protein detected by dys-2, an antibody recognizing the 17 C-terminal amino acids of dystrophin (Fig. 6). No dystrophin expression was detected in infected myoblasts by western analysis, indicating that the muscle creatine kinase promoter functions minimally, if at all, within the Ad5 β dys EAM prior to terminal differentiation of these cells. Dystrophin expression in *mdx* cells infected with EAMs was confirmed by immunofluorescence studies using N-terminal dystrophin antibodies. In agreement with the western analysis, dystrophin expression from the muscle creatine kinase (MCK) promoter was detected only in differentiated *mdx* myotubes infected by Ad5 β dys EAMs (Fig. 7).

DISCUSSION

Several genetic complementation strategies have been proposed as potential therapeutic treatments for DMD, including the transplantation of donor myoblasts (22), delivery of recombinant dystrophin expression cassettes by intramuscular injection of naked DNA (23) or using retroviral (24) and adenoviral vectors

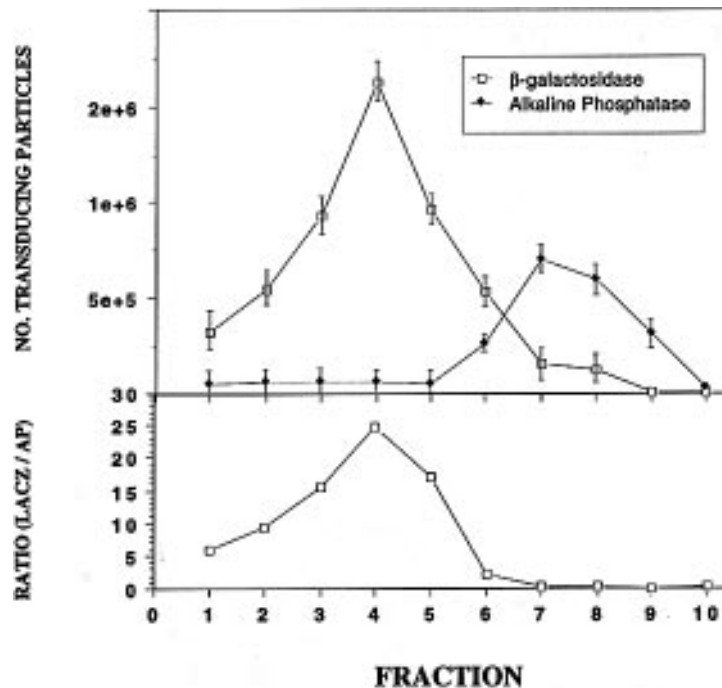


Figure 5. Contamination of Ad5 β dys EAMs by hpAP virions obtained from passage 6. Following four cycles of CsCl purification, aliquots were removed from the top of the centrifugation tube (see Materials and Methods) and used for infection of 293 cells, which were fixed 24 h later and assayed for β -galactosidase or alkaline phosphatase activity. The number of transducing particles are an underestimation of the actual totals as, in some cases, a positive cell may have been infected by more than one transducing particle. The ratio of the two types of virions—Ad5 β dys EAMs (LacZ) or hpAP (AP)—in each fraction is indicated in the lower graph.

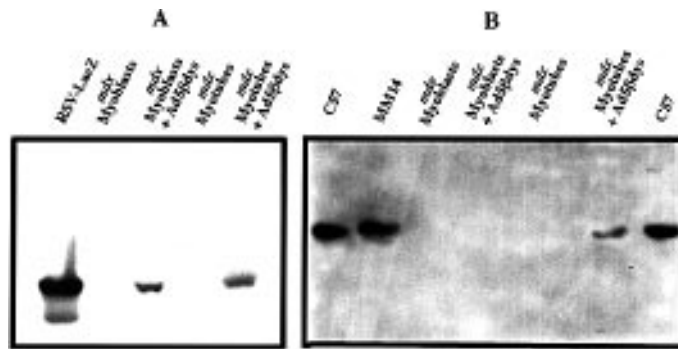


Figure 6. Immunoblots of protein extracts from *mdx* myoblasts and myotubes demonstrating the expression of β -galactosidase (A) and dystrophin (B) in cells infected with Ad5 β dys EAMs. Total protein was extracted 3 days post-infection in all cases. Myotubes were infected at 3 days following a switch to differentiation media (47). (A) Lane 1 contains total protein extract from 293 cells infected with a virus expressing β -galactosidase as a control. (B) Lanes 1 and 7 contain total protein from mouse muscle (C57) while lane 2 contains protein from wild-type MM14 myotubes, as controls.

