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Dystrophin Expression in Muscle Following Gene Transfer with a Fully Deleted ("Gutted") Adenovirus Is Markedly Improved by Trans-Acting Adenoviral Gene Products

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ABSTRACT

Helper-dependent adenoviruses (HDAd) are Ad vectors lacking all or most viral genes. They hold great promise for gene therapy of diseases such as Duchenne muscular dystrophy (DMD), because they are less immunogenic than E1/E3-deleted Ad (first-generation Ad or FGAd) and can carry the full-length (Fl) dystrophin (dys) cDNA (12 kb). We have compared the transgene expression of a HDAd (HDAdCMVDysFl) and a FGAd (FGAdCMV-dys) in cell culture (HeLa, C2C12 myotubes) and in the muscle of mdx mice (the mouse model for DMD). Both vectors encoded dystrophin regulated by the same cytomegalovirus (CMV) promoter. We demonstrate that the amount of dystrophin expressed was significantly higher after gene transfer with FGAd-CMV-dys compared to HDAdCMVDysFl both *in vitro* and *in vivo*. However, gene transfer with HDAd-CMVDysFl in the presence of a FGAd resulted in a significant increase of dystrophin expression indicating that gene products synthesized by the FGAd increase, *in trans*, the amount of dystrophin produced. This enhancement occurred in cell culture and after gene transfer in the muscle of mdx mice and dystrophic golden retriever (GRMD) dogs, another animal model for DMD. The E4 region of Ad is required for the enhancement, because no increase of dystrophin expression from HDAdCMVDysFl was observed in the presence of an E1/E4-deleted Ad *in vitro* and *in vivo*. The characterization of these enhancing gene products followed by their inclusion into an HDAd may be required to produce sufficient dystrophin to mitigate the pathology of DMD by HDAd-mediated gene transfer.

OVERVIEW SUMMARY

Adenovirus (Ad) deleted of all viral genes (helper-dependent Ad, HDAd) are less immunogenic then E1-deleted Ad (first-generation, FGAd). HDAd are consequently viewed as better vectors to treat disease such as Duchenne muscular dystrophy (DMD). In this report we demonstrate that the expression level of dystrophin carried by a HDAd is significantly lower compared to a FGAd. This observation was made in cell culture and after gene transfer in muscles of two animal models of DMD. We further show that FGAd synthesizes gene products that enhance its own transgene expression, as well as the transgene expression of HDAd when provided *in trans*. The E4 region was essential for this enhancement, because no increase of dystrophin expression by a HDAd occurred in the presence of an E1/E4-deleted Ad. The characterization and inclusion of these enhancing factors into a HDAd might be necessary for efficient treatment of DMD by HDAd-mediated gene therapy.

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INTRODUCTION

Replication-incompetent adenovirus (Ad) rendered defective because of deletion of the essential E1 region, also known as first generation Ad (FGAd) possess several properties that make them a very attractive vector for gene therapy application. For example, they can easily be produced to very high titer, they have a wide tropism, they can transduce both mitotic and post-mitotic cells and, depending of the tissue or the animal model under investigation, they can provide very high transduction level (reviewed in Karpati *et al.*, 1997; Ragot *et al.*, 1997). Unfortunately, gene transfer using FGAd in immunocompetent animals resulted only in temporary expression of the transgene, because of an immune response of the host that eliminates the transduced cells or that silences the transgene expression (Dai *et al.*, 1995; Gilgenkrantz *et al.*, 1995; Lochmuller *et al.*, 1996; Yang *et al.*, 1996; Guibinga *et al.*, 1998; Howell *et al.*, 1998). The transgene antigenicity (Tripathy *et al.*, 1996; Michou *et al.*, 1997; Yang *et al.*, 1996) as well as low grade synthesis of Ad gene products (Yang *et al.*, 1994a,b), have been shown to be responsible for triggering the deleterious immune response.

One approach to reduce the immune response caused by the leaky expression of viral gene products was to delete additional viral genes such as E4 (Gao *et al.*, 1996; Armentano *et al.*, 1997; Brough *et al.*, 1997; Dedieu *et al.*, 1997; Wang *et al.*, 1997; Lusky *et al.*, 1998) or E2 (Engelhardt *et al.*, 1994; Yang *et al.*, 1994b; Hu *et al.*, 1999; Hodges *et al.*, 2000). A more drastic approach has been to remove all viral sequences except for the inverted terminal repeats (ITR) and the packaging signal (Fisher *et al.*, 1996; Kochanek *et al.*, 1996; Kumar-Singh and Chamberlain, 1996; Parks *et al.*, 1996; Kochanek, 1999). Such completely deleted Ad vectors are often referred to as "gutted Ad," large capacity Ad or helper-dependent Ad (HDAd). Deletion of additional Ad genes in these vectors has been associated with a reduction of cellular immune response, which has often lead to an improvement in the duration of the transgene expression (Gao *et al.*, 1996; Wang *et al.*, 1997; Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Chen *et al.*, 1999; Hu *et al.*, 1999; Maione *et al.*, 2000). However, in immunodeficient animals, several reports have documented a reduction or a rapid loss of transgene expression after gene transfer with Ad vectors lacking, in addition to E1, the E4 or E2 regions (Lieber *et al.*, 1996; Armentano *et al.*, 1997; Brough *et al.*, 1997; Lieber *et al.*, 1997; Dedieu *et al.*, 1997; Lusky *et al.*, 1998, 1999). Another advantage of using Ad with large deletion, is the proportional gain in their transport capacity, which, in the case of a fully deleted HDAd has increased to approximately 36 kb.

Duchenne muscular dystrophy (DMD) is a fatal disease characterized by a progressive loss of skeletal muscle fibers leading to death usually by the age of 25 (Engel *et al.*, 1994). DMD, which affects 1 of 3,500 male births, is caused by mutation of the gene encoding dystrophin, a rod-shaped cytoskeletal protein associated with the inner face of the plasma membrane of muscle (Sadoulet-Puccio and Kunkel, 1996). Although no effective treatment exists for DMD, one promising approach for treating this disease is gene replacement by Ad-mediated dystrophin gene transfer. Several groups have demonstrated the potential usefulness of this approach in preclinical studies using mdx mice, the mouse model for DMD (Vincent *et al.*, 1993; Acsadi *et al.*, 1996; Deconinck *et al.*, 1996; Yang *et al.*, 1998), and in the dystrophin-deficient golden retriever (GRMD) dog model (Howell *et al.*, 1998). We have also demonstrated that Ad-mediated utrophin gene transfer, utrophin being a functional dystrophin homologue, could equally well mitigate the dystrophic phenotype of the mdx muscle (Gilbert *et al.*, 1999; Ebihara *et al.*, 2000; Wakefield *et al.*, 2000). However, as discussed above, dystrophin and utrophin expression after gene transfer with FGAd in immunocompetent animals was temporary due in large measure to the host cellular immune response against transduced fibers.

In an attempt to improve the duration of dystrophin expression after gene transfer in muscle, we have constructed a HDAd deleted of all viral genes and encoding dystrophin regulated by the cytomegalovirus (CMV) promoter (HDAdCMVDysFl). Surprisingly, in comparison to a FGAd encoding the dystrophin regulated by the same promoter (FGAdCMV-dys), the amount of dystrophin produced was significantly lower after gene transfer with HDAdCMVDysFl *in vitro* and in the muscle *in vivo*. We demonstrate that a marked increased of dystrophin production by HDAdCMVDysFl occurs in the presence of Ad gene products in cell culture and in the muscle of mdx mice and GRMD dogs, two animal models for DMD. We show that the E4 region is required for the enhancement, because no increase of dystrophin expression was observed in the presence of an E1/E4-deleted Ad. These observations have important implications for the design of Ad vectors for potential use in clinical trials for DMD or for other genetic diseases requiring abundant expression of a therapeutic transgene in muscle. In the case of DMD, the characterization of these adenoviral gene products, followed by their inclusion into the backbone of a HDAd, may be needed to produce sufficient dystrophin to mitigate the dystrophic phenotype of the diseased muscle.

MATERIALS AND METHODS

First- and second-generation adenovirus

Unless stated otherwise, all cells were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Burlington, Ontario, Canada) supplemented with 30 μg/ml gentamicin, 20 m*M* L-glutamine and 10% fetal bovine serum (BioWhittaker, Walkersville, MD) under an atmosphere of 5% CO_2.

