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A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase

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ABSTRACT **Adenoviral vector-mediated gene transfer offers significant potential for gene therapy of many human diseases. However, progress has been slowed by several limitations. First, the insert capacity of currently available adenoviral vectors is limited to 8 kb of foreign DNA. Second, the expression of viral proteins in infected cells is believed to trigger a cellular immune response that results in inflammation and in only transient expresssion of the transferred gene. We report the development of a new adenoviral vector that has all viral coding sequences removed. Thus, large inserts are accommodated and expression of all viral proteins is eliminated. The first application of this vector system carries a dual expression cassette comprising 28.2 kb of nonviral DNA that includes the full-length murine dystrophin cDNA under control of a large muscle-specific promoter and a lacZ reporter construct. Using this vector, we demonstrate independent expression of both genes in primary *mdx* (dystrophin-deficient) muscle cells.**

Broad application of *in vivo* gene transfer for the treatment of human inherited or acquired diseases will require development of new viral or nonviral vector systems or a substantial improvement of existing systems. Although adenoviral vectors are highly effective for transfer of genes into tissues *in vivo*, several limitations have prevented their general use thus far. First, the expression of viral proteins in infected cells is believed to trigger a cellular immune response that precludes long-term expression of the transferred gene (1–9). Second, the insert capacity of currently available adenoviral vectors is limited to 8 kb of foreign DNA (10).

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are allelic, lethal degenerative muscle diseases with an incidence of 1:3500 male births (11). In DMD, mutations in the dystrophin gene usually result in the absence of dystrophin, a cytoskeletal protein in skeletal and cardiac muscle. In BMD, dystrophin is usually expressed in muscle but at a reduced level and/or as a shorter, internally deleted form, resulting in a milder phenotype. No effective treatment is available for DMD or BMD at this time. Currently, viral vector gene delivery represents the most promising technology for gene transfer into muscle *in vivo*. Because of the limited capacity of first-generation vectors, truncated forms of the dystrophin cDNA, either derived from patients with mild BMD or generated recombinantly, have been used in the initial studies of adenoviral vector-mediated gene transfer for DMD (12, 13).

We have constructed a new adenoviral vector that has no viral coding sequences and possesses a very high insert capacity. Using this adenoviral vector system, we demonstrate gene transfer of both the 13.8-kb full-length dystrophin cDNA under transcriptional control of the 6.5-kb muscle creatine kinase (MCK) promoter and the *Escherichia coli* lacZ gene as an independently expressed reporter element into primary myoblasts.

MATERIALS AND METHODS

Construction of the Plasmid pDYSβgal. A plasmid STK3 was constructed by inserting an oligonucleotide with a restriction endonuclease *Pme*I recognition sequence into the *BstX*I site of Bluescript II KS (Stratagene) and another oligonucleotide with an *Avr*II recognition sequence into the *Eco*RV site. A 298-bp *Xho*I/*Xba*I poly(A) signal from the bovine growth hormone gene was inserted into the *Cla*I site of STK 3. A 13.8-kb *Sma*I fragment of pCCL-DMD containing the full-length dystrophin cDNA (14) was cloned into the *Sal*I site of STK 3. A 6.5-kb *Xho*I fragment containing the murine MCK promoter, including the first intron (15), was inserted into the *Avr*II site. A 4.6-kb *Eco*RI/*Sal*I fragment containing a cytomegalovirus (CMV) promoter-lacZ gene cassette (16) was inserted into the *Sma*I site. In these cloning steps noncompatible ends had been made blunt by using the Klenow fragment of DNA polymerase I and dNTPs in fill-in reactions. The final construct pDYSβgal is depicted in Fig. 1*A*. The total size of the circular plasmid is ≈28.2 kb.

Generation of the Adenoviral Mutant SV5. 293 cells were transfected with pFG140 (17). FG140 virus was plaque-purified and propagated, and DNA was purified. FG140 virus DNA was cleaved with *Xba*I, and the 34.6-kb DNA fragment extending from nucleotide 1339 to the right viral terminus was isolated by agarose gel electrophoresis and electroelution. A 389-bp left terminal fragment, amplified by PCR with primer 6328 (5′-**GGGCCC**ATCATCAATAATATACCTTATTTTG-3′) encompassing the left viral terminus and including an *Apa*I recognition sequence (boldface type) and primer Ad5-pack-*Xba*I-457–480 (5′-TGA**TCTAGA**CCGGGTATAAATACAC-TACACGTC-3′) that extends from nucleotide 480 to nucleotide 457 and includes an *Xba*I recognition sequence (boldface type) using the packaging impaired adenoviral mutant dl309-267/358 (18) as the template, was subcloned into Bluescript II KS. The adenoviral mutant, dl309-267/358, is deleted for three of the five elements constituting the packaging signal of adenovirus type 5 (Ad5) and is therefore packaging-impaired and can be grown in cell culture only to an ≈90-fold reduced titer when compared to wild-type Ad5 (18). The subcloned viral left terminus derived from dl309-267/358 was removed from the plasmid by cleavage with *Apa*I and *Xba*I, agarose gel electro-

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Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; MCK, muscle creatine kinase; CMV, cytomegalovirus; X-Gal, 5-bromo-4-chloro-3-indoyl β-D-galactoside; β-gal; β-galactosidase; moi, multiplicity of infection; Ad5, adenovirus type 5.

