Development of helper-dependent adenovirus for gene expression in muscle

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ABSTRACT

Duchenne muscular dystrophy (DMD) is characterized by necrosis and progressive loss of muscle fibers. DMD patients have a mutation in the gene encoding dystrophin, a large membrane-associated cytoskeletal protein on the cytoplasmic side of the sarcolemma. Gene therapy using fully deleted adenoviral vectors shows great potential for the eventual treatment of DMD and other genetic diseases. These vectors are less immunogenic than their predecessors and have the capacity to carry large DNA inserts such as the full-length dystrophin (12 kb). However, the lack of viral genes results in a weakened and subsiding (short) transgene expression in muscle. Findings in the lung and liver have shown the adenoviral E4 region, in particular E4 open reading frame 3 (ORF3) to contribute to the maintenance of transgene expression. We constructed an adenovirus in which E4 ORF3 was reintroduced into a fully-deleted adenovirus along with full-length dystrophin (AdCBDysORF3). Dystrophin levels produced by AdCBDysORF3 were found to be not sustained in *mdx* mice, dropping significantly by day 90. However, expression levels did increase when AdCBDysORF3 was complemented with other viral proteins such as E1B. Likewise, increasing the expression of the primary adenovirus receptor (CAR) in muscle also resulted in a higher initial dystrophin expression in myofibers.

RÉSUMÉ

La dystrophie musculaire de Duchenne (DMD) est caractérisée par la nécrose et la perte progressive des fibres musculaires. La DMD est causée par une mutation dans le gène de la dystrophine, une protéine qui est retrouvée du côté cytoplasmique du sarcolemme. Avec l'utilisation de vecteurs d'adénovirus complètement délétés de tous gènes viraux, la thérapie génique démontre beaucoup de potentiel pour le traitement des maladies géniques comme la DMD. Ces vecteurs sont moins immunogènes que leurs prédécesseurs et ils ont la capacité de transporter de grosses insertions d'ADN tel que la dystrophine (12 kb). Toutefois, à cause du manque de gènes viraux, l'expression du transgène est faible et de courte durée dans les muscles. Des études dans les poumons et le foie ont révélé que la région adénovirale E4, en particulier E4 open reading frame 3 (ORF3) contribue au maintien d'expression du transgène. Nous avons généré un adénovirus dans lequel le E4 ORF3 est réintroduit avec la dystrophine (AdCBDysORF3). Les niveaux de dystrophine obtenus avec AdCBDysORF3 n'ont pas étaient maintenus dans les souris *mdx* et ont chuté considérablement à 90 jours. Cependant, les niveaux d'expression ont augmenté avec la complémentation d'AdCBDysORF3 avec d'autres protéines virales comme le E1B. De plus, une augmentation du nombre de récepteurs primaires de l'adénovirus a aussi accru l'expression initiale de la dystrophine.

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ABBREVIATIONS

BFU = blue forming units

BMD = Becker Muscular Dystrophy

CAR = Coxsackie / adenoviral receptor

CAT = chloroamphenicoltransferase

CB = chicken β-actin

CFTR = cystic fibrosis transmembrane conductance regulator

CMV = cytomegalovirus

DAP = dystrophin-associated proteins

DBP = DNA binding protein

DMD = Duchennne Muscular Dystrophy

HAGA = human α-galactosidase A

IL-8 = human interleukin 8

ITR = inverted terminal repeat

kb = kilobases

lacZ = β-galactosidase gene

MCK = muscle creatine kinase

MOI = multiplicity of infection

MUC-1 = human mucin I

ORF = open reading frame

PGK = phosphoglycerate kinase

Pol = polymerase

pTP = terminal protein

RSV = Rous sarcoma virus

TA = tibialis anterior

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INTRODUCTION

Muscular Dystrophy

Duchenne muscular dystrophy (DMD), a muscle disorder, is characterized by the progressive weakness and wasting of voluntary muscles that control body movement. During the course of this disease, fatty and connective tissue replace muscle tissue (Engel et al., 1994). DMD occurs almost exclusively in boys with an incidence rate of 1 in 3500 newborn males (Emery, 1993).