The FGAd (E1/E3-deleted) encoding either β-galactosidase (FGAdCMV-lacZ) or the minidystrophin cDNA modeled on the Becker gene (FGAdCMV-dys) have been described previously (Acsadi *et al.*, 1994, 1996; Jani *et al.*, 1997). Ad5/δE1(β-gal)δE4 is an E1/E4-deleted Ad that expresses β-galactosidase regulated by the phosphoglycerate kinase promoter (Wang *et al.*, 1995). The E4 region of Ad5/δE1(β-gal)δE4 is only partially deleted, because open reading frame 4 (ORF4) is expressed. The Ad vectors were isolated by centrifugation on two consecutive discontinuous CsCl gradients using standard method of purification (Acsadi *et al.*, 1994). The CsCl was removed by chromatography on Sephadex G25 columns (Amersham Pharmacia Biotech Inc, Piscataway, NJ) and eluted with phosphate-buffered saline (PBS). The titer (virus particles/milliliter) was determined from the optical density at 260 nm (Mittereder *et al.*, 1996). When not used the same day, the viral

preparations were kept at −80°C in PBS supplemented with 10% glycerol, 0.1% bovine serum albumin (BSA), 10 m*M* Tris-HCl (pH 7.2), and 5 m*M* $MgCl_2$. Before injection, the glycerol was removed by chromatography on Sephadex-G25. The infectious titer of FGAdCMV-lacZ and Ad5/δE1(β-gal)δE4 was determined by calculating the number of β-galactosidase positive cells (blue-forming unit, BFU) at 20 hr following infection of 293A cells in 30-mm dishes with grids (Sarstedt, Newton, NC), as described previously (Wang *et al*., 1995). The infectious titer of FGAdCMV-dys was determined by measuring the cytopathic effect after serial dilution on 293A cells (Nyberg-Hoffman *et al*., 1997) and by in situ hybridization (see below). The ratio of virus particles to infectious particles was 50:1 for both FGAdCMV-lacZ and Ad5/δE1(β-gal)δE4, and 100:1 for FGAdCMV-dys.

Construction of HDAdCMVDysFl

To construct a HDAd expressing the full-length human dystrophin regulated by the CMV promoter and enhancer (HDAd-CMVDysFl), plasmids pCMVDysFl and pUCλI were first generated using standard methods of molecular biology (Sambrook *et al*., 1989). Plasmid pCMVDysFl was made by inserting the following DNA fragments into the multiple cloning site of pBluescript (Stratagene, La Jolla, CA): a unique *Not* I site, the ITR and the packaging sequence of Ad type V, as well as the rabbit β-globin poly(A) signal derived from pAdCMV-dys (Acsadi *et al*., 1996; Jani *et al*., 1997), the full-length human dystrophin cDNA (Acsadi *et al*., 1991), the immediate early promoter and enhancer of human CMV (position -508 to +70 from the transcription initiation site), and a unique *Sa*l I restriction site. The CMV promoter was obtained by PCR of p-CMVLacZ (Acsadi *et al*., 1994) using primers 5′-TGCACGTCGA-CATAACTTACGGTAAATGG-3′ and 5′-GCTAACTAGTG-GAGGCTGGATCGGTC-3′. Digestion of pCMVDysFl with *No*t I and *Sal* I releases the left ITR and the dystrophin expression cassette. Plasmid pUCλI was constructed by inserting the following DNA fragments into pUC19 (MBI Fermentas, Burlington, Ontario, Canada), a unique *Xho* I site, the 16.8-kb *Bam* HI fragment of lambda DNA (position 5,505—22,346), the last 250-bp of Ad type 5 encoding the right ITR followed by a unique *Hin*d III site. The right ITR was obtained by PCR of purified DNA (Graham and Prevec, 1991) of FGAd-CMV-lacZ using primers 5′-TACGTGTCGACACAACTTC-CTCAAATCG-3′ and 5′-CGAGCCTCGAGCATCATCAAT-AATATACC-3′. The lambda DNA stuffer and the right ITR can be released by digesting pUCλI with *Xho* I and *Hin*d III. HDAdCMVDysFl was generated by ligating 7 μg of purified *No*t I/*Sal* I fragment of pCMVDysFl with 14 μg of purified *Xho* I/*Hin*d III fragment of pUCλI overnight at 16°C in 100 μl, and extracted by phenol/chloroform followed by ethanol precipitation. Before ligation, the *Not* I, *Xho* I and *Hin*d III ends were dephosphorylated by treatment with calf alkaline phosphatase (New England BioLabs Inc, Mississauga, Ontario, Canada) and purified by electrophoresis on low-melting-point agarose followed by phenol/chloroform extraction and ethanol precipitation. Six micrograms of ligation product was used to transfect a 60-mm dish of 293Cre4 cells (Parks *et al*., 1996) with Lipo-fectAMINE™ (Life Technologies, Burlington, Ontario, Canada) according to the manufacturer's recommendations. The next day, the cells were infected with the helper virus (AdLC8cluc) (Parks *et al*., 1996) and harvested 2 days later. The vector was amplified by consecutive passages on 293Cre4 cells as described previously (Parks *et al*., 1996). The presence of HDAdCMVDysFl in the cell lysate was verified by dot blot hybridization (Jani *et al*., 1997) and by *in situ* hybridization (see below) using probe specific for dystrophin or the lambda stuffer. The virus was purified by two rounds of CsCl buoyant density centrifugation as described (Hitt *et al*., 1995). The CsCl was removed by chromatography on Sephadex G25 columns (Amersham Pharmacia Biotech Inc, Piscataway, NJ) and eluted with PBS. The titer (virus particles/ml) was determined from the optical density at 260 nm (Mittereder *et al*., 1996). The contamination level by AdLC8cluc, which was 0.1% or less depending on the viral preparation, was determined by measuring the cytopatic effect after serial dilution on 293A cells (Nyberg-Hoffman *et al*., 1997). The infectious titer of HDAdCMVDysFl was measured by *in situ* hybridization (see below). The ratio of virus particles to infectious particles was 100:1. The structure of the viral DNA was confirmed by restriction analysis of purified DNA (Graham and Prevec, 1991) followed by agarose gel electrophoresis. The gel was stained using SYBR® Gold (Molecular Probes, Inc. Eugene, OR) and analyzed on a phosphoimager (STORM, Molecular Dynamics Inc).

In situ *hybridization*

In situ hybridization was accomplished using modifications of published methods (Guiot and Rahier, 1995). Briefly, eight-well Permanox® Chamber Slide™ (Nalge Nunc International, Naperville, IL) coated with 0.01% collagen (Roche Molecular Biochemicals, Laval, Quebec) were seeded with 3.0×10^4 of 293A cells. The next day, the cells were infected with 100 μl of various virus dilutions in growth medium containing helper virus (AdLC8cluc) at a multiplicity of infection (MOI) of 3 infectious particles. Helper virus was added to promote replication of viral DNA to increase the number of DNA copies and thus the signal intensity. After 1 hr, 200 μl of growth medium was added and the cells were incubated overnight at 37°C. All subsequent manipulations were accomplished at room temperature unless stated otherwise. The cells were fixed with 2% glutaraldehyde for 10 min, permeabilized with 1% Triton X-100 for 10 min, and treated with 30 μg/ml of proteinase K (Roche Molecular Biochemicals, Laval, Quebec) in PBS for 15 min at 37°C. The cells were then treated with 0.023 *N* HCl for 5 min, fixed with 2% glutaraldehyde for 10 min and incubated for 2–4 hr at 37°C with prehybridization buffer [50% (vol/vol.) formamide, 20% (wt/vol) dextran sulfate, 4 × SSC, 2% (vol/vol) Denhardt's solution, 100 ug/ml sheared herring sperm DNA]. Thirty microliters of diluted denatured probe in prehybridization buffer was added to each chamber and incubated overnight at 37°C. The probes (purified DNA fragments of dystrophin cDNA or phage lambda stuffer) were labeled with a random primer fluorescein labeling kit (NEN™ Life Science Products, Boston, MA) according to the manufacturer's recommendations. The next day, the cells were washed with 4x SSC/30% formamide, incubated with a biotin conjugated monoclonal anti-fluorescein isothiocyanate (FITC) antibody (clone FL-D6, Sigma, St. Louis, MO) followed with horseradish peroxidase conjugated to streptavidin (Vector Laboratories, Inc. Bur-

lingame, CA). The peroxidase activity was revealed by immersion in the AEC Turbo Reagent (DAKO Diagnostics, Mississauga, Ontario, Canada), and mounted in Immuno-Mount (Shandon Lipshow, Pittsburgh, PA). The infectious titer was determined by calculating the percentage of positively labeled nuclei multiplied by the dilution factor.

Western blot analysis

HeLa cells were seeded at 0.5×10^5 cells in 3-cm dishes and infected the next day with Ad vectors at various MOI in 1 ml of medium. One milliliter of medium was then added, the cells were incubated for 4 days, lysed with 150 μl of sample buffer consisting of 62 m*M* Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.02% bromophenol blue, 10 μM phenylmethylsulfonyl fluoride (PMSF), 2.5 U aprotinin/ml, 0.5 μg leupeptin/ml and boiled for 4 min. The nuclear DNA was sheared by 20 passages through a 22G1 1/2 needle. The protein concentration was determined and the samples were processed for western blot analysis as described previously (Gilbert *et al*., 1998), using a monoclonal antibody specific for the carboxyl terminus of dystrophin (NCL-DYS2, Novocastra, Newcastle upon Tyne, UK).