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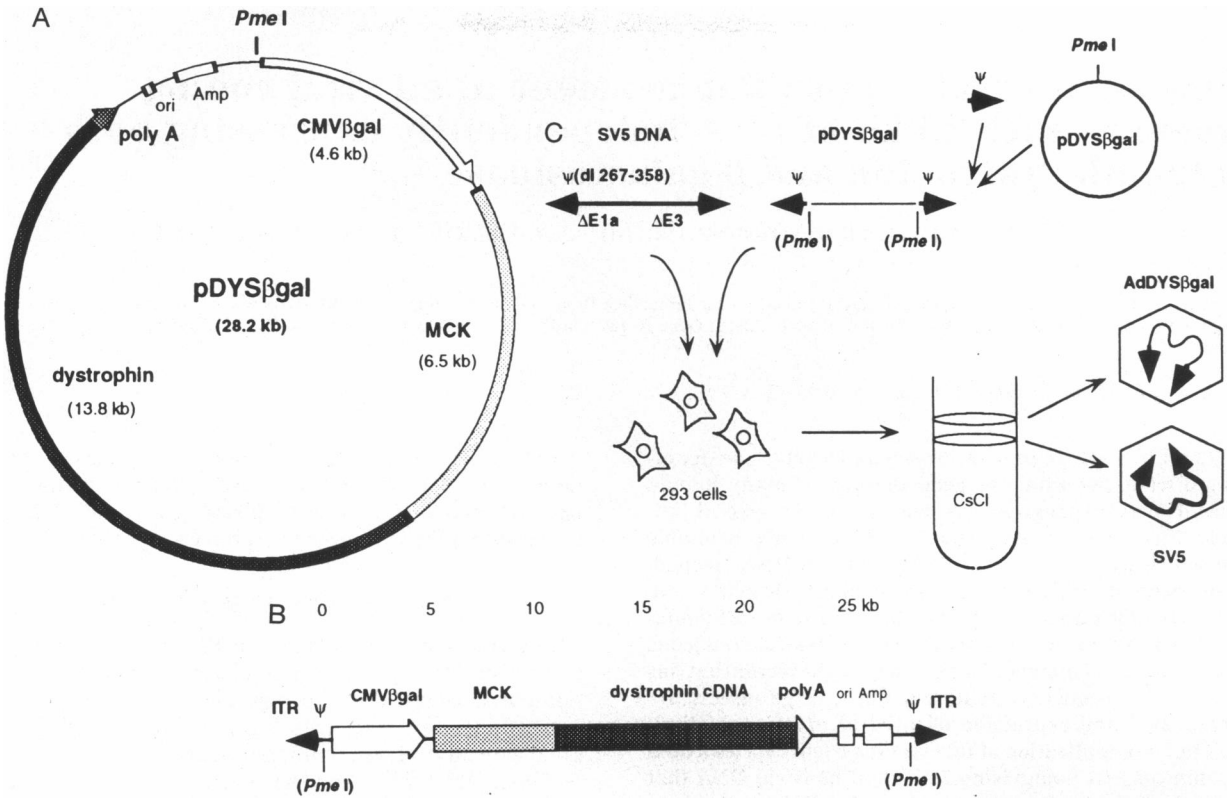

FIG. 1. Rescue, amplification, and purification of the viral vector AdDYSβgal. (*A*) Structure of the 28.2-kb plasmid pDYSβgal containing the 6.5-kb murine MCK promoter, the murine 13.8-kb full-length dystrophin cDNA (dystrophin), the 290-bp polyadenylylation signal of the bovine growth hormone gene [poly(A)], and the *E. coli* lacZ gene under the control of the early CMV promoter (CMVβgal). The bacterial origin of replication (ori) and ampicillin resistance gene (Amp) are indicated. This plasmid has a unique *Pme*I restriction endonuclease cleavage site. (*B*) Structure of pDYSβgal after linearization with *Pme*I and ligation of the left terminus of adenovirus type 5 containing the viral terminal repeat (ITR) and the full-length packaging signal (ψ) to the ends of the linearized DNA using T4 DNA ligase. The *Pme*I site is destroyed during the ligation step. (*C*) Schematic outline of the rescue, amplification, and purification of AdDYSβgal. The linearized pDYSβgal DNA with left adenoviral termini ligated to both ends was cotransfected with DNA/terminal protein complex prepared from the viral mutant SV5 that has a partial deficiency for encapsidation [ψ(dl267-358)] and a deletion within both E1a and E3 (ΔE1a, ΔE3). After plaque purification and serial propagation on 293 cells, a cell lysate was subjected to CsCl equilibrium centrifugation. Two bands were visible after the centrifugation. The upper band (lower buoyant density) contained AdDYSβgal, the lower (higher buoyant density) contained SV5.

phoresis, and electroelution, and was dephosphorylated with calf intestinal phophatase (New England Biolabs) according to the manufacturer's recommendation. The fragment was ligated to the 34.6-kb *Xba*I fragment (nucleotide 1339 to right terminus) of FG140 virus DNA using T4 DNA ligase. The ligation product was transfected into 293 cells by calcium phosphate transfection (19, 20) followed by plaque purification and propapation on 293 cells. This adenoviral mutant was called SV5. The structure of SV5 with a total size of 35 kb was confirmed by amplification of the left viral terminus by using primers flanking the packaging signal and by restriction analysis of SV5 DNA.

