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Intraarterial Delivery of Naked Plasmid DNA Expressing Full-Length Mouse Dystrophin in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

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ABSTRACT

Our previous studies have demonstrated that the intraarterial delivery of naked plasmid DNA leads to high levels of foreign gene expression throughout the muscles of the targeted limb. Although the procedure was first developed in rats and then extended to nonhuman primates, the present study has successfully implemented the procedure in normal mice and the *mdx* mouse model for Duchenne muscular dystrophy. After intraarterial delivery of plasmid DNA expressing the normal, full-length mouse dystrophin from either the cytomegalovirus promoter or a muscle-specific human desmin gene control region, *mdx* mouse muscle stably expressed dystrophin in 1–5% of the myofibers of the injected hind limb for at least 6 months. This expression generated an antibody response but no apparent cellular response.

OVERVIEW SUMMARY

Intravascular administration of naked plasmid DNA could be used to deliver the normal human dystrophin gene to the peripheral limbs of patients with Duchenne muscular dystrophy so as to preserve limb function. Preventing the loss of limb muscles would help maintain quality of life, such as the use of hands for many self-care and communication (i.e., computer) skills. To evaluate this hypothesis in animal models, a criterion for human trials, the present study sought to apply the intravascular naked plasmid DNA approach to the mouse *mdx* model for Duchenne muscular dystrophy. The major question that this study addressed was whether stable expression of full-length, normal mouse dystrophin could be achieved in the *mdx* model by this delivery approach.

INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD) is an important candidate for gene therapy because it is a common, life-threatening disease and one-third of all cases arise *de novo* without carrier status in the mother, limiting the value of population-based screening (Davies, 1997; Biggar *et al*., 2002). Newborn screening is possible so as to enable early intervention (van Ommen and Scheuerbrandt, 1993; Parsons *et al*., 2002, 2003). The molecular and cellular rationale for gene therapy has been provided by transgenic studies in the *mdx* mouse model for DMD, in which prenatal expression of full-length dystrophin was ameliorative (Cox *et al*., 1993; Lee *et al*., 1993; Phelps *et al*., 1995; Wells *et al*., 1995). Using an inducible expression system in transgenic mice, postnatal expression of full-length mouse dystrophin also attenuated *mdx* mouse muscle

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pathology (Ahmad *et al*., 2000). Postnatal gene transfer studies using adenoviral, adeno-associated, and other types of viral vectors have found that exogenous dystrophin expression is sufficient to prevent muscle dystrophy (Wang *et al*., 2000; Dickson *et al*., 2002; Scott *et al*., 2002; Watchko *et al*., 2002; Wells and Wells, 2002). Loss of muscle fibers is also prevented by naked dystrophin DNA transfer (Acsadi *et al*., 1991; Danko *et al*., 1993). Although these studies have established proof of principle, gene therapy for DMD is particularly challenging because the large dystrophin gene must be delivered to at least 10–20% of the muscle cells in many muscle groups throughout the body (Phelps *et al*., 1995; Davies, 1997). Various viral and nonviral methods for gene delivery to muscle are being developed (Wells and Wells, 2002; Lu *et al*., 2003).

Our previous studies have indicated that the intravascular injection of naked plasmid DNA (pDNA) into limb arteries of rats and nonhuman primates (rhesus monkeys) leads to high levels of foreign gene expression in skeletal muscle throughout the limb, with only minimal muscle damage (Budker *et al*., 1998; Zhang *et al*., 2001). Other studies have used an intravascular approach to deliver adenoviral and adeno-associated viral vectors (Greelish *et al*., 1999). It is our hypothesis that intravascular delivery of naked pDNA could be used to deliver the normal human dystrophin gene to the peripheral limbs of patients with Duchenne muscular dystrophy so as to preserve limb function. Preventing the loss of limb muscles would help maintain quality of life, such as the use of hands, for many self-care and communication (i.e., computer) skills. To evaluate this hypothesis in animal models, a criterion for human trials, the present study sought to apply the intravascular naked pDNA approach to the mouse *mdx* model for Duchenne muscular dystrophy.

Previously, we have been unable to perform the intravascular procedure effectively in mice. This is contrary to the usual predicament for a gene therapy approach, wherein it is difficult to extend a procedure from small to large animal models (Jiao *et al*., 1992). In our current study, we have in fact been able to develop the intravascular delivery procedure in *mdx* mice. Although the efficiency is less than what we can achieve in larger animals, its use in the mouse model has enabled us to address two important scientific questions relevant to the procedure in humans: (1) can a large pDNA vector expressing the full-length dystrophin be efficiently delivered via the intravascular route to dystrophic muscle? The effect of pDNA size on expression was also evaluated with luciferase expression vectors; and (2) can long-term expression of normal, full-length mouse dystrophin be achieved in the *mdx* mouse model? A previous study reported expression of full-length mouse dystrophin in the diaphragm muscle of *mdx* mice after naked pDNA expression vector was injected under low pressure into the tail vein and the inferior vena cava was briefly clamped (Liu *et al*., 2001).

Concerning long-term expression, pDNA persists in myofibers in a nonintegrated form once it has entered their postmitotic nuclei (Wolff *et al*., 1992; Danko *et al*., 1993). The rapid shutdown of promoters within pDNA, a phenomenon that occurs after the intravascular delivery of pDNA to the liver, does not appear to occur in muscle (Herweijer *et al*., 2001; Zhang *et al*., 2001; Herweijer and Wolff, 2003; Karpati *et al*., 2003). After direct intramuscular injection of pDNA, persistent expression of low levels of luciferase is possible (Wolff *et al*., 1992). Yet, with the high levels of expression achieved after intravascular delivery of pDNA, long-term expression of reporter genes such as luciferase requires that the animal be immunosuppressed (Zhang *et al*., 2001). Presumably, the higher levels of expression lead to a cellular immune response against the cells expressing the transfected gene. If a muscle-specific promoter is used, long-term expression may require immunosuppression to be only transiently applied at about the time of pDNA administration (Zhang *et al*., 2001). Similar effects of transient immunosuppression or muscle-specific promoters on the proclivity of an immune response have also been observed with viral vectors (Lochmuller *et al*., 1996; Jooss *et al*., 1998; Cordier *et al*., 2001; Yuasa *et al*., 2002).

Previous studies have explored the immune consequences of dystrophin expression resulting from direct, intramuscular injection of naked DNA into mice and humans (Braun *et al*., 2000; Ferrer *et al*., 2000; Romero *et al*., 2002; Thioudellet *et al*., 2002). A previous study by one of us (S.B.) found that the direct, intramuscular injection of a full-length, human dystrophin–cytomegalovirus (CMV) promoter construct into notexin-treated muscles of *mdx* mice led to transient dystrophin expression (Braun *et al*., 2000). This was accompanied by anti-human dystrophin antibodies and a transient myositis indicative of a cellular response. Another study also found that multiple injections into untreated *mdx* muscles of constructs expressing full-length human dystrophin from the CMV promoter led to anti-human dystrophin antibodies and accumulations of CD8-positive cells around dystrophin-positive myofibers (Ferrer *et al*., 2000). A similar immune response was also observed with constructs expressing a human minidystrophin from either the CMV promoter or the muscle-specific α-skeletal actin promoter (Ferrer *et al*., 2000). In contrast, neither a humoral nor cellular response was detected after multiple injections of constructs expressing mini- or full-length mouse dystrophin from the muscle-specific muscle creatine kinase (MCK) promoter (Ferrer *et al*., 2000). Accordingly, stable expression of mouse dystrophin occurred in immunocompetent, *mdx* mice.