For infection of culture of muscle cells, C2C12 myoblasts were plated in 3-cm dishes at a density of 1.5×10^5 cells/dish. The next day, the medium was replaced with differentiation medium (DMEM supplemented with 30 μg/ml gentamicin, 20 m*M* L-glutamine, and 2% horse serum) and incubated for 4 days. At that time, well-differentiated myotubes had formed (although there were still nondifferentiated myoblasts in the culture) and the cells were infected with various MOI of Ad vectors in 1 ml of differentiation medium. It was assumed that 3.0×10^5 cell nuclei were present at the time of infection. One hour later, 1 ml of differentiation medium was added and the cells were incubated for 4 days, before being processed for western blot analysis as described above using a monoclonal antibody specific for the human dystrophin (NCL-DYS3, Novocastra, Newcastle upon Tyne, UK).

Isolation of viral DNA and quantification by dot blot analysis and by PCR

HeLa cells or C2C12 myotubes were infected as described above. At 4 days post-infection, the viral DNA was isolated (Hirt, 1967) and processed for dot blot analysis (Jani *et al*., 1997) using a ^{32}P-labeled lambda stuffer DNA fragment as a probe. The signal was visualized and quantified using a phosphoimager (STORM, Molecular Dynamics).

The quantity of vector DNA after gene transfer in the muscle was determined by semiquantitative PCR as described previously (Chen *et al*., 1999). Briefly, DNA samples were extracted from 50 cryostat sections of muscles injected with HDAdCMVDysFl, with a mixture of HDAdCMVDysFl/FGAdCMV-lacZ , or with a mixture of HDAdCMVDysFl/Ad5/δE1(β-gal)δE4. A total of 50 ng of DNA was amplified by *Taq* DNA polymerase and two primer sets. One primer set was specific for the lambda stuffer or for β-galactosidase, the other primer set was specific for the mouse adipsin gene, which is an internal control of input DNA. A standard curve was made using 50 ng of DNA of PBS-injected muscle spiked with known amount of pUCλI or pCMVβ (Clontech Laboratories, Inc., Palo Alto, CA) and amplified in parallel. The lambda primer pair, 5′-GGCGTACTGACGGATTC-3′ and 5′-CAGTTCAAGACGACGCAG-3′, was specific for a sequence between nucleotides 19,542 and 19,973 of bacteriophage lambda, giving a 432-bp PCR product (GenBank accession number J02459). The β-galactosidase primer pair, 5′-TGCTGCGTTGGAGTGAC-3′ and 5′-CAGTTCAACCACCGCAC-3′, was specific for a sequence between nucleotide 1,507 and 1,889 of the β-galactosidase gene giving a 383-bp PCR product (GenBank accession number U02451). PCR reactions were performed in 25 μl containing 1 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 8.8), 50 m*M* KCl, 0.08% Nonidet P40, 4 μM of each primer, 0.2 m*M* of each dNTP, and 1.25 units of *Taq* DNA polymerase. Amplification was carried out for 28 cycles at 94°C for 30 sec, 57°C for 1 min , 72°C for 1 min, followed by a single extension at 72°C for 10 min. The DNA was analyzed by electrophoresis on 1.8% agarose gel, stained with SYBR® Gold (Molecular Probes, Inc. Eugene, OR), and analyzed on a phosphoimager (STORM, Molecular Dynamics Inc). SYBR® Gold is a fluorescent nucleic acid stain that can be used to quantify DNA after agarose gel electrophoresis by gel scanner systems (Tuma *et al*., 1999; Even-Chen and Barenholz, 2000). The standard curve was linear over the range of DNA used in the study and all the values were normalized to the adipsin signal.

Intramuscular injection and histochemistry

All animal experiments were performed according to McGill and Murdoch universities guidelines for animal care. For experiments using mdx mice, the left and right tibialis anterior muscles (TA) of neonatal (2- to 4-day-old) or young adult (4-week-old) mdx mice (C57BL/10ScSn-mdx/J; The Jackson Laboratory, Bar Harbor, ME) were injected once at a single site with 10 or 30 μl, respectively, of Ad vectors in PBS at a titer ranging from 1.0×10^{12} to 1.5×10^{12} virus particles/ml, as described previously (Acsadi *et al*., 1996). At 10 days post-injection, the mice were euthanized by an overdose of pentobarbital, and the TA was removed and frozen in liquid nitrogen-cooled isopentane. Cryostat sections were stained for dystrophin by immunohistochemistry and for histochemical detection of β-galactosidase as described previously (Acsadi *et al*., 1994, 1996). When monoclonal antibodies (see below) were used on the mouse tissues, the endogenous mouse immunoglobulins were first blocked by incubation with an affinity purified goat anti-mouse IgG (Fab fragment, Jackson Immuno Research, Mississauga, Ontario, Canada). For each injected muscle, the total number of transduced fibers (dystrophin or β-galactosidase positive) on a single cryostat section, which spans the entire TA cross section, was then determined. For experiments using GRMD dogs, four 4-day-old dogs (2 normals, 1 carrier and 1 affected) were injected in the right TA with 100 μl of a mixture of HDAdCMVDysFl and FGAdCMV-lacZ at a titer of 1.5×10^{11} and 1.3×10^{11} virus particles/ml respectively. The left extensor carpi radialis (ECR) was injected with 100 μl of HDAdCMVDysFl alone at a titer of 3.0×10^{11} virus particles/ml. At 10 days post-injection the injected areas of the muscle were removed and consecutive cryostat sections were stained for dystrophin and counterstained with hematoxylin, or processed for detection of β-galactosidase expression. The injection, biopsies, and immunohistochemistry

were performed as described previously (Howell *et al.*, 1998). For each injected muscle site, the transduction level was determined by counting the total number of transduced fibers in the cryostat sections having the greatest amount of transgene expression. The following dystrophin antibodies were used for immunohistochemistry: a rabbit polyclonal antibody raised against the carboxyl terminus of human dystrophin that recognizes murine, canine, and human dystrophin (Acsadi *et al.*, 1996); a monoclonal antibody specific for the amino terminus of human dystrophin (NCL-DYS3, Novocastra, Newcastle upon Tyne, UK); a monoclonal antibody that recognizes the rod domain of the murine, canine and human dystrophin (NCL-DYS1, Novocastra).

Statistical analysis

The data were analyzed using an unpaired two-tailed *t*-test. Statistical significance was set at $p < 0.05$.

RESULTS

Weak dystrophin expression after gene transfer with HDAd

For the study of dystrophin expression in muscle after gene transfer with an Ad vector deleted of all viral genes, a HDAd encoding the cDNA of the full-length human dystrophin regulated by the CMV promoter was constructed and called HDAd-CMVDysFl (Fig. 1A). The only viral sequences contained in this vector are the left and right ITR, as well as the packaging signal of Ad type 5. To maintain the genome of HDAd-CMVDysFl to a minimum size that is necessary for efficient packaging into virus particles (Parks and Graham, 1997), a fragment of 17-kb derived from bacteriophage lambda (lambda DNA) was included, thus increasing the total vector size to 31 kb. HDAdCMVDysFl was produced and amplified using the 293Cre-*lox*P system as described previously (Parks *et al.*, 1996). The DNA of HDAdCMVDysFl was isolated from purified virus particles and analyzed by digestion with restriction enzymes (Fig. 2). Apart from minor DNA bands corresponding to less than 6% of the total DNA (Fig. 2B, arrowheads), the pattern of DNA fragments matches exactly the predicted structure of HDAdCMVDysFl. The exact nature of the minor contaminating bands is not clear. They could be derived from a small population of vectors generated by recombination of HDAdCMVDysFl with itself or with the helper virus, a process known to occur during the production of HDAd (Fisher *et al.*, 1996; Sandig *et al.*, 2000). The infectious titer of HDAd-CMVDysFl was measured by infecting 293A cells with various vector dilutions. At 20 hr post-infection, the percentage of cells with vector DNA was determined by *in situ* hybridization using probes specific for dystrophin (Fig. 3). As a control, cells were also infected with FGAdCMV-dys, a FGAd encoding the minidystrophin cDNA (Fig. 1B) (Acsadi *et al.*, 1996). We found that the ratio of virus particles, as determined by the optical density at 260 nm, to the infectious particles, as determined by in situ hybridization (virus particles:infectious particles) was comparable (100:1) for both vectors. Similar infectious titers for HDAdCMVDysFl were obtained using a probe specific for the lambda DNA (data not shown). Western blot analysis of a lysate of cells infected with HDAdCMVDysFl demonstrated that the dystrophin expression cassette carried by this vector was functional because a dystrophin band of 400 kDa was detected on the blots (see below, Fig. 5).