Rescue of the AdDYSβgal Vector. The 358-bp left terminal *Sac*II fragment of Ad5 that has an *Apa*I recognition site added at the left terminus and that includes the complete packaging signal of Ad5 was subcloned into the *Apa*I and *Hin*dIII sites of Bluescript II KS, yielding STK17. This fragment can be removed from STK17 by cleavage with *Apa*I and *Eco*RV. The 358-bp *Apa*I–*Eco*RV fragment of STK17 with the left terminal repeat and the complete packaging signal of Ad5 was isolated by agarose gel electrophoresis and electroelution and was dephosphorylated with calf intestinal phophatase. Five micrograms of pDYSβgal DNA linearized with *Pme*I (Fig. 1*A*) was ligated to a 3-fold molar excess of the dephosphorylated *Apa*I–*Eco*RV fragment using T4 DNA ligase for 16 h at room temperature with rATP at a concentration of 100 μM. The ligation reaction was purified by phenol/chloroform extraction followed by ethanol precipitation, and the DNA was cotransfected together with SV5 virus DNA/terminal protein complex (see below) into 80% confluent 293 cells grown in minimal essential medium/10% newborn calf serum by using the calcium phosphate method (19). The SV5 virus DNA/terminal protein complex was prepared as follows: the SV5 virus mutant was propagated on 293 cells and cell extract was prepared by three freeze–thaw cycles. The virus DNA, which retains the terminal protein attached at the termini, was freed from the virus capsids by incubating SV5 virus freeze–thaw extract with an equal volume of 8 M guanidine hydrochloride, 10 mM Tris (pH7.5), 2 mM EDTA, and 4 mM 2-mercaptoethanol on ice for 20 min. This mixture was used directly for calcium phosphate transfection reactions. Transfection efficiency was ≈100-fold higher than with DNA without the terminal protein attached. The optimal amount of cell extract used in transfection experiments resulting in ≈50–100 plaques per 60-mm dish (≈1.5 × 10^6 293 cells per 60-mm cell culture dish) had been determined previously in titration experiments. After a 5-h transfection incubation period the medium was changed and after another 12 h the monolayers were overlaid with 10 ml 0.5% agarose containing minimal essential medium with 5% newborn calf serum. After plaques became visible, individual plaques were isolated and resuspended in 250 μl phosphate-buffered saline (PBS) containing 10% glycerol and were subjected to a single freeze–thaw cycle. Thirty microliters of this suspension was used to infect COS-7 cells. Sixteen hours postinfection cells were fixed with 0.5% (vol/vol) glutaraldehyde in PBS and stained with 5-bromo-4-chloro-3-indoyl β-D-

galactoside (X-Gal) as described (21). Five plaque isolates (≈2% of the analyzed plaques) gave a higher number of β-galactosidase (β-gal) expressing COS-7 (40–400 β-gal positive cells from 30 μl of the primary plaque isolate) and were used to infect 293 cells in 24-well dishes with 1×10^4 cells per well. Three of these could be serially propagated and contained the expected insert as evaluated by Southern blot analysis (22). One was chosen for further propagation. The AdDYSβgal vector together with the helper SV5 virus were serially propagated on 293 cells and then purified by CsCl equilibrium centrifugation.

After CsCl equilibrium centrifugation, 20 μl of the purified particles from the upper and the lower band, respectively, were incubated in 50 mM Tris, 20 mM EDTA, 0.5% SDS, and 200 μg of proteinase K per ml in a final volume of 400 μl at 37°C for 4 h followed by phenol/chloroform extraction and ethanol precipitation. The DNA was resuspended in 20 μl 10 mM Tris (pH 7.5) and 1 mM EDTA. An aliquot was digested with *Bam*HI followed by agarose gel electrophoresis, staining with ethidium bromide, and Southern blot analysis by using ^{32}P-labeled pDYSβgal DNA or adenoviral DNA as the probe (23) (see Fig. 2). Prehybridization and hybridization were performed in 1.5 × standard saline phosphate/EDTA (SSPE), 1% sodium dodecyl sulfate (SDS), 1% nonfat milk, and 20% dextran sulfate at 65°C for 12–16 h. The membranes were washed twice for 15 min with 2× standard saline citrate (SSC)/0.5% SDS, and three times for 20 min with 0.1× SSC/0.5% SDS at 65°C followed by exposure to Kodak X-Omat AR x-ray films for 1–12 h. In different experiments, aliquots were digested with *Bam*HI, *Hin*dIII, *Pvu*II, *Xba*I, or *Not*I and were subjected to Southern blot analysis using either pDYSβgal DNA or the 358-bp left terminal *Sac*II fragment of Ad5 as probes (data not shown), confirming that the overall structure of AdDYSβgal had been preserved during the rescue of pDYSβgal with the viral left terminal DNA linked to the *Pme*I sites of the linear pDYSβgal molecule.