It is postulated that the immune response against exogenous dystrophin expression is influenced by the gene delivery vehicle, route of administration, the particular *mdx* mouse strain, the specifics of the dystrophin, pDNA expression vector, and treatment of the muscle before gene delivery. These factors would have a bearing on the extent and type of immune response by determining the amount and time course of gene expression and the type of cells that are expressing. Dystrophic muscle may provide a more immunogenic environment (Yuasa *et al*., 2002). Because human Duchenne patients have various dystrophin mutations (~70% have deletions) resulting in different types and amounts of residual dystrophin expression and revertants, we used two *mdx* strains, mdx^{4Cv} and mdx^{5Cv}. Although both these strains lack full-length dystrophin (dystrophin isoform protein [Dp] 427) expression and have few revertants, mdx^{5Cv} expresses Dp260, Dp140, Dp116, and Dp70, whereas mdx^{4Cv} expresses only Dp116 and Dp70 (Danko *et al*., 1992; Im *et al*., 1996). The decreased expression of dystrophin revertants and dystrophin isoform protein in these two strains as compared with other *mdx* strains would make an immune response to exogenous dystrophin expression more likely; thus serving as a more critical test for immune complications.

Furthermore, the increased levels of foreign reporter gene expression enabled by the intravascular/naked DNA delivery approach heighten the immune response as compared with direct, intramuscular injection (Zhang *et al*., 2001). Thus, the present intravascular/naked DNA experiments provided another test for the stability of full-length mouse dystrophin expression and the induction of humoral and cellular immune responses. Two mouse dystrophin pDNA expression vectors using a CMV or muscle-specific promoter were delivered intraarterially to *mdx* mouse muscle and the stability of expression was evaluated. Humoral and cellular responses to this foreign dystrophin expression were evaluated.

MATERIALS AND METHODS

Mouse procedures

ICR (Hsd:ICR [CD-1]) and C57 (C57BL/10ScNHsd or C57BL/6Nhsd) mice were obtained from Harlan Laboratories (Indianapolis, IN). The two Duchenne muscular dystrophy models, mdx^{4Cv} (B6Ros.Cg-DMD$^{mdx\text{-}4Cv}$) and mdx^{5Cv} (B6Ros.Cg-DMD$^{mdx\text{-}5Cv}$), were obtained from Jackson Laboratory (Bar Harbor, ME). All mice had a body weight greater than 25 g and were approximately 3 to 6 months in age. The *mdx* strains were on a C57 background whereas the ICR mice were outbred.

Mice were anesthetized by intramuscular injection (into the back) of ketamine (100 mg/kg) and xylazine (2 mg/kg) before performing intraarterial injections, and anesthetized with inhaled isoflurane (Abbott Laboratories, Abbott Park, IL) before performing tail vein injections. The surgical procedure in mice was essentially the same as described previously for rats (Budker *et al*., 1998), except that a modified 30-gauge butterfly needle was used. The needle was modified by replacing the 27-gauge needle on a butterfly catheter (Abbott Laboratories) with a 30-gauge needle (BD Medical Systems, Franklin Lakes, NJ). The tip of the needle was threaded down to just above the beginning of the femoral artery. In addition, the preinjection volume was decreased to 0.6 ml of 0.017% papaverine (Sigma, St. Louis, MO) in normal saline. The following formula was used to determine the injection volume of the pDNA solution: DNA solution volume (ml) = (body weight $\times$ 0.1) $-$ 0.2. Given that the pDNA concentration of 100 μg/3 ml was kept fixed, the amount of pDNA injected per mouse body weight was kept constant, but the actual amount of pDNA injected per animal varied. A modification from the rat procedure was to use a Harvard PHD 2000 programmable pump (Harvard Apparatus, Holliston, MA) to consistently inject the pDNA solution at a rate of 30 ml/min. The pump was required because of the small injection volumes and because the *mdx* mice were more sensitive to variations in injection speed. In other words, the *mdx* mice did not tolerate increased volumes of injection as well as the ICR mice did. In addition, the blood vessels were more prone to damage in *mdx* and C57 mice than in ICR mice. Injections were deemed "successful" if the injection caused substantial leg swelling and the artery was not damaged. Animals that did not receive successful injections were excluded from the study at the time of the injection and did not undergo further analyses. Approximately 60 and 80% of the injections were successful in *mdx* mice and ICR mice, respectively.

Tail vein injections under increased pressure were done with 50 to 100 μg of pDNA in ~2.5 ml injected within 7 to 10 sec, as previously reported (Zhang *et al*., 1999).

Plasmid expression vectors

Plasmids pCILuc and pCILacZ use the human immediate-early CMV promoter to express reporter genes encoding luciferase and β-galactosidase, respectively (Budker *et al*., 1998). To construct CMV-luciferase expression vectors larger than the 5688-bp pCILuc, DNA fragments were obtained by polymerase chain reaction (PCR) from sequences 12593–18298 and 6878–18298 of λ phage and inserted into the *Ngo*M IV site of pCILuc to form larger sized pCILuc derivatives pCILuc-11.4 (11,394 kb) and pCILuc-17.1 (17,109 bp). The *Ngo*M IV site of pCILuc is located 312 bp downstream of the poly(A) addition site. The orientation of the insert was not determined.

The pCMV-mDys construct (21.8 kb in size) expressing the full-length mouse dystrophin from the CMV promoter was constructed by insertion of the full-length mouse dystrophin cDNA (a kind gift from P. Clemens, University of Pittsburgh) into the backbone pTG11022 (described in Braun *et al*., 2000). The pDCR-mDys construct (26.6 kb in size) uses the DCR (desmin control region) promoter generated from sequences isolated from cosmid MA281, which extends from nucleotides $-$18662 to $+$60 and contains the 5′ desmin region (positions $-$18662 to $-$1785), the enhancer (positions $-$1784 to $-$538), the promoter, and a short stretch of exonic sequences (positions $-$537 to $+$60) of the human desmin gene (Raguz *et al*., 1998; Antoniou *et al.*, submitted). We have generated a shorter version of this region, with a 2.4-kb deletion downstream at position $-$18662 and a 6.8-kb deletion upstream at position $-$1874, that we linked to the desmin enhancer-promoter. This new promoter, named DCR, was placed upstream of the full-length mouse dystrophin cDNA in a plasmid backbone that differs from pTG11022 by replacement of the HMGCR intron with the hybrid intron 16S/19S of simian virus 40 (SV40) (Okayama and Berg, 1983).

Muscle analyses

After the mice were killed by administration of isoflurane and cardiac aspiration, the muscles of the leg were divided into five groups: anterior, posterior, and medial groups of the upper hind limb (thigh), and anterior and posterior groups of the lower leg. Cryostat sections (6 μm thick) were made for each group of muscle and stained immunohistochemically for dystrophin protein expression. Frozen muscle sections were dried and washed with phosphate-buffered saline (PBS) twice. For mouse dystrophin expression, the primary antibody was a 1:100 diluted serum containing polyclonal mouse anti-dystrophin antibody (produced in our laboratory). Detection of the primary antibody was accomplished by either immunofluorescence or horseradish peroxidase staining, using mouse-on-mouse (MOM) immunodetection kits (Vector Laboratories, Burlingame, CA).