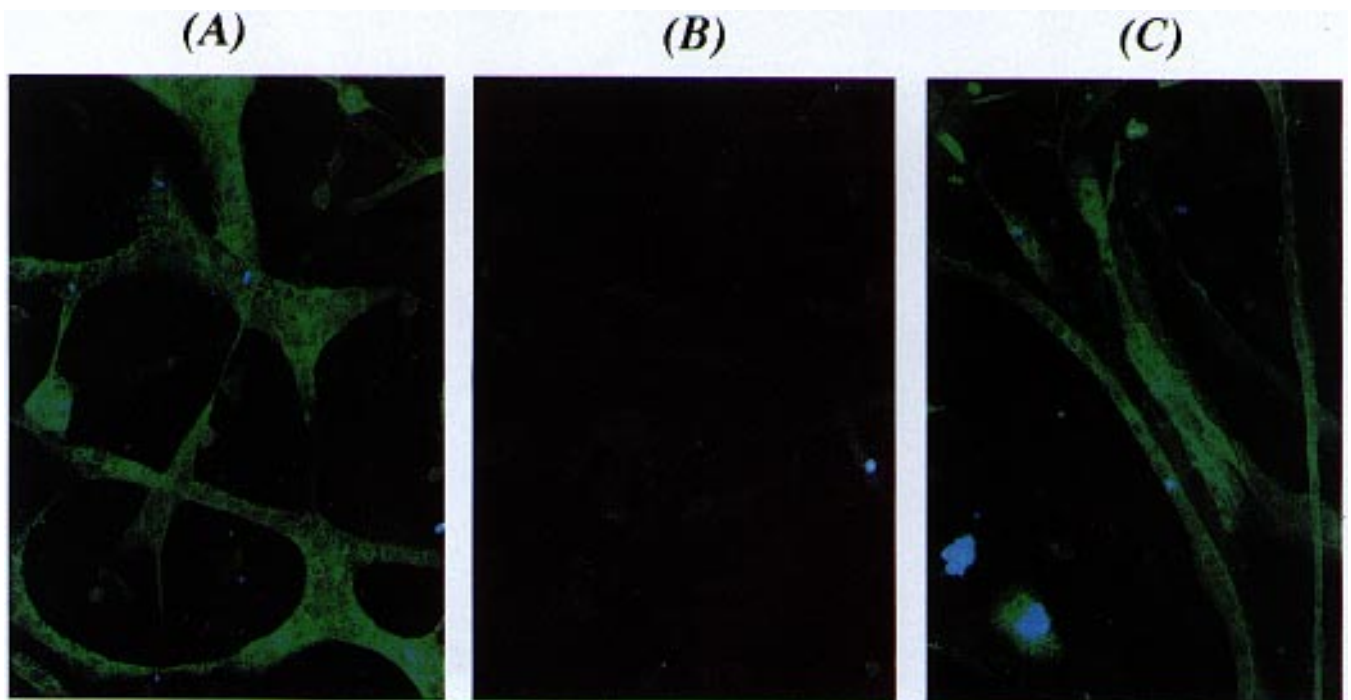


Figure 7. Immunofluorescence of dystrophin in wild-type MM14 myotubes (A), uninfected *mdx* (B) and infected *mdx* myotubes (C) indicating transfer and expression of recombinant dystrophin to differentiated *mdx* cells by Ad5 β dys EAMs.

(25). Each strategy has met with severe limitations. Although 30–80% of muscle fibers of nude/*mdx* mice are dystrophin-positive following myoblast transplantation, few dystrophin-positive fibers have been obtained using these methods in human trials (22,26–28). Intramuscular injection of plasmid DNA transduces only 1–3% of myofibers in the quadriceps of *mdx* mice, a scenario unlikely to be effective in clinical trials (23). Retroviral vectors only infect dividing cells, limiting their application to post-mitotic muscle fibers and neurons, the target tissues for DMD (29). Adenovirus-mediated transfer of a minidystrophin gene to newborn *mdx* quadriceps has produced a reduction of dystrophic degeneration (25). However, at high doses of virus administration to immunocompetent animals, an inflammatory response results from the low-level

expression of viral proteins (14,15). Hence, considerable improvement in vector technology will be needed to overcome the limitations of cloning capacity and viral gene expression before clinical trials of gene therapy for DMD could be considered.