After CsCl equilibrium centrifugation and collection of the upper band containing the AdDYSβgal vector, serial dilutions were used to infect 293 cells. After 16 h the cell monolayer was fixed with 0.5% (vol/vol) glutaraldehyde in PBS and stained with X-Gal. The number of cells expressing β-gal was visually determined and the titer of AdDYSβgal was calculated.

Growth and Infection of *mdx* Myoblasts. Primary dystrophin-deficient myoblasts were isolated from skeletal muscle of a 7-day *mdx* mouse according to the method of Rando and Blau (24). Myoblasts were propagated on collagen-coated dishes in growth medium consisting of Ham's nutrient mixture F-10 supplemented with 20% (vol/vol) fetal bovine serum, 100 units of penicillin G per ml, and 100 μg of streptomycin per ml. Myoblasts were plated at a density of 2×10^5 cells per 3.5 cm collagen-coated dish and infected with purified viral vector over a range of multiplicities of infection (moi) as determined by infection of 293 cells. Twenty-four hours postinfection, growth medium was replaced with fusion medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (vol/vol) horse serum, 2.5×10^{-6} M dexamethasone, and 10^{-6} M insulin. Fusion medium was changed daily until the cells were stained for β-gal expression or collected for dystrophin expression analysis. For dystrophin analysis, monolayers were rinsed three times with PBS and three times with a solution of 50 mM Tris·HCl (pH 8), 5 mM EDTA, and 5 mM EGTA (TEE). Cells were collected in a small volume of TEE and centrifuged at 14,000 rpm for 5 min at 4°C. The cell pellet was resuspended in TEE with 0.3% SDS and incubated on ice for 20 min. Brief sonication of each sample reduced viscosity. Total protein concentration was assayed using the bicinchoninic acid protein assay reagent (Pierce). Samples of protein (25 μg) were separated by electrophoresis on a SDS/5% PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and 5% goat serum in Tris-buffered saline-Tween (TBS-T) for 1 h. Immunostaining was performed according to the protocol for ECL Western blotting detection reagents (Amersham) by using DYS2 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), a mouse monoclonal antibody directed against the carboxyl-terminal 17 amino acids of dystrophin, diluted 1:100 in TBS-T as the primary antibody, and horseradish peroxidase-conjugated anti-mouse antibody (Kirkegaard & Perry Laboratories) diluted 1:5000 in TBS-T as the secondary antibody. The chemiluminescent signal was detected by a 30-sec exposure to autoradiography film.

Transfection of C2C12 Myoblasts with pDYSβgal. C2C12 myoblasts (25) were plated at a density of 4×10^4 cells per 3.5-cm collagen-coated dish in DMEM growth medium with 10% (vol/vol) fetal bovine serum, 100 units of penicillin G per ml, and 100 μg of streptomycin per ml. Forty-eight hours later lipofection was performed according to the manufacturer's directions using 3 μg pDYSβgal and 10 μl lipofectamine (GIBCO/BRL) per dish. Some transfected monolayers were stained with X-Gal 24 h posttransfection, demonstrating β-gal expression in 17% of myoblasts. Others were switched to fusion medium that was changed daily for 7 days before collection for dystrophin protein expression analysis.

RESULTS AND DISCUSSION

A plasmid, pDYSβgal, was constructed to contain two transcription units: (*i*) the 13.8-kb full-length murine dystrophin cDNA (14) under the control of the 6.5-kb murine MCK promoter that is active only in skeletal and cardiac muscle (15) and (*ii*) the *E. coli* lacZ gene under the control of the human CMV promoter (16) (Fig. 1*A*). This construct was rescued as an adenoviral vector by a procedure involving (*i*) linearization of the plasmid at a unique *Pme*I restriction endonuclease cleavage site, (*ii*) ligation of 358 bp of the left terminus of Ad5 containing the viral origin of replication and the full-length packaging signal of Ad5 (18, 26) to both ends of the linear DNA, and (*iii*) cotransfection of the ligation product with DNA of the helper adenoviral mutant, SV5, into 293 cells (Fig. 1 *B* and *C*). SV5 is deficient for E1a and E3 functions and, more importantly, has a 91-bp deletion within the left terminal packaging signal (18, 26), thereby reducing the efficiency of packaging of helper viral genome into viral capsids. After cotransfection of linear pDYSβgal ligated to the 358-bp Ad5 left terminus together with SV5 DNA/terminal protein complex into 293 cells, the cell monolayer was overlaid with agarose-containing medium. Individual plaques were isolated after 5–7 days and tested for β-gal expression by infection of COS-7 cells. A plaque isolate that yielded a higher number of COS-7 cells expressing β-gal (see *Materials and Methods*) was subjected to serial propagation on 293 cells. After six serial propagations, 4×10^7 293 cells were infected. Sixty hours postinfection the freeze–thaw cell extract was centrifuged to equilibrium in a continuous CsCl gradient, resulting in two distinct bands visible in the middle of the centrifugation tube. Each band was isolated and further purified by two additional CsCl equilibrium centrifugations. DNA was isolated from small aliquots of each band, digested with different restriction endonucleases, separated by agarose gel electrophoresis and analyzed by ethidium bromide staining (Fig. 2*A*) and Southern blot analysis (Fig. 2 *B* and *C*). The lower band (higher buoyant density) corresponded to the helper virus, SV5, and the upper band (lower buoyant density) to the adenoviral vector, AdDYSβgal. These results demonstrated the successful rescue and amplification of an adenoviral vector having a 28.2-kb nonviral DNA insert and lacking any viral DNA except for the 358-bp viral left terminus with the essential elements for viral replication and packaging. Furthermore, AdDYSβgal was physically separated from SV5 based on the different buoyant densities of the two types of particles. Our studies directly prove the previous suggestion that a small segment from the left terminus of Ad5 is sufficient for replication and packaging