Quantitative determination of the percentage of dystrophin-positive myofibers was done by randomly picking one cross-section in the center of each muscle group. For each cross-sec-

tion, pictures were taken electronically, using a cross-pattern within the entire cross-section. The total number of cells, including the number of positive cells, was counted and a percentage of positive cells was calculated. These percentages (number of positive myofibers per total number of myofibers) are represented for each muscle group or for the entire limb.

Muscle sections were stained for β-galactosidase activity and counterstained with hematoxylin and eosin as previously described (Budker *et al*., 1998).

Toxicity and immune assays

Serum creatine phosphokinase (CPK) analyses were done with an Ektachem DT60 analyzer (Eastman Kodak, Rochester, NY). The presence of anti-mouse dystrophin antibodies was detected by using the mouse serum as the primary antibody (in the above-described immunohistochemical procedure) on HeLa cells transfected with pCMV-mDys (using LT-1; Mirus, Madison, WI) and normal ICR muscle. Immunoblots were also done to determine the presence of anti-dystrophin antibodies, using a protocol adapted from Braun *et al*. (2000). Briefly, cellular extracts from chick embryo fibroblasts infected with a full-length human dystrophin or an empty poxviral vector were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a linear gradient of 4–12% polyacrylamide gel (Novex, Carlsbad, CA) and transferred to a nitrocellulose sheet for 5 hr at 55 V in 50 m*M* Tris, 380 m*M* glycine, and 0.01% SDS, using a wet-transfer apparatus. After blocking with 5% nonfat dry milk in TBST (Tris-buffered saline with Tween 20) for 45 min, two-well strips were incubated for 1.5 hr with 1 ml of mouse serum diluted 1:50 with 5% milk–TBST. After extensive washing (three times for 1 min each and three times for 15 min each) with 10 ml of TBST per strip, the membrane was probed for 1 hr with 5 ml of peroxidase-conjugated rabbit anti-mouse whole immunoglobulins (DakoCytomation, Carpinteria, CA) diluted 1:5000 in 5% milk–TBST and washed again. Antibody–antigen complexes were detected with the enhanced chemiluminescence (ECL) system from Amersham Biosciences (Piscataway, NJ).

CD4- and CD8-positive cells were immunohistochemically detected in muscle sections with monoclonal, rat anti-mouse CD4 (clone RM4-5; BD Biosciences Pharmingen, San Diego, CA) or anti-mouse CD8 (clone 53-6.7; BD Biosciences Pharmingen) as primary antibodies and the rat ExtrAvidin peroxidase staining kit (Extra-6; Sigma) as the secondary antibody system. For dystrophin immunostaining, 3,3′-diaminobenzidine (DAB) without nickel was used as a substrate for the peroxidase reaction to produce a brown color. For CD4 and CD8 immunostaining, DAB with nickel was used as a substrate for the peroxidase reaction to produce a black color. Immunohistochemical staining for β-galactosidase was done with a mouse polyclonal anti-β-galactosidase antibody, VECTASTAIN MOM immunodetection kits (Vector Laboratories), and 3-amino-9-ethylcarbazole (AEC) as a substrate to produce a red color from the peroxidase reaction.

Statistical analyses

All presented p values are two-sided and based on a nonparametric Wilcoxon signed-rank test.

RESULTS

Effect of mouse strain on efficiency of plasmid DNA expression

To use the *mdx* mouse model we needed to develop the intraarterial technique for mice. Previously, we had worked out the intravascular method for delivering naked DNA to limb muscles in larger animals such as rats, dogs, and rhesus monkeys. In contrast to other gene therapy approaches, intraarterial delivery is more challenging in mice, given its requirement for manipulating small vessels and limbs. The first step was to use reporter genes such as luciferase and LacZ to develop and evaluate the technique in normal mice (ICR and C57 strains) and *mdx* mice, which are on a C57 background.

Approximately 100 μg of pCILuc (in which luciferase is expressed from the human CMV promoter) in normal saline was injected into the surgically exposed external iliac artery at a rate of 30 ml/min. One week after injection, all the hind limb muscles were excised and divided into five groups that were analyzed for luciferase activity (Fig. 1). The efficiency of luciferase expression was similar in ICR, C57, and *mdx* mouse strains under the injection conditions detailed above. Average luciferase of all the muscles in the hind limbs was 181, 198, and 174 ng/g muscle in ICR, C57, and mdx^{5Cv} mice, respectively. In addition, ICR mice were able to tolerate larger injection volumes and injection speeds that yielded approximately 1000 ng of luciferase per gram of muscle (data not shown). For comparison, larger injection volumes and more rapid injections in rats yielded 500 to 1000 ng of luciferase per gram of muscle (Budker *et al*., 1998; and additional unpublished data). In all these rodents and under various injection conditions, the upper/medial and lower/posterior muscle groups yielded higher luciferase levels than did the other muscle groups (as in Fig. 1).

Similar results were also obtained with the β-galactosidase reporter system (Fig. 2). One week after intraarterial injection of pCILacZ, the percentage of β-galactosidase-positive fibers was similar in the three mouse strains. Although the best sec-

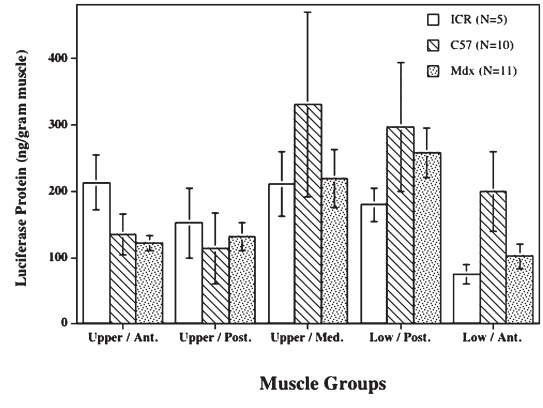


FIG. 1. Mean luciferase expression in various hind limb muscle groups 1 week after ~100 μg of pCILuc (33.3 μg/ml) was injected intraarterially at a rate of 30 ml/min into ICR, C57, and mdx^{5Cv} mice. N, number of mice analyzed (one injection and limb per mouse). Error bars indicate the standard error.

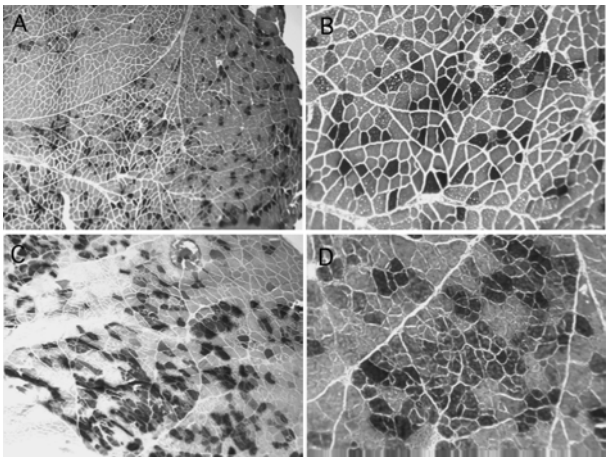


FIG. 2. β-Galactosidase expression in various hind limb muscle groups 1 week after ~100 μg of pCILacZ was injected intraarterially into C57 (**A** and **B**) and *mdx*^{5Cv} mice (**C** and **D**), as in Fig. 1. Fields demonstrating some of the highest percentages are shown. Images were captured with a ×4 objective in (**A**) and (**C**) and with a ×10 objective in (**B**) and (**D**).

tions are shown in Fig. 2, we estimated that on average 5% of the muscle fibers were β-galactosidase positive. These percentages of positive cells are consistent with the luciferase levels and our previous experience in rats concerning the relationship between luciferase and percentage of β-galactosidase-positive myofibers (Budker *et al*., 1998).