Dystrophin expression following gene transfer with HDAd-CMVDysFl in the dystrophin deficient muscle of the mdx mice was then investigated. The TA of neonatal and adult mdx mice was injected with 10 or 30 μl, respectively, of a pure preparation of HDAdCMVDysFl at a titer of 1.0×10^{12} virus particles/ml. As control, the TA of adult and neonatal mdx mice were injected with the same volume and the same titer of FGAd-CMV-dys. At 10 days post-injection, the injected muscles were stained for dystrophin and the number of dystrophin-positive fibers was determined. Unexpectedly, the number of muscle fibers expressing dystrophin in both neonatal and adult mdx mice was dramatically higher after the gene transfer with FGAdCMV-dys than in the HDAdCMVDysFl-injected muscles (Fig. 4). In addition, after gene transfer with FGAdCMV-dys, the sarcolemmal signal intensity was stronger, and several muscle fibers contained additional diffuse sarcoplasmic dystrophin

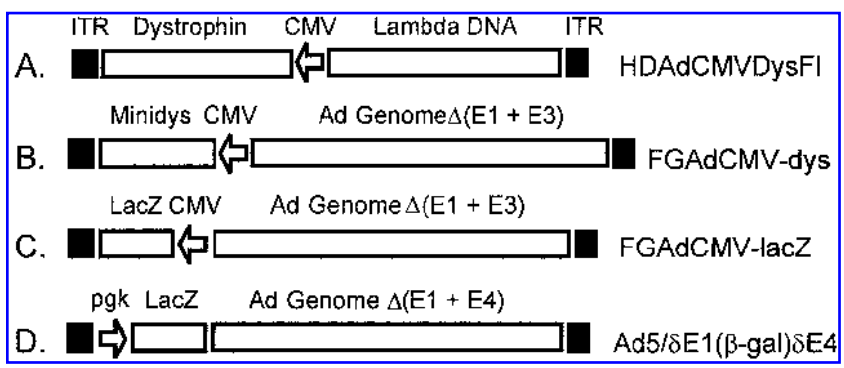

FIG. 1. Structure of the vectors used in the present study. HDAdCMVDysFl is a HDAd containing a stuffer DNA derived from bacteriophage lambda and expressing the cDNA of the full-length human dystrophin regulated by the CMV promoter. FGAd-CMV-dys and FGAdCMV-lacZ are two E1/E3-deleted Ad expressing minidystrophin (Minidys) or β-galactosidase (LacZ), respectively, both transgenes being regulated by the CMV promoter. Ad5/δE1(β-gal)δE4 is an E1/E4-deleted Ad that encodes β-galactosidase regulated by the mouse phosphoglycerate kinase (pgk) promoter. ITR, Position of the inverted terminal repeats.

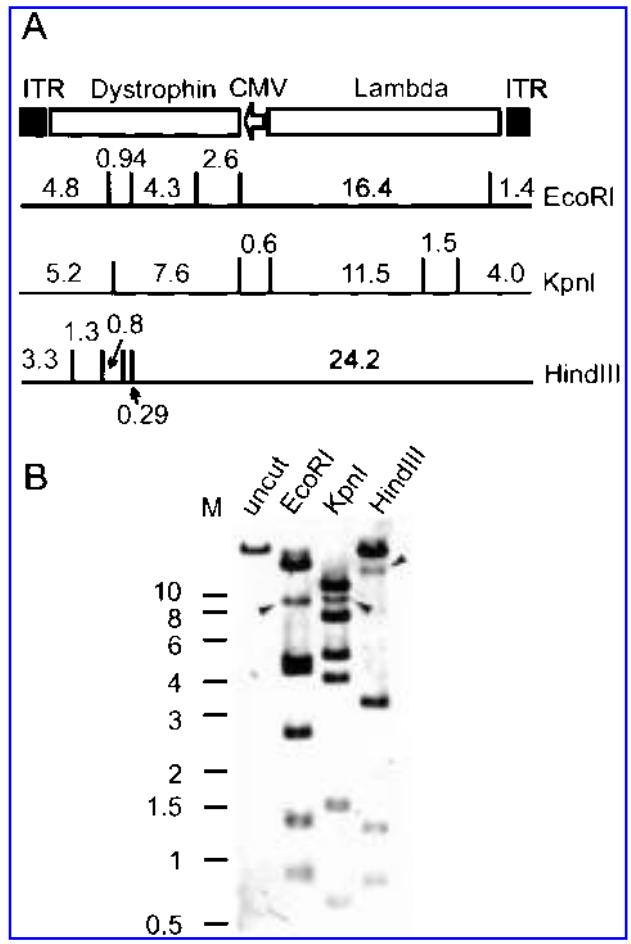

FIG. 2. Characterization of HDAdCMVDysFl by restriction analysis. (**A**) Position of the cleavage sites for *Eco* RI, *Kpn* I, and *Hin*d III on the genome of HDAdCMVDysFl. The numbers above and below the bars correspond to the size of the fragments in kb after digestion with the restriction enzymes. (**B**) Agarose gel of undigested (uncut) DNA of HDAdCMVDysFl or digested with *Eco* RI, *Kpn* I, and *Hin*d III. M, DNA size marker in kilobases; arrowheads, position of uncharacterized DNA fragments.

staining, a sign of marked dystrophin overexpression (arrows, Fig. 4A). Such intense diffuse sarcoplasmic dystrophin staining was not observed in muscles injected with HDAdCMVDysFl. The few dystrophin-positive fibers observed after injection with HDAdCMVDysFl were not revertant fibers of the mdx muscle that express dystrophin (Hoffman *et al.*, 1990), because they were stained with a monoclonal antibody that recognizes the human but not the murine dystrophin (Fig. 4).

Increased dystrophin expression in the presence of Ad gene products

The previous observations demonstrated that dystrophin was more efficiently expressed by FGAdCMV-dys, suggesting that viral gene products encoded by FGAdCMV-dys markedly augment dystrophin expression. To test this hypothesis, HeLa cells and C2C12 myotubes were infected with HDAdCMVDysFl alone or together with FGAd encoding β-galactosidase (FGAdCMV-lacZ; Fig. 1C). As a control, cells were infected with FGAdCMV-dys alone or in the presence of FGAdCMV-lacZ at the same titer. At 4 days post-infection, the cells were lysed and analyzed by western blot analysis for the expression of dystrophin. Co-infection with FGAdCMV-lacZ dramatically increased the amount of dystrophin produced by HDAdCMVDysFl in HeLa cells and C2C12 myotubes (Fig. 5). This observation suggests that one or more gene product(s) synthesized by FGAdCMV-lacZ could enhance the transgene expression of an Ad devoid of all viral genes such as HDAdCMVDysFl.

To determine if an increase in the number of DNA templates was responsible for the enhanced dystrophin expression in the presence of FGAdCMV-lacZ, low-molecular-weight DNA was isolated from HeLa cells and C2C12 myotubes that were previously infected 4 days earlier with only HDAdCMVDysFl, or with HDAdCMVDysFl and FGAdCMV-lacZ. The relative abundance of vector DNA was then determined by dot blot analysis using a radio-labeled probe specific for the lambda stuffer followed by quantification on a phosphoimager (Table 1). Significantly more HDAdCMVDysFl DNA was detected in HeLa cells after co-infection with FGAdCMV-lacZ, whereas no augmentation occurred in C2C12 myotubes. This data indicate that the enhanced dystrophin expression observed in C2C12 my-

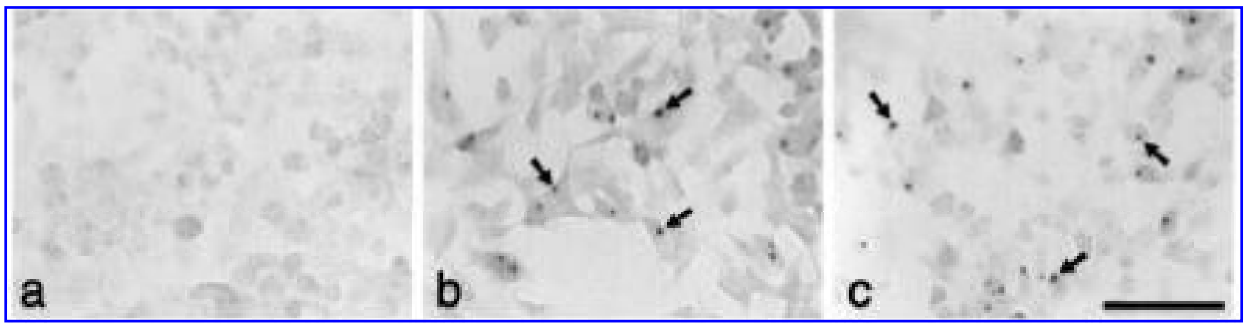

FIG. 3. Titration of Ad vectors by *in situ* hybridization. Mock-infected cells (**a**) or cells infected with FGAdCMV-dys (**b**) or HDAdCMVDysFl (**c**) were processed for *in situ* hybridization using a probe specific for dystrophin. Arrows, Examples of nuclei labeled with viral DNA. Scale bar = 85 μm.

otubes in the presence of FGAdCMV-lacZ is not due to an increase in the number of dystrophin DNA templates but is the result of either enhanced transcription, or RNA transport, or other post-transcriptional mechanisms. The fact that significantly more DNA copies were observed in HeLa cells in the presence of FGAdCMV-lacZ is in agreement with previous studies that demonstrated some replication of FGAd in this cell type because of the existence of E1-like factors. (Dedieu *et al.*, 1997; Steinwaerder *et al.*, 2000). In addition, the previous data would suggest that no significant replication of FGAd occurs in post-mitotic culture of C2C12 myotubes. In the latter cell line, slightly less DNA was detected in the presence of FGAdCMV-lacZ, most likely because of the competition between FGAdCMV-lacZ and HDAdCMVDysFl for limited number of receptors at the cell surface. In HeLa cells slightly more dystrophin were produced by FGAdCMV-dys in the presence of FGAdCMV-lacZ (Fig. 5A). This is probably due to the fact that more efficient DNA replication occurs in HeLa cells at higher MOI (Steinwaerder *et al.*, 2000), resulting in a proportionally increase of DNA templates for dystrophin synthesis.