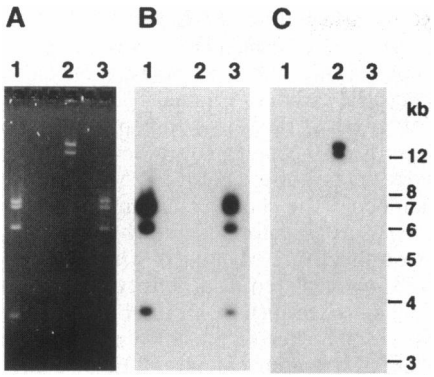

FIG. 2. Structural analysis of AdDYSβgal and SV5 after CsCl equilibrium centrifugation. (*A*) DNA aliquots prepared from the upper (lane 3) and lower (lane 2) viral bands and from pDYSβgal (lane 1) were cleaved with *Bam*HI, separated by agarose gel electrophoresis, and stained with ethidium bromide. The upper part of the gel is shown. The uppermost band in lane 3 of this gel corresponds to a 7580-bp fragment that includes one of the viral termini and hybridizes to the 358-bp left terminal *Sac*II fragment of Ad5 (data not shown). The uppermost band in lane 1 corresponds to a 7270 bp fragment of pDYSβgal that does not hybridize to this probe (data not shown) because the plasmid does not contain the viral termini. (*B* and *C*) The DNAs were transferred to a nylon membrane, hybridized with ^{32}P-labeled probes derived from either pDYSβgal DNA (*B*) or adenoviral DNA (*C*), and exposed to x-ray films. After long exposure, faint bands were also detectable in lane 3, which corresponded to *Bam*HI-cleaved SV5 DNA and in lane 2, which corresponded to *Bam*HI-cleaved AdDYSβgal DNA (data not shown).

of DNA into viral capsids, if all other viral functions are provided by trans-complementation (18, 26). There is evidence that this also may hold true for other adenovirus serotypes, and is also indirectly supported by the observation that adenovirus/simian virus 40 hybrids can be propagated under certain conditions in cell culture (27–33).

The titer of the purified AdDYSβgal (Fig. 3*B*) achieved in these experiments as determined by infection of 293 cells and X-Gal staining was 1.4×10^{10}/ml with a total yield of 4.9×10^9 infectious particles obtained from 1.6×10^8 293 cells. The contamination of AdDYSβgal with SV5 virus was not more than 1% as determined by plaque assay on 293 cells and by comparing relative DNA amounts by Southern blot analysis. The integrity of the vector particles was investigated by electron microscopy (34). AdDYSβgal particles were morphologically identical to SV5 particles (Fig. 3 *C* and *D*).

To demonstrate functional activity of AdDYSβgal, primary myoblasts derived from the *mdx* mouse, a genetic and biochemical model for human DMD disease (35), were infected with different amounts of AdDYSβgal. Staining 24 h after infection with X-Gal demonstrated prominent β-gal expression (Fig. 4*A*). In parallel, other myoblast monolayers were induced to differentiate into myotubes 24 h after infection. Four days after induction of cell fusion, myotubes stained with X-Gal demonstrated very strong expression and accumulation of β-gal in the transduced primary *mdx* myotubes (Fig. 4*B*). Parallel monolayers of myotubes were analyzed for dystrophin expression. Western blot analysis of protein extracts using a mouse monoclonal antibody directed against the carboxyl terminus of dystrophin demonstrated expression of the full-length protein (Fig. 4*C*). A specific band of 427 kDa was detected in primary myotubes transduced by AdDYSβgal (lanes 2 and 3) and in the C2C12 muscle cell line transfected with pDYSβgal (lane 4), but not in untransduced primary myotubes (lane 1). The transferred dystrophin was shown to be full-length by comparison with dystrophin expressed in myotubes derived from normal mouse muscle (lane 5). The dystrophin gene was not expressed in AdDYSβgal-infected myoblasts before differentiation into myotubes (data not shown). These findings demonstrate that dystrophin expression from this vector is under control of the MCK promoter as desired, not from the CMV promoter located further upstream.

The ultimate goal of somatic gene transfer for DMD and BMD is correction of the sarcolemmal defect that leads to muscle degeneration. Transgenic *mdx* mouse lines expressing the dystrophin cDNA in cardiac and skeletal muscle (36, 37) provide a germ-line gene correction model demonstrating that the functional defect in *mdx* mice can be corrected by gene transfer of the dystrophin cDNA. The dystrophin construct under the control of the MCK promoter in these transgenic studies is nearly identical to the construct used in the experiments reported here. Therefore, we anticipate that the achievement of a sufficient level of somatic gene transfer using