To assess toxicity of the procedure in mice, we analyzed serum creatine phosphokinase (CPK) values before and after injection (Fig. 3). As expected, CPK levels were higher in *mdx* mice than in C57 animals before injection and did not significantly increase after injection. In fact, the CPK levels in *mdx* mice went down after surgery, possibly because they were less active after anesthesia and surgery. Histologic examination of the muscles also did not reveal increased muscle damage (Fig. 2).

Effect of plasmid size on efficiency of expression of luciferase constructs

Given that plasmid expression vectors with the full-length dystrophin construct would be larger than most other plasmid vectors, we wanted to explore the effect of plasmid size on the efficiency of luciferase expression (Fig. 4). Luciferase expression vectors of increasing size were constructed by inserting stuffer DNA of different sizes from λ phage DNA 3′ of the polyadenylation site of pCILuc. The same number (moles) of pDNA molecules was injected for each expression vector and muscles were analyzed for luciferase expression 1 week after injection. Using tail vein injections, which deliver DNA mostly to liver, we found that vector size per se, or λ DNA sequences, did not affect luciferase expression (Fig. 4, left). But with pDNA delivered intravascularly to muscle, there appeared to be a small effect in which the 17.1-kb pDNA expressed approximately 2-fold less than the 5.7-kb fragment (Fig. 4, left). A similar size effect was seen in *mdx* mice as well (data not shown). In addition, an effect of pDNA size on expression efficiency was also observed when the pDNA was injected directly into mouse muscle (intramuscular) or transfected into HeLa cells *in vitro* (Fig. 4, middle and right).

Dystrophin expression in mdx *mice*

Two different full-length mouse dystrophin expression vectors were constructed: pDCR-mDys using regulatory promoter sequences of the desmin gene that are capable of driving full physiological levels of desmin gene expression in all muscle cell types (Raguz *et al.*, 1998; Antoniou *et al.*, submitted) (26.6 kb in size) and pCMV-mDys, using the CMV promoter (21.8 kb in size). The hind limbs of either *mdx*^{4Cv} or *mdx*^{5Cv} mice were injected intraarterially with ~300 μg of these plasmid constructs and the muscles were analyzed at various times afterward (Figs. 5 and 6, and Table 1). Low- and high-power views are shown from sections with relatively high percentages of dystrophin-positive myofibers (Fig. 5). Quantitative analyses indicated that the procedure enabled up to 5% of entire muscle groups and limbs to be positive for dystrophin-positive cells (Fig. 6 and Table 1). The percentage of dystrophin-positive cells was similar in both *mdx*^{4Cv} and *mdx*^{5Cv} strains (Table 1). The DCR promoter construct (pDCR-mDys) yielded percentages of positive cells similar to those produced by the CMV promoter construct (pCMV-mDys) (Table 1). The intensity of dystrophin staining was also similar from the pCMV-mDys and pDCR-mDys constructs (data not shown). However, immunoblot analysis is in progress to determine exactly how total dystrophin expression from the different constructs compares.

Given the phenomenon of revertants, two controls were used. In mice injected with dystrophin constructs, the contralateral muscle was not injected because it is infeasible to inject both hind limbs of the same mouse. The average percentage of dystrophin-positive myofibers in these noninjected muscles was about 0.2%. The percentage of dystrophin-positive cells was significantly greater in experimental muscles injected with dystrophin constructs than in noninjected, control muscles ($p < 0.005$). The number of revertants in control muscles did not increase over the time of this study. As a second control, hind limbs were injected with pCILuc in order to determine whether

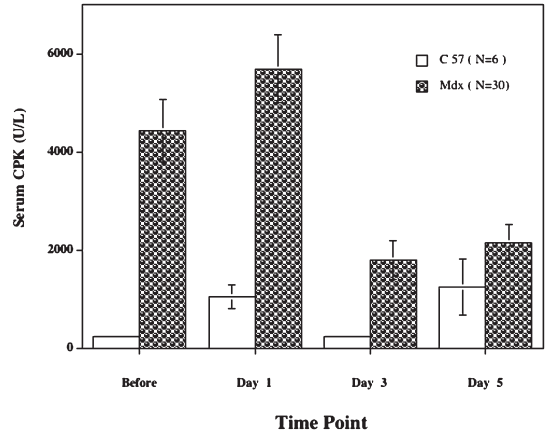


FIG. 3. Mean serum creatine phosphokinase (CPK) in *mdx* and C57 mice at various times after intraarterial injection done as in Fig. 1. N = number of mice analyzed (one injection and limb per mouse). Error bars indicate the standard error.

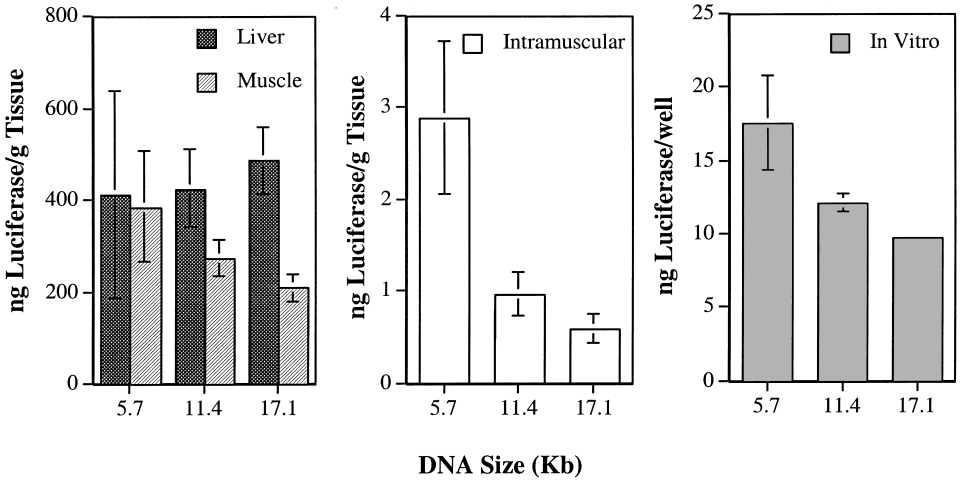


FIG. 4. Mean expression of luciferase after intravascular delivery of three plasmids of different size to either liver or muscle in ICR mice (*left*), direct injection into quadriceps muscles of C57 mice (*middle*), or transfection using LT-1 (Mirus) into HeLa cells *in vitro* (*right*). The plasmids have the same expression cassette as pCILuc. Forty micrograms of pCILuc (5.7 kb in size), 80 μg of pCILuc-11.4, and 120 μg of pCILuc-17.1 were injected so as to deliver the same molar amount of each pDNA. Liver and HeLa cell expression was analyzed 1 day after injection, whereas muscles were analyzed 1 week afterward. $n = 3$–5 for each data point, and error bars indicate the standard error.