The approach described in this study makes use of the positive characteristics of adenovirus, i.e. a high infectivity rate and hence efficient transduction of target cells while eliminating the viral genome, expression of which contributes to the development of a strong host immune response. Our strategy depends on the observation that Ad DNA is packaged into virions in a polar, left-to-right fashion and is dependent on *cis*-acting packaging elements. The Ad5 packaging domain extends from nucleotide 194 to 358 and is composed of five distinct elements that are

functionally redundant (30,31). Theoretically, any molecule containing the Ad5 origin of replication and packaging elements should replicate and be packaged into mature virions in the presence of non-defective helper virus. It has already been shown that Ad origins of replication are functional on autonomously replicating minichromosomes (32). Minichromosomes containing two cloned inverted Ad termini require non-defective Ad as a helper. DNA synthesis is initiated at the correct nucleotide even when the origins are not located at molecular ends. The activity of embedded origins leads to the generation of linear minichromosomes from circular molecules (16). Furthermore, it has been shown previously that viruses rendered defective by replacement of a small part of the genome with a reporter gene cassette can be rescued in a helper-dependent manner (33).

These data suggest that an artificial, infectious virus could be constructed containing no viral genes yet maintaining a high infection efficiency due to the viral capsid. To test this hypothesis, we have constructed encapsidated adenovirus minichromosomes comprised of a full-length dystrophin cDNA under the control of a 3.3 kb MCK enhancer/promoter, a bacterial β -galactosidase (β -gal) gene under control of a CMV enhancer/promoter and the Ad origin of replication together with *cis*-acting viral packaging signals. EAMs were propagated and amplified by serial passage in 293 cells with the use of a *trans*-acting E1-deleted helper virus. The obvious advantage of these vectors is that they should theoretically have a cloning capacity up to 35 kb and yet be unable to express any virally encoded genes.

The choice of hpAP as a helper virus has allowed monitoring of the relative populations of helper to EAMs at the various stages of serial propagation. We have shown that the smaller genome of the EAM (27.8 kb) has a packaging advantage over that of the helper virus (~36 kb) as evidenced by the net accumulation of EAMs at a rate higher than that of the helper virus after several rounds of replication. Separation of EAMs from helper virus was achieved by repeated centrifugation of viral lysates on a continuous CsCl gradient. Similar conditions have been used to achieve partial separation of viruses differing in size by only 200 bp (33). Our results indicate that EAMs can be propagated efficiently to high titers in the presence of hpAP as a helper and that EAMs can be separated from helper virus on CsCl gradients. We have shown that the lysate can be fractionated to yield an enriched fraction for EAMs, which before CsCl purification represented a minor amount of the total viral preparation.

Temporal specific expression of the recombinant genes has been examined by comparing expression of β -galactosidase and dystrophin in *mdx* myogenic cultures at various stages of differentiation following infection by Ad5 β dys EAMs. The endogenous MCK promoter has been shown previously to be inactive in myoblasts, and is transcriptionally activated only upon terminal differentiation of myoblasts into myocytes and myotubes (34,35). Since the Ad5 β dys EAMs contain the E1 enhancer, it was of interest to determine whether the MCK promoter/enhancer would be active in myoblasts. However, we did not detect dystrophin in myoblast cultures following infection with Ad5 β dys. In contrast, dystrophin synthesis was readily observed following infection of differentiated *mdx* myofibers, using both indirect immunofluorescence and western analysis with dystrophin antibodies.

A similar strategy to deliver a full-length dystrophin cDNA has been reported recently by Kochanek and colleagues (36). However, in that study, the ITRs were ligated onto a linear

plasmid immediately prior to co-transfection of 293 cells with helper viral DNA. Our approach incorporates the ITRs directly into the plasmid vector, simplifying preparation of EAMs by direct plasmid propagation.