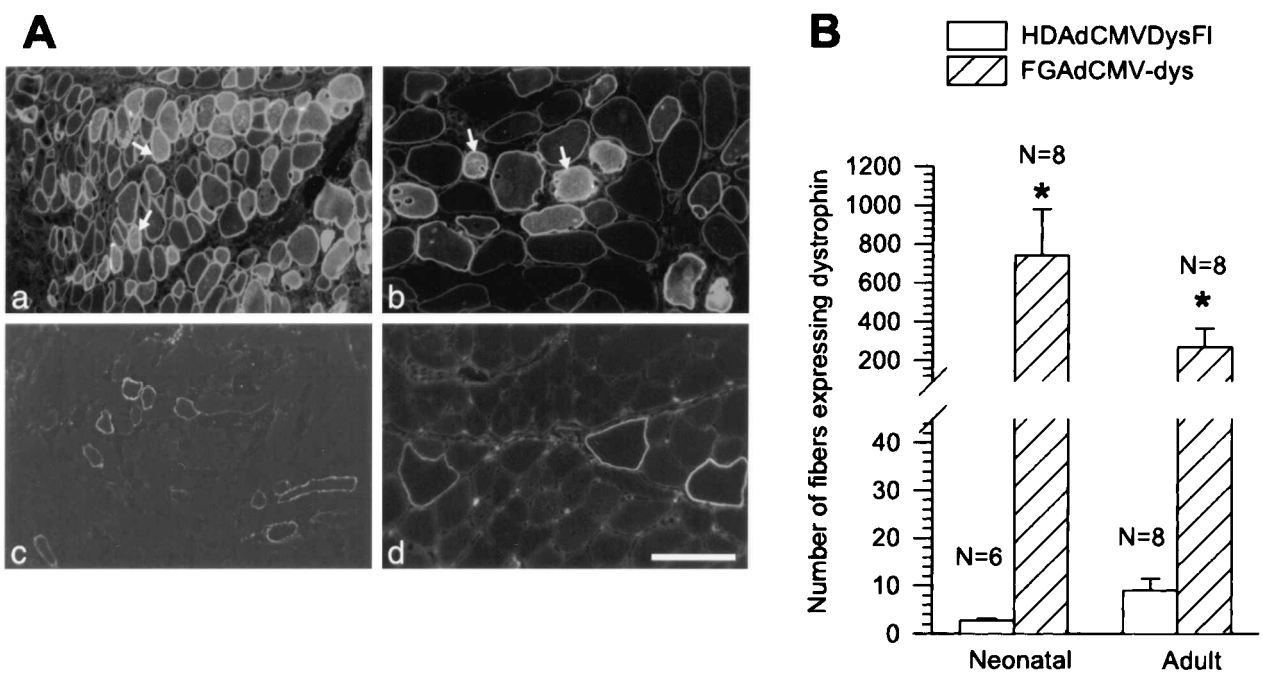

FIG. 4. Higher dystrophin expression *in vivo* after gene transfer with FGAdCMV-dys compared to HDAdCMVDysFl. The TA of neonatal and adult mdx mice was injected with 1.0×10^{12} virus particles/ml of either FGAdCMV-dys or HDAdCMVDysFl. At 10 days post-injection, the expression of dystrophin was analyzed by immunofluorescence using a monoclonal antibody specific for the human dystrophin. (**A**) Cryostat sections of neonatal (**a**,**c**) and adult (**b**,**d**) muscles injected with FGAdCMV-dys (**a**,**b**) or HDAdCMVDysFl (**c**,**d**). Arrows, Muscle fibers with an intense cytoplasmic positive signal (in addition to show sarcolemmal immunostaining) demonstrating marked dystrophin overexpression. Scale bar = 100 μm. (**B**) Quantitative analysis of the number of dystrophin-positive fibers. Bars correspond to the mean ± SEM. N, Number of muscles used for the analysis; *, means significantly different, $p < 0.05$.

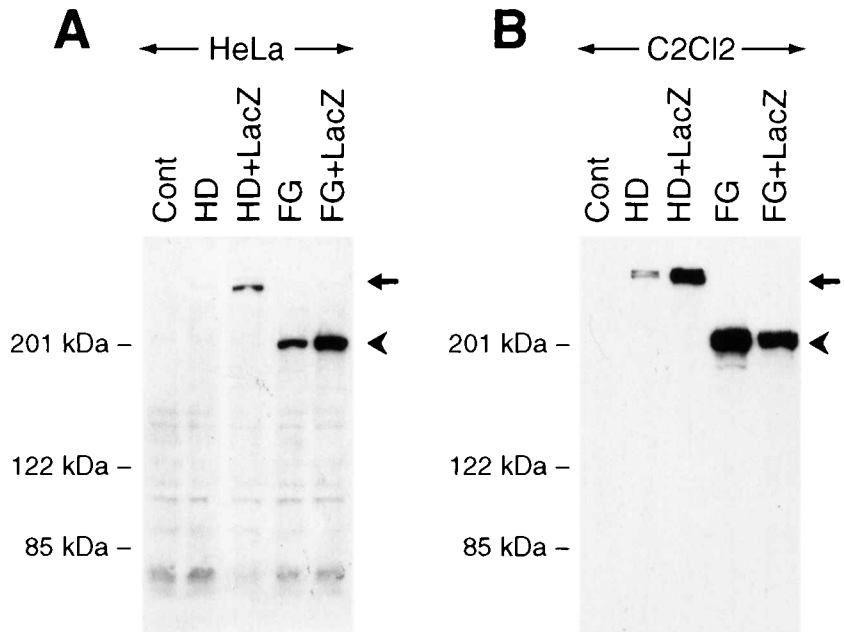

FIG. 5. Ad gene products increase dystrophin expression of HDAdCMVDysFl in cell culture. HeLa cells (**A**) or C2C12 myotubes (**B**) were infected with HDAdCMVDysFl (HD), HDAdCMVDysFl and FGAdCMV-lacZ (HD+LacZ), FGAdCMV-dys (FG), or FGAdCMV-dys and FGAdCMV-lacZ (FG+LacZ). In HeLa cells, the MOI was 2,000 virus particles for HDAdCMVDysFl and FGAdCMV-dys, and 10,000 virus particles for FGAdCMV-lacZ. In C2C12, the MOI was the same (10,000 virus particles) for each vector. At 4 days post-infection, the cells were lysed, and 15 (HeLa) or 30 μg (C2C12) of total protein was analyzed by western blot using a dystrophin antibody. Cont, Mock-infected cells; arrow, full-length dystrophin; arrowhead, minidystrophin. The position of molecular weight marker is indicated on the left of each gel.

Having shown that Ad gene products could increase dystrophin expression of HDAdCMVDysFl *in vitro*, we then investigated if the same applies *in vivo*. The TA muscle of neonatal mdx mice was injected with the same concentration of HDAdCMVDysFl (1.0×10^{12} virus particles/ml) spikes with increased concentration of FGAdCMV-lacZ. At 10 days post-injection, serial sections of the injected muscles were analyzed by histochemistry for dystrophin and β-galactosidase expression and the number of dystrophin positive fibers was determined (Fig. 6). In agreement with the observation made *in vitro*, co-infection with FGAdCMV-lacZ resulted in a significant augmentation in the number of dystrophin-positive fibers. The level of enhancement was proportional to the amount of FGAdCMV-lacZ in the preparation and no significant increase was apparent when less than 5% of FGAdCMV-lacZ was present (Fig. 6B). In some muscles, at the highest concentration of FGAdCMV-lacZ employed, more than 50% of the muscle fibers were dystrophin positive and several fibers had, in addition to the

TABLE 1. AD GENE PRODUCTS INCREASE THE AMOUNT OF HDADCMVDYSFL IN HELA CELLS BUT NOT IN C2C12 MYOTUBES[a]

	Amount of HDAdCMVDysFl DNA (arbitrary units)	
Vector tested	*HeLa cells (mean ± SEM,* n = *3)*	*C2C12 myotubes (mean ± SEM,* n = *3)*
HDAdCMVDysFl	3.1 ± 0.06	7.8 ± 0.39
HDAdCMVDysFl + FGAdCMV-lacZ	593 ± 143[b]	5.3 ± 0.30[b]

[a]HeLa cells or C2C12 myotubes were infected with HDAdCMVDysFl alone or mixed with FGAdCMV-lacZ. In HeLa cells, the MOI was 20,000 and 10,000 virus particles for HDAdCMVDysFl and FGAdCMV-lacZ, respectively. In C2C12 myotubes, the MOI was 10,000 virus particles for both vectors. At 4 days post-infection the small nuclear DNA was extracted and quantified by dot blot analysis using a probe specific for the lambda stuffer of HDAdCMVDysFl.