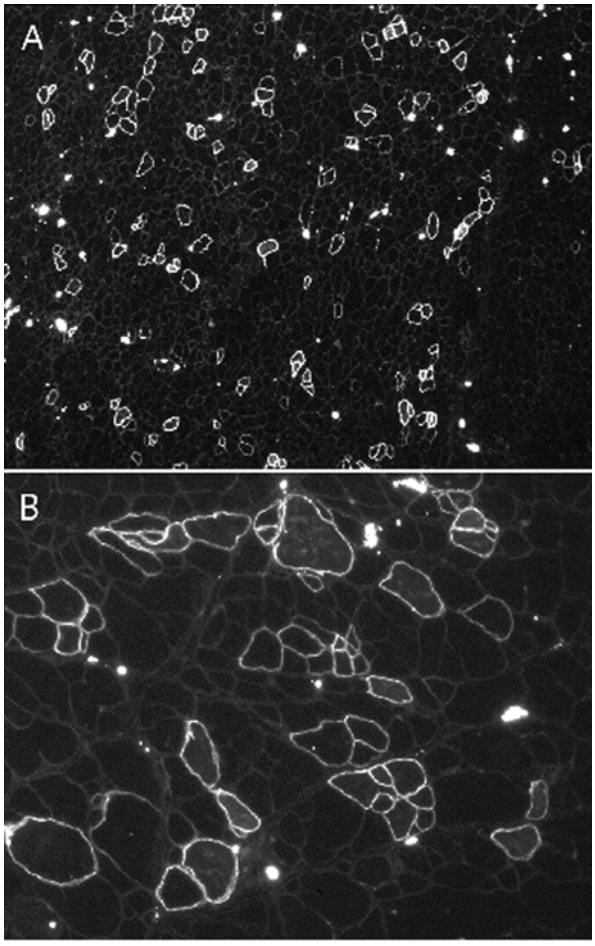


FIG. 5. Immunohistochemical staining for mouse dystrophin expression in *mdx*^{4Cv} mouse muscle 1 month after intraarterial injection of ~300 μg of pDCR-mDys. Images were captured with a ×4 objective in (**A**) and with a ×10 objective in (**B**).

the injection process itself can lead to an increased number of revertants. Quantitative analyses indicated that these control (pCILuc) muscles (Table 2) had similar numbers of dystrophin-positive (revertant) fibers compared with control muscles (Table 1).

Furthermore, the time course of dystrophin expression was determined (Fig. 6 and Table 1). In the *mdx*^{4Cv} strain, the percentage of dystrophin-positive cells was stable for at least 6 months after injection with the pDCR-mDys construct and for at least 3 months after injection with the pCMV-mDys construct. Similar results were obtained in the *mdx*^{5Cv} strain. The time courses of dystrophin expression are shown for specific muscle groups in Fig. 6.

Immunological studies

A humoral response to full-length, mouse dystrophin expression was looked for by testing the sera of mice for the presence of anti-dystrophin antibodies at the time of sacrifice for muscle dystrophin expression analysis (Table 1 and Fig. 7). By 1 month after either pCMV-mDys or pDCR-mDys injection, more than half the mice had detectable anti-mouse dystrophin antibodies. The incidence of a humoral response was similar in both *mdx* strains and did not correlate with the amount of dystrophin expression (Table 1). Both immunoblot and immunohistochemical assays gave nearly identical results except in two animals.

A cellular response was investigated by a variety of methods. In hematoxylin–eosin histologic analyses, no muscles expressing foreign dystrophin presented evidence of myofiber damage. Muscle sections were also immunostained for CD4- or CD8-positive cells. The number of myofiber-associated T cells was determine on randomly-selected muscle fields 3 months after pCMV-mDys injection. For each mouse, 1,000 to 2,000 myofibers were evaluated. In four mice, the mean numbers of CD4-positive T cells per 100 myofibers were 14.7 ($\pm$1.4) and 14.4 ($\pm$0.6) in muscles injected with pCMV-mDys and in control,

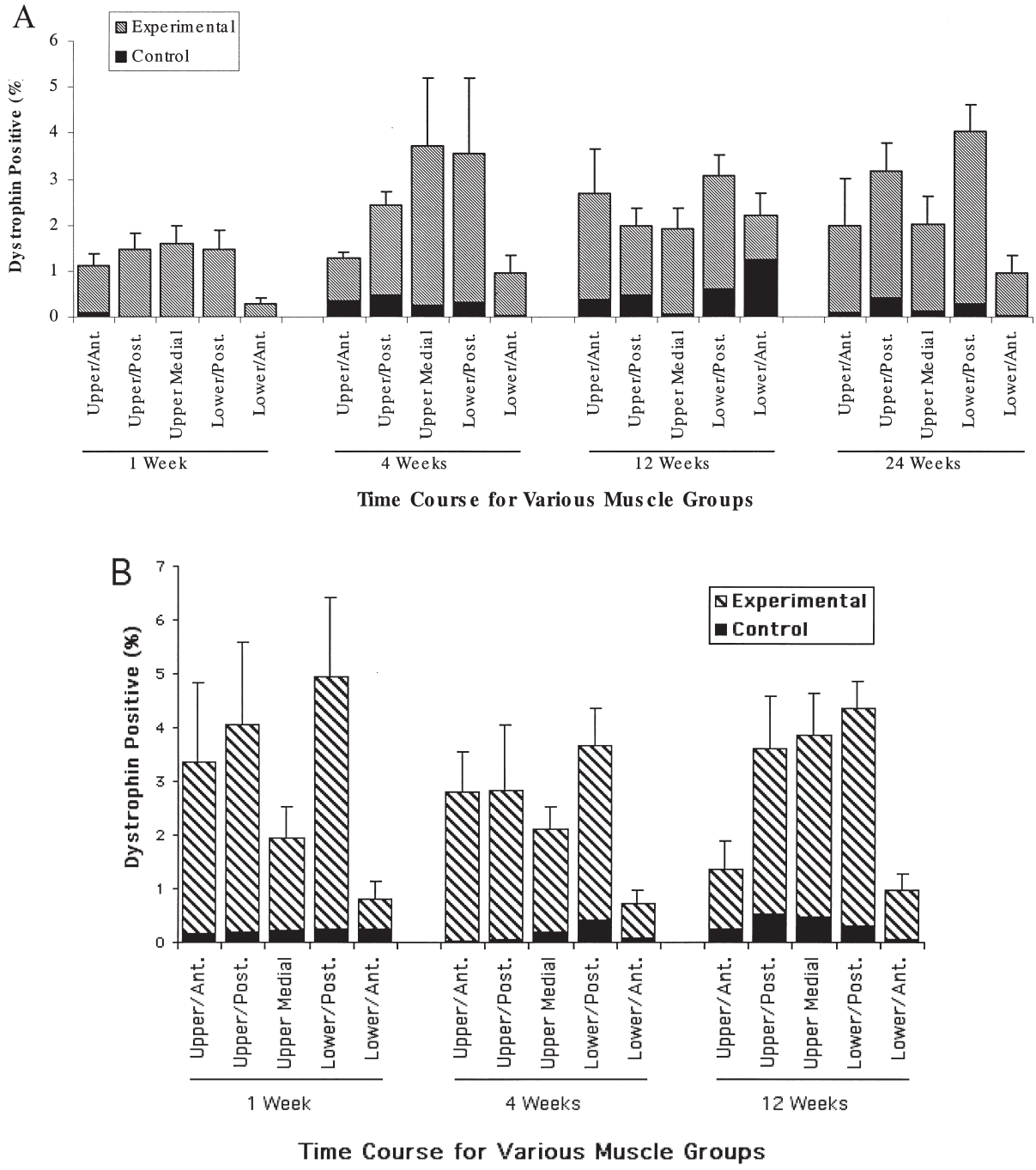


FIG. 6. Mean percentage of dystrophin-positive cells in the indicated hind limb muscle groups at various times after intraarterial injection of ~300 μg of pDCR-mDys into *mdx*^{4Cv} mice (**A**) or ~300 μg of pCMV-mDys into *mdx*^{4Cv} mice (**B**). The mice were 3–6 months of age at the time of injection. Solid areas indicate the number of dystrophin-positive fibers (revertants) in the control muscles (from the contralateral, uninjected muscles). n = 3–5 for each data point, and error bars indicate the standard error.