Several key issues need to be addressed prior to the introduction of EAMs into *mdx* mice or other animal models of human disease. Firstly, contamination of EAMs with helper virions will be deleterious by contributing towards adenoviral gene expression. Secondly, the emergence of defective virions other than EAMs at later passages allows for the possibility of co-migration of EAMs and defectives in the CsCl gradient. Such defectives may contain parts of the Ad genome capable of expressing some or all of the adenoviral genes by *trans* complementation. During the course of this study, we have been able to obtain fractions with a maximum contamination by helper of 4%. Reduction in the size of pAd5 β dys by removal of the β -galactosidase gene cassette should further increase the physical separation between the two types of virions and hence enhance the purity of Ad5 β dys EAMs. The vector prepared by Kochanek and colleagues incorporated a slightly larger amount of DNA than our vector, yet those authors were able to isolate viral fractions with reduced amounts of helper (36). The difference in helper contamination obtained in the two studies probably reflects differences in CsCl purification protocols, although it will be important to determine the effect of genome size on both vector stability and separation from helper virus. A systematic test of materials with potentially better resolving powers than CsCl at $\rho = 1.3$, such as rubidium chloride and potassium bromide (37), may result in further enhancement of the purity of Ad5 β dys EAMs. Furthermore, although Ad5 β dys EAMs seem to have a packaging advantage over hpAP, titers of the Ad5 β dys EAM might be increased further by using several strategies including the use of helper viruses that approach the packaging limits of Ad5 (10), the use of viruses with mutations in E4 or E2 genes (38–40), inclusion of non-lethal mutations in the packaging signals of hpAP virions (41) or a combination of these strategies. Strategies may also be developed where fractions enriched for Ad5 β dys EAMs are used for co-propagation of the two viruses, reducing the total multiplicity of infection and hence the probability of recombination events.

Purification of high titer EAM stocks will enable *in vivo* experiments to test the potential immunogenicity of these vectors. Previous studies documenting transient gene expression from adenoviral vectors suggest that a major limitation of these delivery systems originates from low-level viral gene expression resulting in an immune response against infected cells (10–12). The deletion of almost the entire Ad genome from these viruses removes the possibility of Ad gene expression *in vivo* and could result in a greatly reduced or absent host immune response as compared with current generation Ad vectors. However, detailed experiments in animals will be needed to characterize fully the potential immunogenicity of these vectors.

In summary, we have shown that embedded inverted Ad origins of replication coupled to an encapsidation signal can convert circular DNA molecules to linear forms in the presence of helper virus and that these genomes can be encapsidated efficiently and propagated to high titers. Such viruses can be purified on a CsCl gradient and maintain their ability to transduce cells *in vitro*, and their increased cloning capacity allows the inclusion of large genes and tissue-specific gene regulatory elements. We have also shown that the dystrophin gene is expressed in cells transduced by such viruses and that the protein product is correctly localized

to the cell membrane. Our method for preparing EAMs theoretically enables virtually any gene of interest to be inserted into an infectious minichromosome by conventional cloning in plasmid vectors, followed by co-transfection with helper viral DNA in 293 cells. This approach should prove useful for a variety of gene transfer studies *in vitro*. While considerable work remains to optimize the propagation and purification of Ad minichromosome vectors, the observation that vectors completely lacking viral genes can be used to transfer a full-length dystrophin cDNA into myogenic cells indicates the potential for this method to contribute to the development of effective gene therapy for DMD.

MATERIALS AND METHODS

Construction of pAd5 β dys

Cloning and *in vitro* mutagenesis were carried out according to standard techniques (42). A modified version of BluescriptII KS+ (Stratagene) was used as the backbone for construction of pAd5 β dys. The inverted fused Ad5 origin of replication and five encapsidation signals were excised as a *Pst*I–*Xba*I fragment from pAd5ori, a plasmid containing the 6 kb *Hind*III fragment from pFG140 (16). This strategy also introduces a 290 bp fragment from Ad5 corresponding to map units 6.97–7.77 adjacent to the right inverted repeat (see Fig. 1). The *Escherichia coli* β -galactosidase gene regulated by the human CMV immediate early promoter/enhancer expression cassette was derived as an *Eco*RI–*Hind*III fragment from pCMV β (43). The murine dystrophin expression cassette was derived as a *Bss*HIII fragment from pCVAA, and contains a 3.3 kb MCK promoter/enhancer element (6).