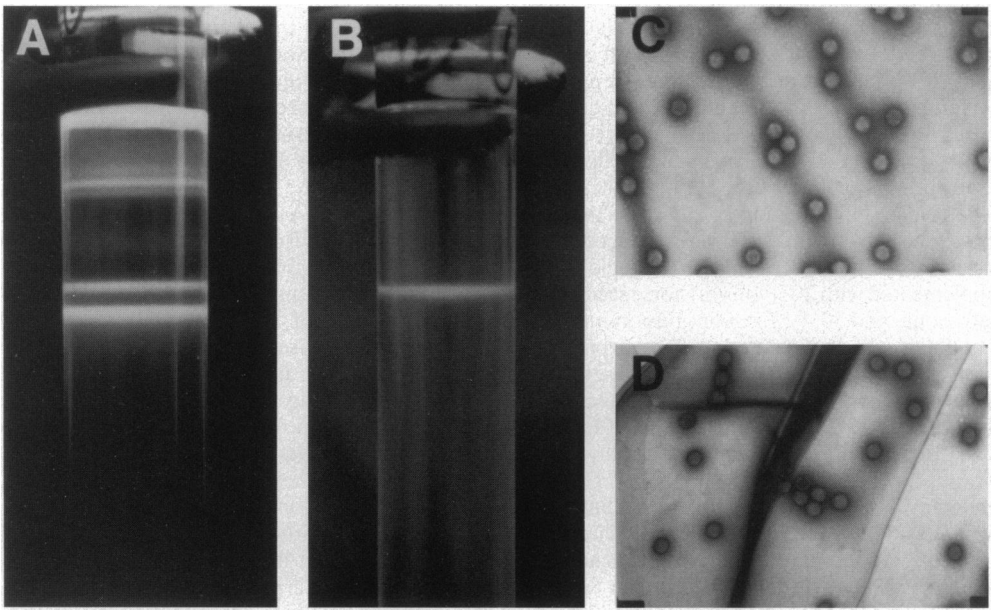

FIG. 3. Purification and electron microscopic analysis of AdDYSβgal. (*A*) Two bands were visible after CsCl equilibrium centrifugation. (*B*) Both bands were individually isolated and were subjected to two additional CsCl centrifugations. The upper band after purification is shown. Particles from the upper band (*C*) and from the lower band (*D*) were analyzed by electron microscopy (34). (×35,800.)