contralateral muscles, respectively. Similarly, the mean numbers of CD8-positive T cells per 100 myofibers were 10.2 ($\pm$4.3) and 10.5 ($\pm$3.9) in muscles injected with pCMV-mDys and in control, contralateral muscles, respectively. Thus, foreign dystrophin expression did not increase the total number of T cells in muscles. In addition, muscles were costained for dystrophin-positive myofibers and either CD4- or CD8-positive cells. None of the muscles at 1, 3, or 6 months after injection had an increased number of such T cells colocalizing around dystrophin-positive myofibers or other myofibers (Fig. 8A and B). Muscles expressing β-galactosidase served as a positive control for these experiments and caused myositis accompanied by increased numbers of T cells around β-galactosidase-positive myofibers (Fig. 8C and D).

Table 1. Percentages of Dystrophin-Positive Myofibers Throughout the Injected Limbs and Contralateral, Noninjected Limbs of *mdx*^{4Cv} and *mdx*^{5Cv} Mice at Various Times After Injection with Either pCMV-mDys or pDCR-mDys pDNA and the Contralateral, Noninjected Limb[b]

	pCMV-mDys				*pDCR-mDys*			
Time (weeks)	*Animal no.*	*Injected limb*	*Control limb*	*Abs*[a]	*Animal no.*	*Injected limb*	*Control limb*	*Abs*[a]
				A. *mdx*^{4Cv}				
1	387	2.0	0.2	ND	196	1.0	0.0	ND
	389	2.6	0.2	ND	197	0.8	0.2	ND
	390	2.3	0.3	ND	198	1.3		ND
	391	4.9	0.2	ND	199	1.1	0.0	ND
4	379	2.0	0.1	+	200	3.6	0.3	–
	380	2.7	0.3	+	201	1.8	0.3	–
	381	1.5	0.1	+	202	1.7	0.4	ND
	395	2.5	0.2	–				
12	382	2.8	0.3	+	203	1.7	0.6	+
	383	3.6	0.4	+	204	1.8	0.2	+
	384	1.8	0.6	+	205	1.3	0.8	+
	393	2.7	0.3	–				
	394	2.9	0.2	+				
24					207	3.2	0.2	–
					208	2.4	0.3	+
					210	1.5	0.2	+
				B. *mdx*^{5Cv}				
1	246	1.1	0.0	ND	220	1.3	0.1	ND
	247	1.3	0.2	ND	222	0.8	0.1	ND
	249	0.8	0.1		227	0.5	0.1	ND
4					211	1.1	0.0	–
					212	2.1	0.0	ND
					213	2.7	0.0	ND
12	244	1.5	0.0	±[b]	214	1.4	0.2	–
	245	0.6	0.1	+	224	3.5	0.5	±[c]
					225	1.5	0.1	+
24					228	2.1	0.0	±
					239	0.9	0.1	–

Abbreviation: ND, not determined.
[a]Abs, Antibodies (detected either by immunohistochemical staining or on immunoblots) at the time of sacrifice.
[b]For mouse 244, antibody was not detected by the IHC method but was slightly positive by the immunoblot method.
[c]For mouse 224, antibody was barely detected by the IHC method but was negative by the immunoblot method.

Table 2. Percentage of Dystrophin-Positive Myofibers in the Limbs of *mdx*^{5Cv} Mice at Various Times After Injection with pCILuc

Time (weeks)	*Animal no.*	*Percent positive myofibers*
4	425	0.04
	426	0.29
	427	0.12
	428	0.08
24	286	0.19
	287	0.17
	288	0.05
	289	0.10

DISCUSSION

The present study successfully developed the intraarterial approach in mice so as to address several issues relevant to its potential use for gene therapy of people with Duchenne muscular dystrophy (DMD). For one, it demonstrated that the intravascular delivery of naked pDNA can enable full-length dystrophin expression throughout the limb muscles of a dystrophin-deficient animal model for DMD. Using either the CMV or the complete muscle-specific desmin gene control region, the percentages of dystrophin-positive fibers throughout all the muscles of the entire hind limb were 1 to 5% at various times after pDNA administration (Fig. 6 and Table 1). Including both *mdx* strains and all time points, the overall average percentage

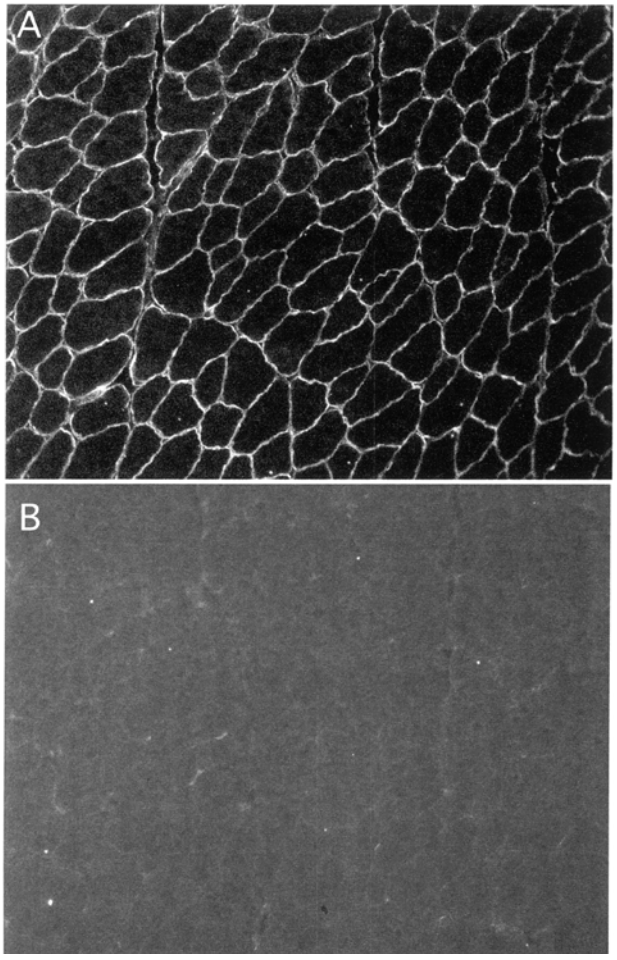
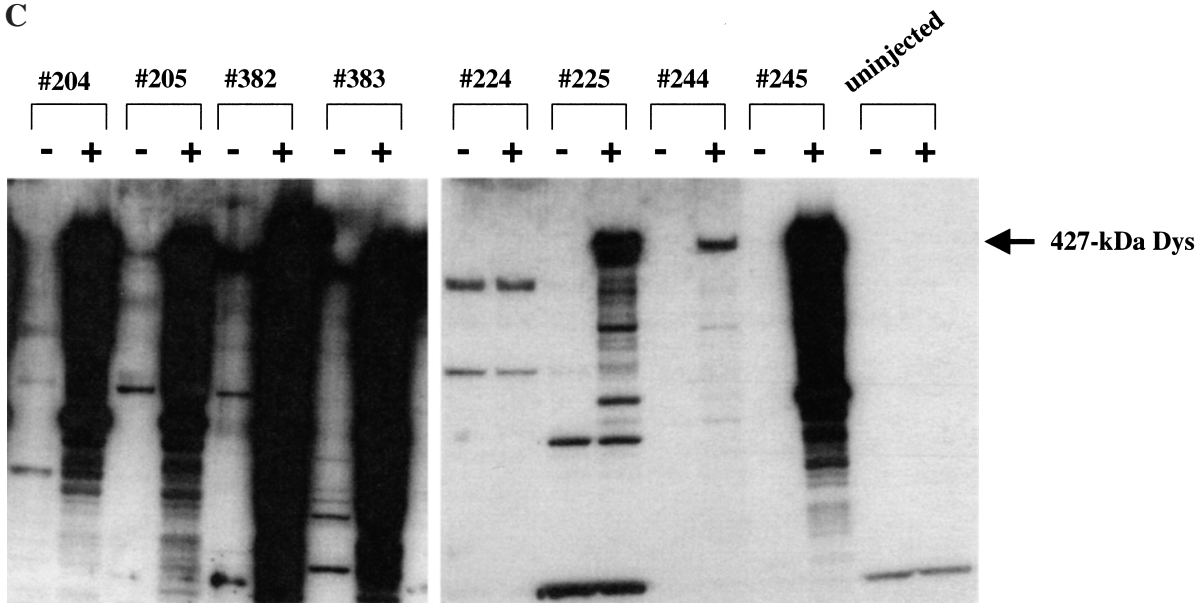