Transfection of 293 cells

Low passage 293 cells (Microbix Biosystems) were grown and passaged as suggested by the supplier. pAd5 β dys and hpAP DNA (5 and 0.5 μ g, respectively) were dissolved in 70 μ l of 20 mM HEPES buffer (pH 7.4) and incubated with 30 μ l of DOTAP (BMB) for 15 min at room temperature. This mixture was resuspended in 2 ml of DMEM supplemented with 2% fetal calf serum (FCS) and added dropwise to a 60 mm plate of 293 cells at 80% confluency. Four hours post-transfection the media was replaced by DMEM with 10% FCS. CPE was observed 6–12 days post-transfection.

Infection and serial passaging of 293 cells

Lysate from one 60 mm plate of transfected 293 cells was prepared by vigorously washing the cells from the plate and centrifuging at 1000 r.p.m. in a clinical centrifuge. Cells were resuspended in DMEM and 2% FCS, freeze-thawed in a dry ice–ethanol bath, cell debris removed by centrifugation, and ~75% of the crude lysate was used to infect 293 cells in DMEM supplemented with 2% FCS for 1 h and then supplemented with 10% FCS thereafter. Infection was allowed to proceed for 18–20 h before harvesting the virus. The total number of cells infected in each serial passage is indicated in Figure 2.

CsCl purification of encapsidated adenovirus minichromosomes

Approximately 25% of the lysate prepared from various passages during serial infections was used to purify virions. Freeze-thawed lysate was centrifuged to remove the cell debris. The cleared lysate was extracted twice with 1,1,2 trichlorotrifluoroethane (Sigma) and applied to CsCl step and self-forming gradients. Purification of virus was initially achieved by passing it twice through CsCl step gradients with densities of $\rho = 1.45$ and $\rho = 1.20$ in a SW28 rotor. After isolation of the major band in the lower gradient, the virus was passed through a self-forming gradient (initial $\rho = 1.334$) at 37 000 r.p.m. for 24 h followed by a relaxation of the gradient by reducing the speed to 10 000 r.p.m. for 10 h in a SW41 rotor at 12°C (44). The upper band from the gradient (composed mainly of Ad5 β dys virions) was isolated using an 18 gauge needle, reloaded on a fourth CsCl gradient ($\rho = 1.334$) and purified at 37 000 r.p.m. for 24 h followed by 10 000 r.p.m. for 10 h at 12°C. The Ad5 β dys-containing CsCl band was removed in 100 μ l fractions from the top of the centrifugation tube and CsCl was removed by chromatography on Sephadex G-50. Aliquots from each fraction were used to infect 293 cells followed by β -galactosidase and alkaline phosphatase assays to quantitate the level of contamination by hpAP virions in the final viral isolate.

Alkaline phosphatase and β -galactosidase assays

For detection of alkaline phosphatase, infected 293 cells on Petri dishes were rinsed twice with phosphate-buffered saline (PBS) and fixed for 10 min in 0.5% glutaraldehyde in PBS. Cells were again rinsed twice with PBS for 10 min followed by inactivation of endogenous alkaline phosphatase activity at 65°C for 1 h in PBS prior to the addition of the chromogenic substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate) at 0.15 mg/ml and nitro blue tetrazolium at 0.3 mg/ml. Cells were incubated at 37°C in darkness for 3–24 h. For β -galactosidase assays, the cells were fixed and washed as above, then assayed as described previously (45).

Propagation and infection of muscle cells

MM14 and *mdx* myogenic cell lines were kindly provided by S. Hauschka (University of Washington) and were cultured as previously described (46). Myoblasts or differentiated myotubes (3 days post-switching) were infected at a multiplicity of infection of 2.2 Ad5 β dys EAMs per cell. Fractions containing minimal contamination with hpAP virions (3, 4 and 5 of passage 6) were used for western and immunofluorescence analysis. Infection was allowed to proceed for 3 days for both the myoblasts and myotubes before harvesting cells.