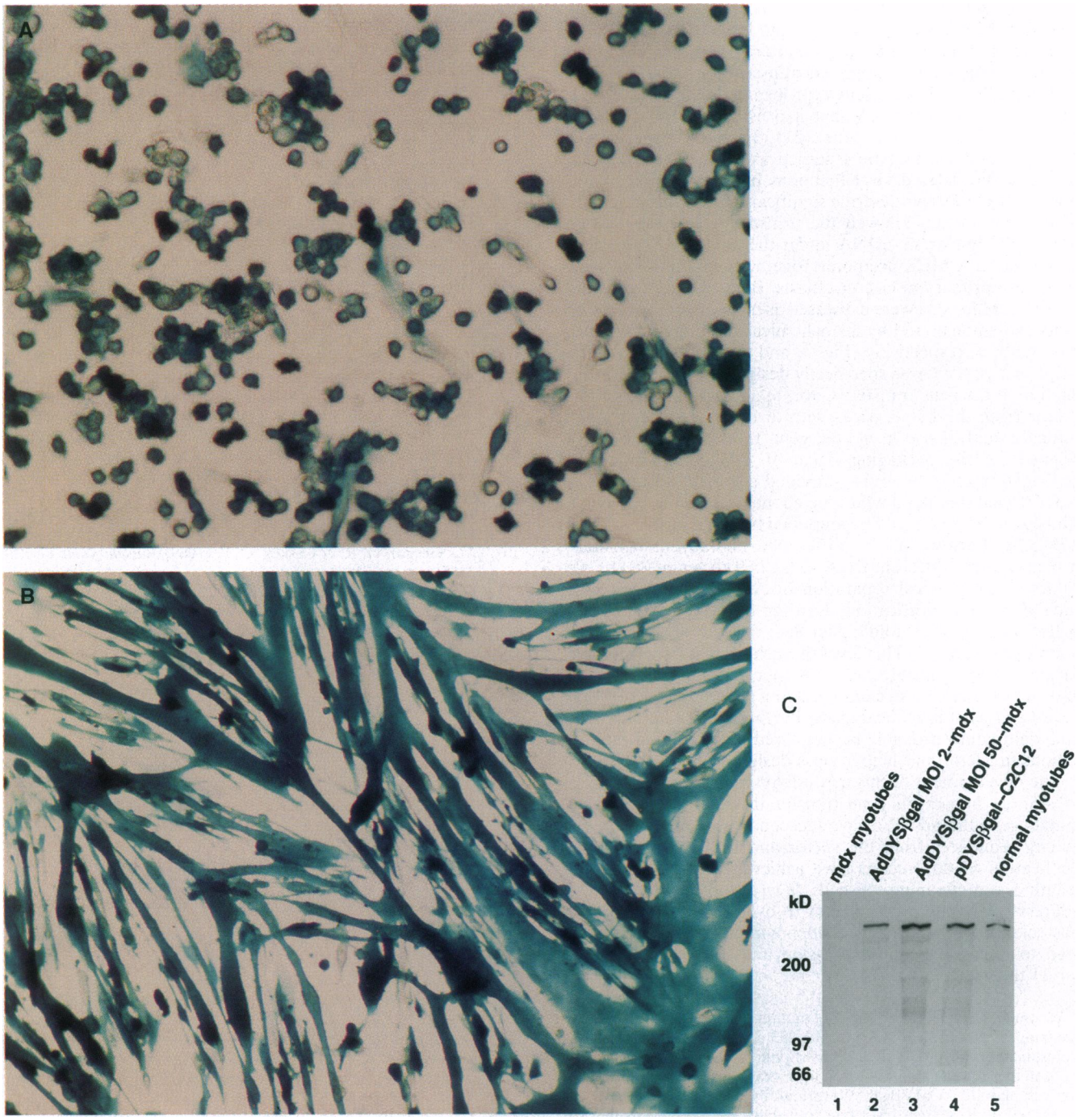

FIG. 4. Functional activity of AdDYSβgal. (*A*) X-Gal staining of primary *mdx* myoblasts 24 h after infection with AdDYSβgal with a moi of 5. (*B*) *mdx* myoblasts were infected with AdDYSβgal with an moi of 1, and myotube formation was induced 24 h after infection. Three days later the myotubes were fixed and stained with X-Gal. (*C*) Expression of full-length dystrophin in primary *mdx* myotubes after infection with AdDYSβgal. Primary myoblasts were infected with AdDYSβgal at different moi. Myotube formation was induced 24 h after infection and myotubes were harvested 3 days later. Western blot analysis of protein extracts was performed by using a mouse monoclonal antibody directed against the 17 carboxyl-terminal amino acids of the dystrophin protein. The individual lanes are as follows: control *mdx* myotubes that were not infected (lane 1), *mdx* myotubes after infection with AdDYSβgal at moi of 2 (lane 2) or 50 (lane 3), C2C12 myotubes after lipofection with pDYSβgal (lane 4), and positive control myotubes fused from primary myoblasts derived from normal mouse skeletal muscle (lane 5).

AdDYSβgal will result in functional correction of the cytoskeletal defect in DMD.

We employed a helper virus strategy for this new adenoviral vector system to address two significant problems posed by the application of adenoviral vector-mediated gene transfer. First, this viral vector lacks all of the protein-encoding viral DNA that may lead to a cellular immune response (1–9), which is believed to prevent long-term expression of the transduced gene. First-generation adenoviral vectors have deletions in the viral E1 and/or E3 regions. Recent efforts have been directed at the deletion of additional early viral functions (E2, E4) with the hope of reducing viral gene expression after transduction of the target cells with the vector (38–42). For propagation of these vectors, cell lines have been developed that can provide the deleted functions. However, on theoretical grounds, it will be difficult to provide all adenoviral functions by a complementing cell line without substantially compromising the high adenoviral titer that is currently one of the major advantages

of adenoviral vectors. Second, the expanded insert capacity provides the versatility to incorporate large cDNAs, endogenous or large tissue-specific promoters, reporter genes, and several independently expressed transcription units.

Previously, we have rescued, propagated, and partially purified an adenoviral DNA that had essential viral elements replaced by a reporter gene (21). In those experiments, wild-type adenovirus type 2 served as the helper virus that provided the deleted viral functions by trans-complementation. In this report we describe significant further development of this system that allowed the transduction of the 13.8-kb full-length dystrophin cDNA under the control of the 6.5-kb muscle-specific MCK promoter, together with the *E. coli* lacZ gene, into primary mouse myoblasts. Both the lacZ gene and the dystrophin gene were expressed as independent expression units as demonstrated by histochemical staining and western blot analysis, respectively (Fig. 4, and data not shown). The helper virus, SV5, was specifically designed to enhance propagation of the gene transfer vector, to allow separation of this vector from the helper virus, and to increase safety for the ultimate goal of use in *in vivo* gene transfer. Deletion of a segment of the packaging signal of SV5 compromised its propagation and, therefore, provided a growth advantage to AdDYSβgal that has a wild-type adenoviral packaging signal. Thus, AdDYSβgal could be amplified by serial propagation in 293 cells. Because of the difference in buoyant densities, probably due to different DNA sizes (28.9 kb versus 35 kb), we achieved the physical separation of AdDYSβgal from SV5, such that after purification the titer of the purified vector AdDYSβgal was 100-fold higher than the titer of the contaminating helper virus. This level of purity might be acceptable for *in vivo* gene transfer since the helper virus is itself defective, and, in fact, is similar to adenoviral first generation vectors that have been used in clinical trials. Furthermore, safety, purity, and yield could probably be improved by additional modifications in vector and helper virus design.

The development of this new adenoviral vector may have a substantial impact on gene transfer in general and also on human gene therapy. We have succeeded in removing all viral coding sequences from the vector and in the first practical application of this vector, have achieved expression of full-length dystrophin in muscle cells. *In vivo* studies are needed to address the ability of this vector to overcome the significant obstacles presented by current adenoviral vectors, which could lead to long-term gene expression and reduced toxicity to target tissues.