[b]Means different from cells infected with HDAdCMVDysFl alone, $p < 0.05$.

strong sarcolemmal dystrophin signal, a diffuse cytoplasmic internal staining, a sign of marked dystrophin overexpression (arrows Fig. 6A).

To verify that the comparatively lower dystrophin expression in the muscle injected with pure preparation of HDAdCMVDysFl was not due the absence of vector DNA, total DNA was extracted from muscles injected with pure preparation of HDAdCMVDysFl and with preparation containing 60% of FGAdCMV-lacZ. The amount of HDAdCMVDysFl DNA was measured by semiquantitative PCR using primers specific for the lambda DNA stuffer as described previously (Chen *et al.*, 1999). To confirm that the same amount of DNA was used for the analysis, the unique mouse adipsin gene was amplified simultaneously and all values were normalized to the adipsin signal (Fig. 7A). The muscles injected with pure preparations of HDAdCMVDysFl contained half the amount of vector DNA compared to the muscles spiked with 60% FGAdCMV-lacZ (Fig. 7B). This two-fold difference in the amount of vector DNA between the two muscle groups is likely to be a negligible factor responsible for the enhanced dystrophin expression in the presence of FGAdCMV-lacZ, since the increase in the number of dystrophin positive fiber was more than one order of magnitude (Fig. 6B).

E4 is required to enhance dystrophin expression

The early region four (E4) of Ad is known to modulate the transgene expression carried by Ad *in vitro* and *in vivo* (Armentano *et al.*, 1997; Brough *et al.*, 1997; Dedieu *et al.*, 1997; Lusky *et al.*, 1998). To test if E4 gene products were involved in enhancing the transgene expression of HDAdCMVDysFl, cultures of HeLa cells and C2C12 myotubes were infected with HDAdCMVDysFl spiked with various quantities of an E1/E4-deleted Ad encoding β-galactosidase (Ad5/δE1(β-gal)δE4, Fig. 1D). As a positive control, cells were also infected with mixtures of HDAdCMVDysFl and FGAdCMV-lacZ. At 4 days post-infection, the cultures were processed by western blots for dystrophin expression (Fig. 8). Increased dystrophin expression

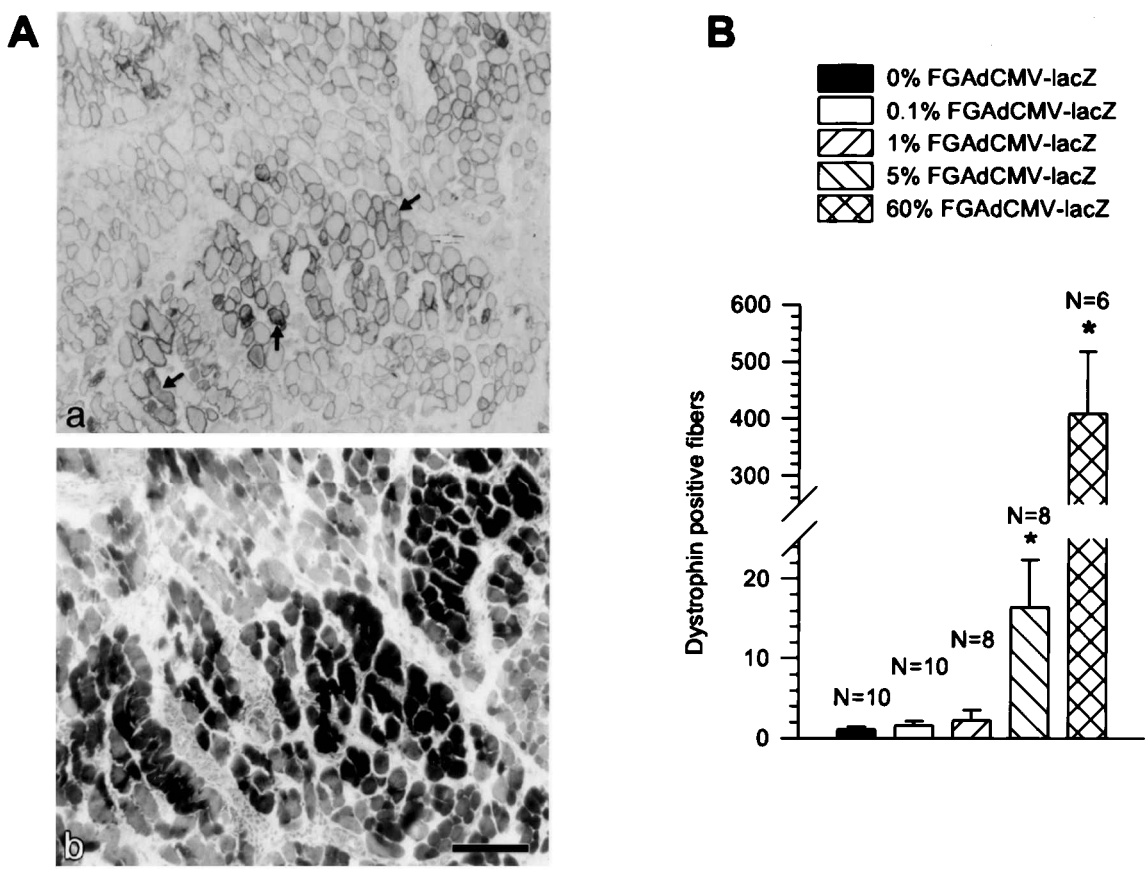

FIG. 6. Ad gene products increase dystrophin expression of HDAdCMVDysFl in mdx muscle. The TA of neonatal mdx mice was injected with various preparations of HDAdCMVDysFl at a titer of $1.0. \times 10^{12}$ virus particles/ml spiked with 0, 0.1, 1, 5, or 60% of virus particles of FGAdCMV-lacZ. At 10 days post-injection the muscles were processed by histochemistry to show dystrophin and β-galactosidase. (**A**) Serial sections of a muscle injected with HDAdCMVDysFl containing 60% FGAdCMV-lacZ stained with an antibody specific for dystrophin (**a**) or processed for β-galactosidase histochemistry (**b**). Arrows, Muscle fibers having an intense cytoplasmic dystrophin staining, a sign of marked dystrophin overexpression. Scale bar = 125 μm. (**B**) Quantitative analysis of the number of dystrophin-positive fibers in each muscle group. Bars correspond to the mean ± SEM. N, Number of muscles used for the analysis in each group; *, mean significantly higher compared to pure preparation of HDAdCMVDysFl, $p < 0.05$.

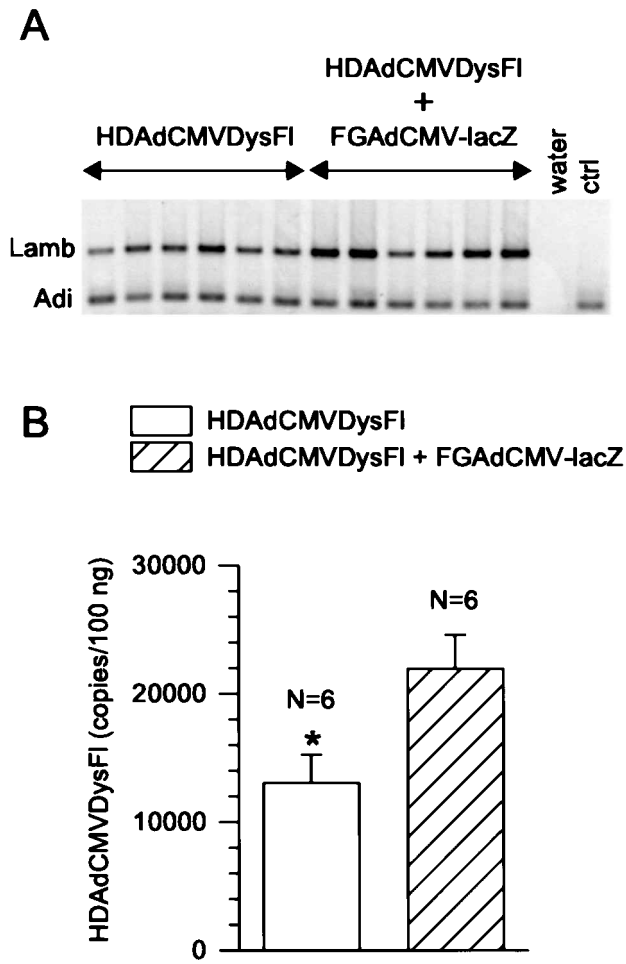

FIG. 7. Lower transgene expression by HDAdCMVDysFl in muscle is not due to loss of vector DNA. Total DNA was isolated from cryostat sections of muscles injected with HDAdCMVDysFl (1.0×10^{12} virus particles/ml) or with the same titer of HDAdCMVDysFl containing 60% of FGAdCMV-lacZ. The same quantity of total DNA from six different muscles in each group was amplified by PCR using primers specific for the lambda DNA stuffer (Lamb) or for the adipsin gene (Adi). (**A**) Agarose gel of the PCR products. Ctrl, PCR reaction using DNA of uninjected muscle; water, PCR reaction without DNA. (**B**) Quantification of the PCR products using primers specific for lambda. Bars correspond to the mean ± SEM. N, Number of muscles used for the analysis; *, mean significantly different, $p < 0.05$.

was detected in the presence of FGAdCMV-lacZ but not in the presence of Ad5/δE1(β-gal)δE4 in both cell types investigated even at the highest MOI tested. This observation demonstrated that E4 is required to enhance dystrophin expression, and that ORF4, the only E4 gene product synthesized by Ad5/δE1(β-gal)δE4 (Wang *et al.*, 1995, 1997) was insufficient for this function.