Total protein extraction and immunoblot analysis

For protein extraction, muscle cells were briefly trypsinized, transferred to a microcentrifuge tube, centrifuged at 14 000 r.p.m. for 3 min at room temp and resuspended twice in PBS. After an additional centrifugation, the cell pellet was resuspended in 80 μ l of RIPA homogenate (42). The sample was sheared briefly using a 22 gauge needle to reduce viscosity, and total protein concentration assayed using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Expression of full-length dystrophin or β -galactosidase in infected *mdx* and MM14 myoblasts or myotubes was analyzed by electrophoresis of 40 μ g

of total protein extract on a 6% SDS-PAGE gel (in 25 mM Tris, 192 mM glycine, 10 mM β -mercaptoethanol, 0.1% SDS). After transferring to Gelman Biotrace NT membrane (in 25 mM Tris, 192 mM glycine, 10 mM β -mercaptoethanol, 0.05% SDS, 20% methanol), the membrane was blocked with 5% non-fat milk and 1% goat-serum in Tris-buffered saline-Tween (TBS-T) for 12 h at 4°C. Immunostaining was done according to the protocol for the ECL western blotting detection reagents (Amersham Life Sciences, Buckingham, UK). The primary antibodies used were Dys-2 (Vector laboratories) and anti- β -galactosidase (BMB, Indianapolis, IN) with a horseradish peroxidase-conjugated anti-mouse secondary antibody.

Immunofluorescence of myogenic cells

Approximately 1.5×10^6 MM14 or *mdx* myoblasts were plated on poly-L-lysine (Sigma) coated glass slides (7×3 cm) which previously had been etched with a 0.05% chromium potassium sulfate and 0.1% gelatin solution. For myotube analysis, the cultures were switched to differentiation media (47) 48 h after plating, immediately infected and then allowed to fuse for 3 days, whereas myoblasts were propagated continuously in proliferation media (47). Cells were washed three times with PBS at room temperature and fixed in 3.7% formaldehyde. For immunostaining, cells were incubated in 0.5% Triton X-100, blocked with 1% normal goat serum and incubated with an affinity-purified antibody against the N-terminus of murine dystrophin (K. Corrado and J.S.C., in preparation) for 2 h followed by extensive washing in PBS and 0.1% Tween-20 with gentle shaking. Cells were incubated with a 1:200 dilution of biotin-conjugated anti-rabbit antibody (Pierce) for 1 h and washed as above. Cells were incubated further with a 1:300 dilution of streptavidin-fluorescein isothiocyanate conjugate (Vectorlabs) for 1 h and washed as above, followed by extensive washing in PBS.

ACKNOWLEDGEMENTS

We thank M. A. Hauser and A. Amalfitano for helpful discussions, and S. D. Hauschka for providing the MM14 and *mdx* myogenic cell lines. This work was supported by grants from the Muscular Dystrophy Association (USA), Harmonize for Hope fund and the Ades family fund.