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1. Stratford-Perricaudet, L. D., Levrero, M., Chasse, J. F., Perricaudet, M. & Briand, P. (1990) *Hum. Gene Ther.* **1,** 241–256.
2. Ginsberg, H. S., Moldaiver, L. L., Sehgal, P. B., Redington, M., Kilian, P. L., Chanock, R. M. & Prince, G. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1651–1655.
3. Jaffe, H. A., Danel, C., Longenecker, G., Metzger, M. & Setoguchi, Y. (1992) *Nat. Genet.* **1,** 372–378.
4. Engelhardt, J. F., Simon, R. H., Yang, Y., Zepeda, M., Weber-Pendleton, S., Doranz, B., Grossman, M. & Wilson, J. M. (1993) *Hum. Gene Ther.* **4,** 759–769.
5. Li, Q., Kay, M. A., Finegold, M., Stratford-Perricaudet, L. D. & Woo, S. L. (1993) *Hum. Gene Ther.* **4,** 403–409.
6. Simon, R. H., Engelhardt, J. F., Yang, Y., Zepeda, M., Weber-Pendleton, S., Grossman, M. & Wilson, J. M. (1993) *Hum. Gene Ther.* **4,** 771–780.
7. Smith, T. A., Mehaffey, M. G., Kayda, D. B. & Saunders, J. M. (1993) *Nat. Genet.* **5,** 397–402.
8. Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E. & Wilson, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4407–4411.
9. Dai, Y., Schwarz, E. M., Gu, D., Zhang, W.-W., Sarvetnick, N. & Verma, I. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1401–1405.
10. Bett, A. J., Haddara, W., Prevec, L. & Graham, F. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8802–8806.
11. Clemens, P. R. & Caskey, C. T. (1994) *Curr. Neurol.* **14,** 29–54.
12. Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J.-C., Perricaudet, M. & Kahn, A. (1993) *Nature (London)* **361,** 647–650.
13. Clemens, P. R., Krause, T. L., Chan, S., Korb, K. E., Graham, F. L. & Caskey, C. T. (1995) *Hum. Gene Ther.* **6,** 1477–1485.
14. Lee, C. C., Pearlman, J. A., Chamberlain, J. S. & Caskey, C. T. (1991) *Nature (London)* **349,** 334–336.
15. Jaynes, J. B., Chamberlain, J. S., Buskin, J. N., Johnson, J. E. & Hauschka, S. D. (1986) *Mol. Cell. Biol.* **6,** 2855–2864.
16. MacGregor, G. R. & Caskey, C. T. (1989) *Nucleic Acids Res.* **17,** 2365.
17. Graham, F. L. (1984) *EMBO J.* **3,** 2917–2922.
18. Graeble, M. & Hearing, P. (1990) *J. Virol.* **64,** 2047–2056.
19. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52,** 456–467.
20. Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. J. (1977) *Gen. Virol.* **36,** 59–74.
21. Mitani, K. Graham, F. L. Caskey, C. T. & Kochanek, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3854–3858.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
23. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
24. Rando, T. A. & Blau, H. M (1994) *J. Cell Biol.* **125,** 1275–1287.
25. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C. & Webster, C. (1985) *Science* **230,** 758–766.
26. Hearing, P., Samulski, R. J., Wishart, W. L. & Shenk T. (1987) *J. Virol.* **61,** 2555–2558.
27. Hassell, J. A., Lukanidin, E., Fey, G. & Sambrook, J. (1978) *J. Mol. Biol.* **120,** 209–247.
28. Hammarskjold, M.-L. & Winberg, G. (1980) *Cell* **20,** 787–795.
29. Deuring, R., Klotz, G. & Doerfler, W. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3142–3146.
30. Doerfler, W. (1982) *Curr. Top. Microbiol. Immunol.* **101,** 127–194.
31. Deuring, R. & Doerfler, W. (1983) *Gene* **26,** 283–289.
32. Gluzman, Y. & van Doren, K. J. (1983) *J. Virol.* **45,** 91–103.
33. Yamada, M., Lewis, J. A. & Grodzicker, T. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3567–3571.
34. Lee, H. & Brown, D. T. (1994) *Virology* **202,** 390–400.
35. Partridge, T. (1991) *Neuropathol. Appl. Neurobiol.* **17,** 353–363.
36. Lee, C. C., Pons, F., Jones, P. G., Bies, R. D., Schlang, A. M., Leger J. J. & Caskey, C. T. (1993) *Hum. Gene Ther.* **4,** 273–281.
37. Cox, G. A., Cole, N. M., Matsumura, K., Phelps, S. F., Hauschka, S. D., Campbell, K. P., Faulkner, J. A. & Chamberlain, J. S. (1993) *Nature (London)* **364,** 725–729.
38. Engelhardt, J. F., Ye, X., Doranz, B. & Wilson, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6196–6200.
39. Yang, Y., Nunes, F. A., Berencsi, K., Gonczol, E., Engelhardt, J. F. & Wilson, J. M. (1994) *Nat. Genet.* **7,** 362–369
40. Armento, D., Sookdeo, C. C., Hehir, K. M., Gregory, R. J., St. George, J. A., Prince, G. A., Wadsworth, S. C. & Smith, A. E. (1995) *Hum. Gene Ther.* **6,** 1343–1353.
41. Krougliak, V. & Graham, F. L. (1995) *Hum. Gene Ther.* **6,** 1575–1586.
42. Wang, Q., Jia, X. & Finer, M. H. (1995) *Gene Ther.* **2,** 775–783.