FIG. 7. The presence of anti-dystrophin antibody in serum was tested at various time points after delivery of the mouse dystrophin gene to muscle. A standard immunofluorescence procedure was employed, except that serum from a plasmid-injected mouse was used instead of primary antibody. (**A**) Example of serum that gives positive dystrophin immunostaining; (**B**) example of serum that gives no dystrophin reaction. (**C**) Examples of immunoblots using sera from injected (numbers indicate animals as per Table 1) or uninjected mdx mice. "+" lanes contained 15 μg of cellular extracts from chick embryo fibroblasts infected with human dystrophin poxviral vector, while "−" lanes contained 15 μg of cellular extracts from chick embryo fibroblasts infected with empty vector.



for dystrophin-positive myofibers was 1.7 % for pDCR-mDys and 2.3% for pCMV-mDys.

The phenomenon of revertants in *mdx* muscle complicates the analysis of dystrophin expression studies. It is practical to perform only one intraarterial injection in *mdx* mice, allowing direct comparison of injected and uninjected contralateral limbs. The percentage of dystrophin-positive fibers was on average 10-fold greater in injected limbs. Thus, it is clear that the procedure enabled substantial dystrophin gene transfer and expression. Nonetheless, to address the possibility that the injection procedure damaged muscle so as to induce regeneration and a greater number of revertants, we also injected mice with pCILuc. However, no increase in the number of revertants was observed (Table 2), indicating that the intraarterial injection of naked pDNA per se did not increase the number of revertants. Also, the mdx^{4Cv} and mdx^{5Cv} strains that were used in this study have far fewer revertants than other *mdx* strains (Danko *et al*., 1992).

Although we were successful in eliciting substantial dystrophin expression, the percentage of dystrophin-positive myofibers was approximately 5-fold less than the percentage of β-galactosidase-positive cells that can be achieved by the intraarterial/naked pDNA procedure in rats and rhesus monkeys (~10% on average) (Budker *et al*., 1998; Zhang *et al*., 2001). There are several reasons for this decreased efficiency. Scaling down the procedure was challenging in mice in general and was particularly difficult for idiosyncratic reasons in C57 mice, the background strain of the *mdx* strains. The arteries of C57 and *mdx* mice are smaller than in the outbred ICR strain and were more likely to become occluded as a result of the procedure. The use of a pump enabled more consistent delivery and reduced the toxicity. Even so, the amount of reporter gene expression (luciferase and β-galactosidase) that was possible without substantial muscle toxicity was less in C57 and *mdx* mice than in ICR mice and rats. The fact that both C57 normal mice and *mdx* strains had decreased expression suggested that the decreased efficiency was not related to the dystrophin deficiency in the *mdx* strains. In accord, previous studies did not find any decrease in expression efficiency of naked pDNA directly injected into the muscles of *mdx* mice (Danko *et al*., 1993; Vilquin *et al*., 2001).

The larger size of the dystrophin constructs compared with the reporter gene constructs is another reason for the decreased transduction efficiency of the dystrophin constructs. The present study systematically determined the effect of plasmid size on naked DNA uptake, using plasmids of different size carrying an identical luciferase expression cassette (Fig. 4). Although a larger pDNA size did not significantly affect luciferase expression in the liver after tail vein injection, it did lower luciferase expression in muscle after intraarterial delivery. One reason for the different effect in liver and muscle is that the liver's vascular system has large fenestrae whereas muscle does not. The lack of fenestrae in the muscle vasculature could impede extravasation of the pDNA. However, larger pDNAs expressed less luciferase even after direct intramuscular injection (Fig. 4, middle) (Danko *et al*., 1993). This suggests that extravascular elements such as the extracellular matrix or intracellular barriers in myofibers impede delivery of large pDNAs. The effect of pDNA size on transfection into cultured cells suggests an intracellular hurdle (Fig. 4, right). In accord, an effect of plasmid size on expression has previously been observed after electrotransfer into skeletal muscle and cationic lipid-mediated transfection of cultured cells (Kreiss *et al*., 1999; Campeau *et al*., 2001; Vilquin *et al*., 2001; Kamiya *et al*., 2002).

The levels of dystrophin expression in the present study were high and consistent enough to determine stability of expression. Using either the muscle-specific DCR promoter or the strong viral CMV promoter, dystrophin expression was stable in both the mdx^{4Cv} and mdx^{5Cv} strains over a period of at least 6 months (Table 1 and Fig. 6). This suggests that neither promoter nor immune effects are problematic when using the intravascular/naked pDNA delivery approach. Unstable expression could occur from loss of myofibers given that the naked pDNA transfects only myofibers and not satellite cells. However, we have previously shown that dystrophin expression in *mdx* muscle prolongs foreign gene expression by preventing *mdx* myofibers from turning over (Danko *et al*., 1993). The stable dystrophin expression observed in the present study is also consistent with prevention of muscle degeneration. Given the restriction of dystrophin to nuclear domains, there is some concern that dystrophin expression needs to be along the entire myofiber so as to prevent increased stress on the dystrophin-negative regions. However, our data argue this theoretical possibility. This study did not address the issue of repetitive injections—a key advantage of naked DNA is that it can be repetitively delivered without generating anti-DNA antibodies (Jiao *et al*., 1992; Zhang *et al*., 2001).

In some of the animals anti-mouse dystrophin antibodies were detected, but this apparently did not interfere with long-term expression. The induction of anti-mouse dystrophin antibodies was not observed when Ferrer *et al*. injected naked pDNA expressing full-length mouse dystrophin into *mdx* mice (Ferrer *et al*., 2000). Our study differed from theirs in several ways. First, we used mdx^{4Cv} and mdx^{5Cv} mice, which express less of the truncated, dystrophin isoforms and have fewer revertants. Second, the intravascular approach achieves higher levels of mouse dystrophin expression throughout the targeted mouse limb. Third, the intravascular procedure may deliver pDNA more effectively to lymph nodes and other regional immune cells. All these differences could account for the increased proclivity to produce antibodies in our study. A less likely reason is the use of different muscle-specific promoters—a DCR promoter in our study and an MCK promoter in the Ferrer *et al*. study—because gene expression was found to be lower from a MCK-dystrophin plasmid as compared with a pDCR-mDys plasmid (Thioudellet *et al.*, 2003).