REFERENCES

- Emery, A.E.H. (1993) *Duchenne Muscular Dystrophy. Oxford Monographs on Medical Genetics No. 24*. Edn 2, Oxford Medical Publications, Oxford.
- Ahn, A.H. and Kunkel, L.M. (1993) The structural and functional diversity of dystrophin. *Nature Genet.*, **3**, 283–291.
- Bulfield, G., Siller, W.G., Wight, P.A. and Moore, K.J. (1984) X chromosome-linked muscular dystrophy (*mdx*) in the mouse. *Proc. Natl Acad. Sci. USA*, **81**, 1189–1192.
- Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G. and Barnard, P.J. (1989) The molecular basis of muscular dystrophy in the *mdx* mouse: a point mutation. *Science*, **244**, 1578–1580.
- Cox, G.A., Cole, N.M., Matsumura, K., Phelps, S.F., Hauschka, S.D., Campbell, K.P., Faulkner, J.A. and Chamberlain, J.S. (1993) Overexpression of dystrophin in transgenic *mdx* mice eliminates dystrophic symptoms without toxicity. *Nature*, **364**, 725–729.
- Phelps, S.F., Hauser, M.A., Cole, N.M., Rafael, J.A., Hinkle, R.T., Faulkner, J.A. and Chamberlain, J.S. (1995) Expression of full-length and truncated dystrophin mini-genes in transgenic *mdx* mice. *Hum. Mol. Genet.*, **4**, 1251–1258.
- Wells, D.J., Wells, K.E., Asante, E.A., Turner, G., Sunada, Y., Campbell, K.P., Walsh, F.S. and Dickson, G. (1995) Expression of human full-length and minidystrophin in transgenic *mdx* mice: implications for gene therapy of Duchenne muscular dystrophy. *Hum. Mol. Genet.*, **4**, 1245–1250.
- Quantin, B., Perricaudet, L.D., Tajbakhsh, S. and Mandel, J.L. (1992) Adenovirus as an expression vector in muscle cells *in vivo*. *Proc. Natl Acad. Sci. USA*, **89**, 2581–2584.
- Graham, F.L. and Prevec, L. (1991) Manipulation of adenovirus vectors. In Murray, E.J. (ed.), *Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols*. The Humana Press Inc., Clifton, NJ, pp. 109–128.
- Bett, A.J., Prevec, L. and Graham, F.L. (1993) Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.*, **67**, 5911–5921.
- Goldman, M.J., Litzky, L.A., Engelhardt, J.F. and Wilson, J.M. (1995) Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2a-defective recombinant adenoviruses: a preclinical toxicology study. *Hum. Gene Ther.*, **6**, 839–851.
- Kass-Eisler, A., Falck-Pedersen, E., Elfenbein, D.H., Alvira, M., Buttrick, P.M. and Leinwand, L.A. (1994) The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. *Gene Therapy*, **1**, 395–402.
- Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E. and Wilson, J.M. (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl Acad. Sci. USA*, **91**, 4407–4411.
- Engelhardt, J.F., Litzky, L. and Wilson, J.M. (1994) Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a. *Hum. Gene Ther.*, **5**, 1217–1229.
- Yang, Y., Nunes, F.A., Berencsi, K., Gönczöl, E., Engelhardt, J.F. and Wilson, J.M. (1994) Inactivation of *E2a* in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nature Genet.*, **7**, 362–369.
- Graham, F.L., Rudy, J. and Brinkley, P. (1989) Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. *EMBO J.*, **8**, 2077–2085.
- Muller, D.W., Gordon, D., San, H., Yang, Z., Pompili, V.J., Nabel, G.J. and Nabel, E.G. (1994) Catheter-mediated pulmonary vascular gene transfer and expression. *Circ. Res.*, **75**, 1039–1049.
- Daniell, E. (1976) Genome structure of incomplete particles of adenovirus. *J. Virol.*, **19**, 685–708.
- Rosenwirth, B., Tjia, S., Westphal, M. and Doerfler, W. (1974) Incomplete particles of adenovirus. II. Kinetics of formation and polypeptide composition of adenovirus type 2. *Virology*, **60**, 431–437.
- Burlingham, B.T., Brown, D.T. and Doerfler, W. (1974) Incomplete particles of adenovirus. I. Characteristics of the DNA associated with incomplete adenovirions of types 2 and 12. *Virology*, **60**, 419–430.
- Lochmüller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G. and Acsadi, G. (1994) Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants ($\Delta E1+\Delta E3$) during multiple passages in 293 cells. *Hum. Gene Ther.*, **5**, 1485–1491.
- Partridge, T.A., Morgan, J.E., Coulton, G.R., Hoffman, E.P. and Kunkel, L.M. (1989) Conversion of *mdx* myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature*, **337**, 176–179.
- Acsadi, G., Dickson, G., Love, D.R., Jani, A., Walsh, F.S., Gurusinghe, A., Wolff, J.A. and Davies, K.E. (1991) Human dystrophin expression in *mdx* mice after intramuscular injection of DNA constructs. *Nature*, **352**, 815–818.
- Dunckley, M.G., Love, D.R., Davies, K.E., Walsh, F.S., Morris, G.E. and Dickson, G. (1992) Retroviral-mediated transfer of a dystrophin minigene into *mdx* mouse myoblasts *in vitro*. *FEBS Lett.*, **296**, 128–134.
- Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilggenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J., Perricaudet, M. and Kahn, A. (1993) Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice. *Nature*, **361**, 647–650.
- Huard, J., Bouchard, J.P., Roy, R., Malouin, F., Dansereau, G., Labrecque, C., Albert, N., Richards, C.L., Lemieux, B. and Tremblay, J.P. (1992) Human myoblast transplantation: preliminary results of 4 cases. *Muscle Nerve*, **15**, 550–560.
- Gussoni, E., Pavlath, G.K., Lanctot, A.M., Sharma, K.R., Miller, R.G., Steinman, L. and Blau, H.M. (1992) Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. *Nature*, **356**, 435–438.