We then tested if E4 products were also required for enhancing the transgene expression of HDAdCMVDysFL *in vivo*. The TA muscle of neonatal mdx mice was injected with either HDAdCMVDysFl alone or with a mixture of HDAdCMVDysFl and Ad5/δE1(β-gal)δE4. At 10 days post-injection, the number of dystrophin- and β-galactosidase-positive fibers was determined. The amount of viral DNA in the injected muscle was also measured by semiquantitative PCR using primer pairs specific for the lambda stuffer of HDAdCMVDysFl or for the *lacZ* gene carried by Ad5/δE1(β-gal)δE4. In agreement with the observations made in cell culture, the presence of Ad5/δE1(β-gal)δE4 did not increase the expression of dystrophin by HDAdCMVDysFl because the same number of dystrophin-positive fibers was detected in the two experimental groups (Table 2). In addition, no difference in the amount of HDAdCMVDysFl DNA was detected between the two groups. The incapacity of Ad5/δE1(β-gal)δE4 to increase dystrophin expression was not due to the lack of vector in the muscle, because β-galactosidase-positive fibers were detected as well as a significant quantity of vector DNA (Table 2).

Ad gene products enhance dystrophin expression from HDAdCMVDysFl in GRMD dogs

Having demonstrated that co-infection with a FGAd increases the expression of dystrophin carried by a HDAd in the muscles of mdx mice, we then investigated if the same result occurs in the muscle of another animal model for DMD, the dystrophin-deficient GRMD dogs. The absence of dystrophin

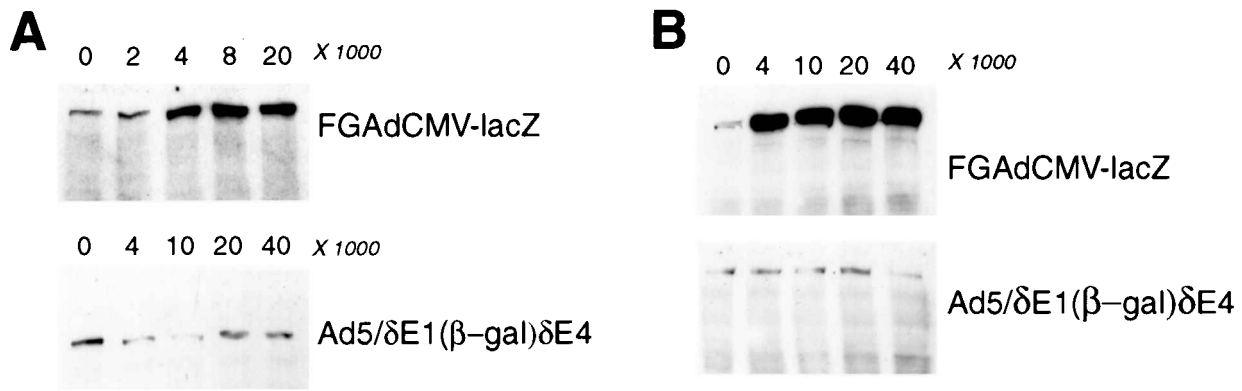

FIG. 8. E4 gene products are essential to increase dystrophin expression from HDAdCMVDysFl in cell culture. HeLa cells (**A**) or C2C12 myotubes (**B**) were infected with HDAdCMVDysFl at an MOI of 10,000 virus particles spiked with various amounts of FGAdCMV-lacZ, or Ad5/δE1(β-gal)δE4 at the indicated MOI in virus particles. At 4 days post-infection, the cells were processed for western blot using a dystrophin-specific antibody.

in this animal model, unlike the mdx mice, leads to a clinicopathological phenotype similar to that of DMD (Valentine *et al.*, 1992). The left extensor carpi radialis (ECR) of a 4-day-old dystrophin deficient GRMD dog was injected with 100 μl of HDAdCMVDysFl and the right TA with 100 μl of a mixture of HDAdCMVDysFl spiked with FGAdCMV-lacZ. At 10 days post-injection, biopsies of the injected sites were taken and analyzed for dystrophin and β-galactosidase expression. The maximal number of dystrophin positive fibers at the injection site was 46 in the muscle injected with only HDAdCMVDysFl, whereas it was 1,101 in the muscle injected with the mixture of the two vectors (Fig. 9). In the latter group, the area containing the dystrophin-positive fibers was also positive for β-galactosidase (Fig. 9C). When the same experiment was carried out in normal and carrier dogs (both animals express dystrophin), muscle fibers expressing the human recombinant dystrophin (total was 39) were detected only in the muscles injected with the mixture of HDAdCMVDysFl and FGAdCMV-lacZ. In the last experiment, the human dystrophin protein was distinguished from the endogenous canine dystrophin by staining the sections with a human-specific antibody (DYS3). In our hands this antibody was less efficient for detecting the transduced fibers in comparison to DYS1, the antibody used to stain the affected dogs. In addition, the transduced fibers in the normal and carrier dogs could have been underestimated because of a competition between the endogenous and recombinant dystrophin for limited binding sites at the sarcolemma. In other words, only the very highly transduced fibers could be detected by immunohistochemistry, because the dystrophin binding sites were already occupied by the endogenous dystrophin. Taken together, these data demonstrate that gene products encoded by FGAd could significantly increase the expression of dystrophin carried by a HDAd in mdx mice as well as the GRMD dog, two animal models of DMD.

DISCUSSION

To be effective as treatment for DMD or other genetic diseases, a therapy based on a gene transfer strategy requires ef-

TABLE 2. E4 IS REQUIRED TO INCREASE DYSTROPHIN EXPRESSION OF HDADCMVDYSFL *IN VIVO*

Vectors tested[a]	*Number of Dys^+ fibers per muscle (mean ± SEM)*	*Number of β-gal^+ fibers per muscle (mean ± SEM)*	*Number of copies of HDAdCMCVDysFl per 100 ng total DNA*[b] *(mean ± SEM)*	*Number of copies of Ad5/δE1(β-gal)δE4 per 100 ng total DNA*[c] *(mean ± SEM)*
HDAdCMVDysFl	54.5 ± 31.6 (n = 12)[d]	Not applicable	7754 ± 1247 (n = 5)	Not applicable
HDAdCMVDysFl + Ad5/δE1(β-gal)δE4	37.8.2 ± 19.4 (n = 12)	60.2 ± 40.4 (n = 12)	7649 ± 1744 (n = 5)	3035 ± 865.6 (n = 5)

[a]The TA of neonatal mdx mice was injected with pure HDAdCMVDysFl at a titer of 1.5×10^{12} virus particles/ml or with a mixture of HDAdCMVDysFl and Ad5/δE1(β-gal)δE4 at the a titer of 1.5×10^{12} and 1.1×10^{12} virus particles/ml, respectively. The injected muscles were analyzed at 10 days post-injection.

[b]As determined by semiquantitative PCR using primers specific for the lambda stuffer.

[c]As determined by semiquantitative PCR using primers specific for β-galactosidase.

[d]Number of muscles used for the analysis.