It is of interest that the elicitation of antibodies against mouse dystrophin did not lead to loss of dystrophin expression. Most likely, the intracellular location of dystrophin makes it inaccessible to antibody-mediated immune effects. The occurrence of persistent dystrophin expression despite the presence of anti-dystrophin antibodies has also been observed after histocompatible myoblast transplantation in immunocompetent *mdx* mice (Bittner *et al*., 1994; Vilquin *et al*., 1995). In contrast, a study using adenoviral vectors correlated loss of dystrophin expression with the emergence of anti-dystrophin antibodies (Gilchrist *et al*., 2002). However, the coexpression of β-galactosidase from the same adenoviral vector and the induction of an immune response against β-galactosidase introduced a complication that weakens the relevance of these observations.

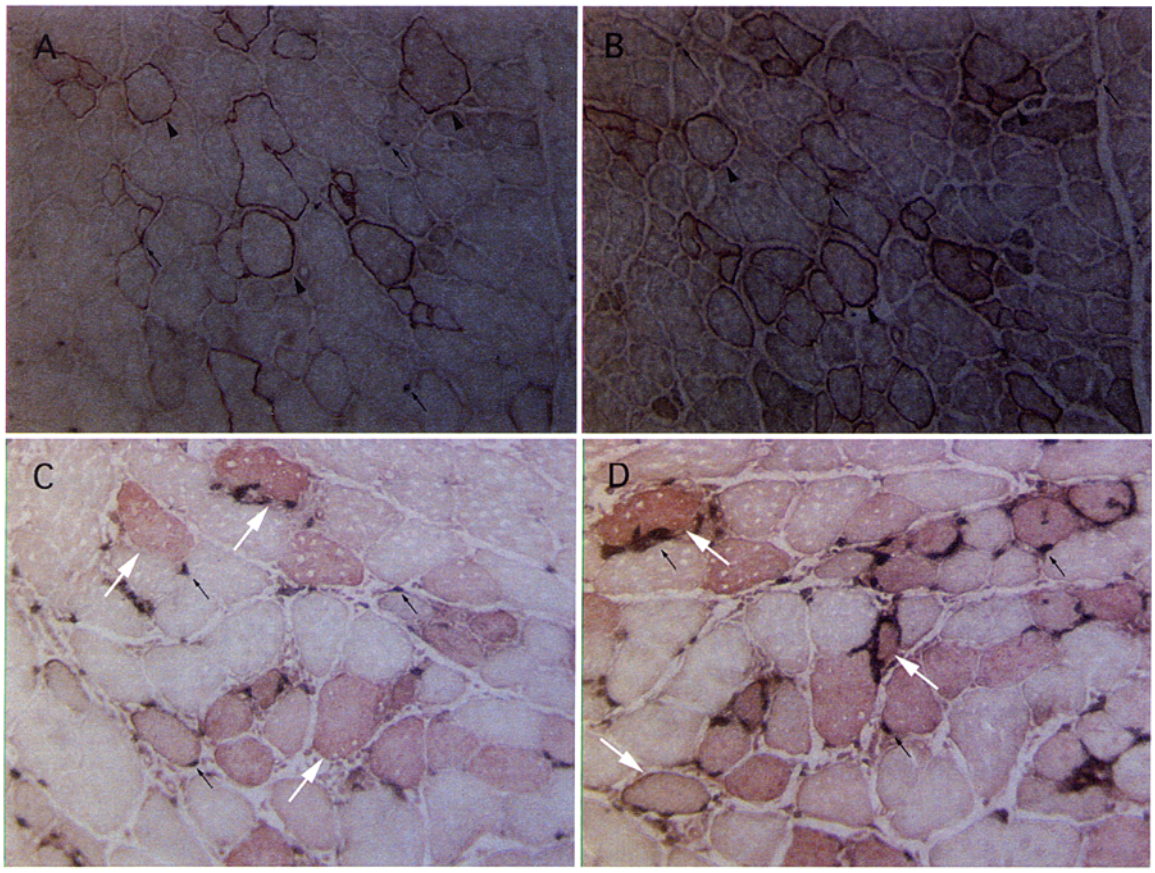


FIG. 8. Immunohistochemical staining for T cell response to foreign gene expression in muscle. (**A** and **B**) One month after intraarterial injection of ~300 μg of pCMV-mDys into *mdx*^{4Cv} mice, muscle was excised and sections were stained for dystrophin (arrowheads, brown staining) and T cells (thin arrows, black staining); CD4 costaining in (**A**) and CD8 costaining in (**B**). (**C** and **D**) Ten days after intraarterial injection of ~100 μg of pCILacZ into C57 mice, muscle was excised and sections were stained for β-galactosidase (white arrows, red staining) and T cells (thin arrows, black staining); CD4 costaining in (**C**) and CD8 costaining in (**D**). Images were captured with a $\times$10 objective.

Increases in the number of CD4- or CD8-positive cells were not observed in *mdx* muscles expressing mouse dystrophin (Fig. 8). This suggests that a cellular immune response was not induced by exogenous dystrophin expression. Without a cellular response, it is unlikely that an antibody response would be sufficient to induce immune-mediated destruction of the myofibers expressing dystrophin, an intracellular protein. A cytotoxic immune response would be needed to kill the dystrophin-expressing myofibers. Correlations between loss of dystrophin expression and a cellular immune response have been observed in myoblast transplantation studies (Vilquin *et al*., 1995; Ohtsuka *et al*., 1998).

In the present study, stable dystrophin expression was observed with constructs containing either the CMV or DCR promoter. Previously, we found that the muscle-specific MCK promoter enabled more prolonged luciferase expression than the CMV promoter after intraarterial pDNA delivery to immunosuppressed rats (Zhang *et al*., 2001). Muscle-specific promoters within adenoviral and AAV vectors have also enhanced stability of muscle expression and their use in the context of the muscle degeneration that accompanies muscle dystrophy may be important (Jooss *et al*., 1998; Cordier *et al*., 2001; Hartigan-O'Connor *et al*., 2001).

The relevance of immune studies in mice to the human situation has been debated in the context of vaccine development and other gene therapies for single gene defects such as hemophilia (Hein and Griebel, 2002; Walsh, 2003). Nonetheless, the results from the present mouse studies form a basis for future human studies and suggest that exogenous full-length dystrophin expressed from intravascularly delivered naked DNA may not cause a destructive immune response in humans. As shown by interim data from a more recent clinical trial, the absence of an immune response in humans after the direct, intramuscular injection of a CMV promoter/full-length human dystrophin construct is also encouraging in this regard (Romero *et al*., 2003). Human experimental studies will eventually be required to determine whether an immune response in the context of our proposed gene transfer approach is problematic or not. These mouse results provide a starting point for designing such clinical studies to address this question. Our ongoing studies in the golden retriever muscular dystrophy (GRMD) dog model for DMD will also be helpful in this regard. In addition,

development and testing of the gene delivery procedure in normal monkeys will provide important preclinical data to determine whether and how to proceed to human clinical trials.

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