28. Mendell, J.R., Kissel, J.T., Amato, A.A., King, W., Signore, L., Prior, T.W., Sahenk, Z., Besson, S., McAndrew, P.E., Rice, R., Nagaraja, H., Stephens, R., Lantry, L., Morris, G.E. and Burghes, A.H.M. (1995) Myoblast transfer in the treatment of Duchenne's muscular dystrophy. *N. Engl. J. Med.*, **333**, 832–838.
29. Duncley, M.G., Wells, D.J., Walsh, F.S. and Dickson, G. (1993) Direct retroviral-mediated transfer of a dystrophin minigene into *mdx* mouse muscle *in vivo*. *Hum. Mol. Genet.*, **2**, 717–723.
30. Grable, M. and Hearing, P. (1990) Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.*, **64**, 2047–2056.
31. Grable, M. and Hearing, P. (1992) *cis* and *trans* requirements for the selective packaging of adenovirus type 5 DNA. *J. Virol.*, **66**, 723–731.
32. Hay, R.T., Stow, N.D. and McDougall, I.M. (1984) Replication of adenovirus mini-chromosomes. *J. Mol. Biol.*, **175**, 493–510.
33. Mitani, K., Graham, F.L., Caskey, C.T. and Kochanek, S. (1995) Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc. Natl Acad. Sci. USA*, **92**, 3854–3858.
34. Chamberlain, J.S., Jaynes, J.B. and Hauschka, S.D. (1985) Regulation of creatine kinase induction in differentiating mouse myoblasts. *Mol. Cell. Biol.*, **5**, 484–492.
35. Jaynes, J.B., Chamberlain, J.S., Buskin, J.N., Johnson, J.E. and Hauschka, S.D. (1986) Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. *Mol. Cell. Biol.*, **6**, 2855–2864.
36. Kochanek, S., Clemens, P.R., Mitani, K. and Caskey, C.T. (1996) A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β -galactosidase. *Proc. Natl Acad. Sci. USA*, in press.
37. Reich, M. and Zarybnicky, V. (1979) Rapid evaluation of the initial and equilibrium parameters of CsCl and RbCl in equilibrium ultracentrifugation. *Anal. Biochem.*, **94**, 193–201.
38. Yeh, P., Dedieu, J.F., Orsini, C., Vigne, E., Deneffe, P. and Perricaudet, M. (1996) Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. *J. Virol.*, **70**, 559–565.
39. Brough, D.E., Cleghon, V. and Klessig, D.F. (1992) Construction, characterization, and utilization of cell lines which inducibly express the adenovirus DNA-binding protein. *Virology*, **190**, 624–634.
40. Amalfitano, A., Begy, C. and Chamberlain, J.S. (1996) Improved adenovirus packaging cell lines to support the growth of replication defective gene delivery vectors. *Proc. Natl Acad. Sci. USA*, **93**, 3352–3356.
41. Imler, J.-L., Bout, A., Dreyer, D., Dieterlé, A., Schultz, H., Valerio, D., Mehtali, M. and Pavirani, A. (1995) *Trans*-complementation of E1-deleted adenovirus: a new vector to reduce the possibility of codissemination of wild-type and recombinant adenoviruses. *Hum. Gene Ther.*, **6**, 711–721.
42. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. MacGregor, G.M. and Caskey, C.T. (1989) Construction of plasmids that express *E. coli* β -galactosidase in mammalian cells. *Nucleic Acids Res.*, **17**, 2365.
44. Anet, R. and Strayer, D.R. (1969) Density gradient relaxation: a method for preparative buoyant density separation of DNA. *Biochem. Biophys. Res. Commun.*, **34**, 328–334.
45. MacGregor, G.M., Nolan, G.P., Fiering, S., Roederer, M. and Herzenberg, L.A. (1991) Use of *E. coli lacZ* (β -galactosidase) as a reporter gene. In Murray, E. (ed.), *Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols*. Human Press Inc., Clifton, NJ, pp. 217–225.
46. Linkhart, T.A., Clegg, C.H. and Hauschka, S.D. (1981) Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromolecular growth factors in the culture medium. *Dev. Biol.*, **86**, 19–30.
47. Clegg, C.H., Linkhart, T.A., Olwin, B.B. and Hauschka, S.D. (1987) Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell. Biol.*, **105**, 949–956.