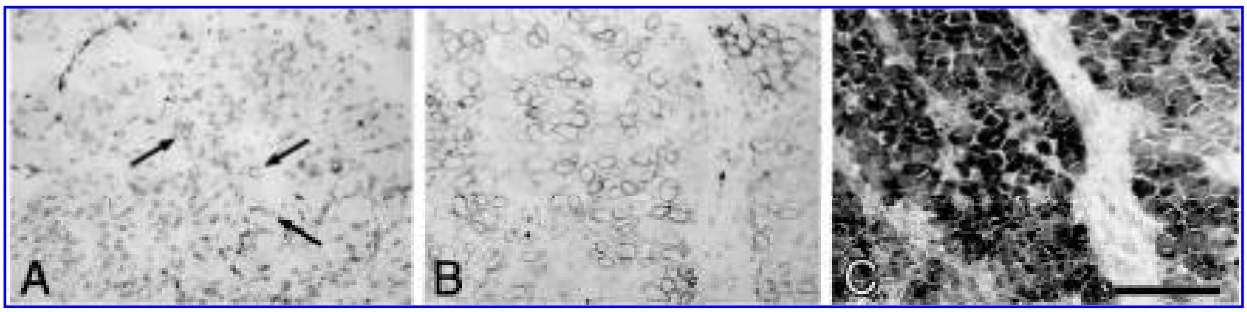

FIG. 9. Ad gene products increase dystrophin expression of HDAdCMVDysFl in muscle of GRMD dog. The left ECR of a 4-day-old affected GRMD dog was injected with HDAdCMVDysFl (3.0×10^{11} virus particles/ml) or the right TA with a mixture of HDAdCMVDysFl and FGAdCMV-lacZ at a titer of 1.5×10^{11} and 1.3×10^{11} virus particles/ml, respectively. At 10 days post-injection, cryostat sections of the injected muscle sites were processed for dystrophin (**A**,**B**) and β-galactosidase (**C**) expression. (**A**) Section of a muscle injected with only HDAdCMVDysFl showing few dystrophin positive fibers (arrows). (**B**) Section of a muscle injected with a mixture of HDAdCMVDysFl and FGAdCMV-lacZ and demonstrating abundant dystrophin positive fibers. (**C**) Section of the same region as **B** demonstrating abundant β-galactosidase expression. Scale bar = 160 μm.

ficient and high-level prolonged transgene expression. Efficient, but temporary, transgene expression has been achieved after gene transfer with FGAd in many tissues of the body, including muscle. The transgene antigenicity and the immune response triggered by low-grade synthesis of Ad gene products have been both demonstrated to be responsible for the transient nature of the transgene expression (Yang *et al.*, 1994a,b; Tripathy *et al.*, 1996; Yang *et al.*, 1996; Michou *et al.*, 1997). In an attempt to solve the problem caused by the low grade viral synthesis, Ad vectors deleted of all viral genes have been developed. The use of these vectors has resulted in as significant improvement in the duration of the transgene expression in various tissues including muscle (Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Chen *et al.*, 1999; Maione *et al.*, 2000; Zou *et al.*, 2000). In the present study, we have compared dystrophin expression after gene transfer with a FGAd (FGAdCMV-dys) and a fully deleted HDAd (HDAdCMVDysFl) carrying the full dystrophin cDNA. Although dystrophin was regulated by the same CMV promoter, significantly more dystrophin was produced after gene transfer with FGAdCMV-dys both *in vitro* and *in vivo*. We then demonstrated that the comparatively higher transgene expression bestowed by FGAdCMV-dys was due to the presence of Ad gene products that could act *in trans* to enhance the transgene expression of HDAdCMVDysFl in cell culture and in the skeletal muscle of mdx mice and GRMD dogs, two animal models for DMD. This ruled out that a defective vector or a defective expression cassette was responsible for the weaker dystrophin expression observed after gene transfer with HDAdCMVDysFl.

Our data are in agreement with the observation made by several groups that a significant reduction in transgene expression occurs after gene transfer with E1/E4-deleted Ad compared to FGAd (Armentano *et al.*, 1997; Brough *et al.*, 1997; Dedieu *et al.*, 1997; Lusky *et al.*, 1998, 1999; Armentano *et al.*, 1999; Grave *et al.*, 2000). A significant loss of transgene expression was also described after gene transfer with a partly deleted mini-Ad in the liver (Lieber *et al.*, 1996,1997). However, it is unclear why similar reduction in transgene expression was not described by many researchers who have compared HDAd and FGAd in various tissues (Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Chen *et al.*, 1999; Maione *et al.*, 2000; Zou *et al.*, 2000). It is possible that the requirement for Ad gene products to augment the transgene expression in HDAd vectors is tissue specific. The nature of the stuffer carried by the HDAd may have also reduced the transgene expression (Parks *et al.*, 1999) (see below). It is also possible that the Ad gene products exert their action mainly when the transgene is regulated by ubiquitous cellular promoters or strong viral promoters such as CMV (Grave *et al.*, 2000). In this respect, a significant increase of dystrophin expression by a fully deleted HDAd regulated by the hybrid CMV enhancer/β-actin promoter, without lambda stuffer, was observed when co-injected with a FGAd in mdx muscle (data not shown). This indicates that other promoters besides the CMV promoter could be up-regulated by *trans*-acting Ad gene products. Experiments are currently in progress to determine if the up-regulation will occur when tissue-specific eukaryotic promoters, such as the muscle creatine kinase promoter, are employed.

An all-important question is the molecular mechanism(s) by which the Ad gene product(s) augment markedly the transgene expression in the context of a HD ("fully gutted") Ad. We observed that co-administration of an E1/E4-deleted Ad (Ad5/δE1(β-gal)δE4) was unable to increase dystrophin expression of HDAdCMVDysFl in cell culture and in muscle *in vivo*. The E4 region contains seven potential open reading frames encoding proteins of various biochemical functions (for review, see Leppard, 1997). One of them, ORF4, had no effect on the transgene expression, because it is encoded by Ad5/δE1(β-gal)δE4, which did not increase the expression of dystrophin by HDAdCMVDysFl *in vitro* and *in vivo*. The mechanism by which E4 gene products enhance the transgene expression is not clear, and several nonexclusive mechanisms are possible. For example, in the lung, E4 gene product (ORF3) has been suggested to stabilize the transgene expression by inactivating a transcriptional repressor of the CMV promoter (Armentano *et al.*, 1999; Yew *et al.*, 1999). In cell culture, a reduction in the initiation of transcription rate of the transgene has been reported in the absence of E4 (Lusky *et al.*, 1999; Grave *et al.*, 2000). ORF6 and ORF3 are known to increase late viral gene expression by favoring the accumulation of late vi-

ral RNA and their transport to the cytoplasm (for review, see Imperiale *et al.*, 1995). Deletion of E4 in the backbone of a FGAd leads to a reduction of early and late protein synthesis (Dedieu *et al.*, 1997; Lusky *et al.*, 1998). Consequently, the weaker transgene expression in the absence of E4 could be due to the secondary absence of early and late adenoviral gene products.

The presence of FGAdCMV-lacZ in C2C12 did not increase the amount of vector DNA. This indicates that the low level of viral replication or stabilization of the vector DNA were not the mechanisms by which dystrophin expression of HDAdCMVDysFl was enhanced. After gene transfer in mdx muscle, about two times more HDAdCMVDysFl DNA was detected in the presence of FGAdCMV-lacZ. The fact that the number of dystrophin-positive fibers was more than one order of magnitude higher in the presence of FGAdCMV-lacZ, indicates that this small increase in DNA templates was not a significant mechanism by which enhanced dystrophin expression was achieved. The augmentation of vector DNA in the presence of FGAdCMV-lacZ could be due to low viral replication level or to the stabilization of vector DNA. Further studies will be required to resolve these issues because both processes have been described *in vivo* (Lieber *et al.*, 1996, 1997). During the course of evolution, it is likely that the naturally occurring Ad has developed complementary mechanisms to optimize the expression of its episomal genome.

The nature of the stuffer has been shown to modulate the transgene expression of a HDAd (Parks *et al.*, 1999). A more robust transgene expression was observed after gene transfer in the liver with a HDAd made with a stuffer DNA derived from a genomic fragment of hypoxanthine-guanine phosphoribosyltransferase (HPRT) in comparison to a stuffer derived from phage lambda. This suggests that some uncharacterized properties of this stuffer could compensate for the absence of adenoviral gene products. Thus, we could speculate that the inclusion of an improved stuffer, in conjunction with certain adenoviral genes, could further improve the efficacy of the transgene expression of HDAd. However we have to be cautious because inclusion of Ad genes in the HDAd vector could result in the reemergence of some deleterious immunogenicity. This could be partly or totally controlled if the viral genes were regulated by weak or muscle specific promoters, or by inducible promoters for transient expression when needed. The experiments of this study were accomplished using young animals whose muscles are transduced more efficiently than adult muscles (Huard *et al.*, 1995). Even with an optimized HDAd, it might be difficult to transduce mature muscle fibers. However, several parameters such as the tropism of the vector (Bouri *et al.*, 1999), the route of administration (Cho *et al.*, 2000), or the quantity of Ad receptors on the muscle fibers (Nalbantoglu *et al.*, 2001), could be modified to ameliorate the transduction level in adult animals.

In conclusion, we have demonstrated that Ad gene products can significantly increase the transgene expression carried by a HDAd. This observation was made in cell culture and in the muscle of mdx mice and GRMD dogs, two animal models for DMD. The characterization of these adenoviral gene products, followed by their inclusion into an optimized HDAd, might be necessary to generate a vector capable of synthesizing enough dystrophin to correct or mitigate the pathology associated with dystrophin deficiencies.

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