DMD is an X-linked recessive disorder in which a mutation exists within the large dystrophin gene, located at position Xp21 (Hoffman et al., 1987). It is transmitted through carrier mothers and passed on from generation to generation. However, 1/3 of cases represent new mutations where the mother is not a carrier and only this one child is affected (Moser, 1984; Emery, 1980; Gardner-Medwin, 1970). The clinical onset is usually observed between the ages of two and five years old. By DNA analysis, the disease can also be detected or ruled out in babies where there is a positive family history. Initial symptoms of DMD include difficulty rising from the floor, climbing stairs, falling easily, a waddling gait and pseudohypertrophy which is a characteristic prominence of the calf muscles (Duchenne de Boulogne, 1973). The progression of the disorder varies from child to child but it is steady with no remissions. A wheelchair is usually necessary by late childhood or early adolescence. Breathing is also affected in the later stages of DMD. The life span is shortened and victims usually die in their 20s from respiratory or cardiac failure.

As mentioned previously, nearly all patients are male. Occasionally, the disease has been observed in females due to Turner (XO) or Turner mosaic (X/XX or X/XX/XXX) syndromes (Ferrier et al., 1965), a structurally abnormal X chromosome (Berg & Conte, 1974), or an X–autosomal translocation (Lindenbaum et al., 1979). Infrequently, some heterozygote females suffer from the disease due to a failure of inactivation of the mutation-bearing X chromosome (Gomez et al., 1977).

A milder dystrophinopathy exists called Becker muscular dystrophy (BMD). It is similar to DMD by the fact that it is X-linked, except that it progresses at a much slower rate. BMD occurs in approximately 1 out of 30 000 male births. Symptoms usually appear in boys at about age 12, or in some cases even later. These individuals stop walking at an average age 25-30. Like DMD, BMD results from mutations in the dystrophin gene. However the genetic defects are different from DMD leading to a truncated or altered protein. The majority of patients (70%) have in-frame mutations which produce internal deletions or duplications within the protein. As a result, people with BMD have some dystrophin, but it is usually insufficient or of poor quality. Having some dystrophin protects the muscles of those with Becker from degenerating as badly or as quickly as those of people with Duchenne (summarized in Rowland, 1995).

Dystrophin

The dystrophin gene is among the largest known, with 2.4 megabases (Mb) of DNA, representing 1% of the X chromosome. However, only 0.6% of the gene encodes mRNA for dystrophin. The entire gene and its 12 kb mRNA transcript have been cloned

(Koenig et al., 1987). To date, five different cell type specific dystrophin transcripts have been identified. Muscle, cortical and Purkinje cell type dystrophins have a molecular weight of 427 kDa. Glial and Schwann cells produce dystrophin transcripts with molecular weights of 71 and 116 kDa (Figure 1) (Ahn & Kunkel, 1993). For the duration of this thesis, the dystrophin in question will be muscle dystrophin.

Dystrophin is a subsarcolemmal cytoskeletal protein with a molecular weight of 427 kDa. It has been found to have a highly conserved protein sequence between humans and rodents. Dystrophin maintains the structural integrity of the plasma membrane during contraction and the resulting structural deformation (Figure 2) (Engel et al., 1994).

Dystrophin accounts for 5% of sarcolemmal cytoskeletal protein in muscle (Ervasti & Campbell, 1993). Dystrophin was predicted to be a rod-shaped cytoskeletal protein after an analysis of its predicted amino acid sequence (Koenig et al., 1988). The protein is composed of four structural domains. The amino-terminal end consists of 240 amino acids that have high homology to the actin binding protein, α-actinin (Hemmings et al., 1992). The second and largest section of the protein consists of 25 repeats of 109 amino acids in the form of a triple helix (Matsumura et al., 1993). The third domain contains 15 cysteines and a section of 142 amino acids that show homology to the carboxyl terminal of α-actinin (Koenig et al., 1988). The final, or C-terminal domain, containing 420 amino acids is highly conserved across species and has considerable homology with utrophin, a dystrophin related protein (Love et al., 1989).

Dystrophin is connected with a large oligomeric complex of sarcolemmal proteins including α and β-dystroglycan, α, β, γ, and δ-sarcoglycan and syntrophin (Figure 3). The amino terminus of dystrophin attaches to cytoskeletal actin while the carboxyl terminus

Figure 1. The dystrophin gene. At least five different promoters drive independent cell-type specific expression of dystrophin. Three promoters express full-length dystrophin, while two promoters near the C terminus express the last domains. The C (cortical), M (muscle), and P (Purkinjie cell) dystrophin, the full length forms, each use their own first exon. The S (Schwann cell) and G (general or glial) dystrophin promoters encode C-terminal proteins of 116 and 71 kDa respectively (Ahn & Kunkel, 1993).

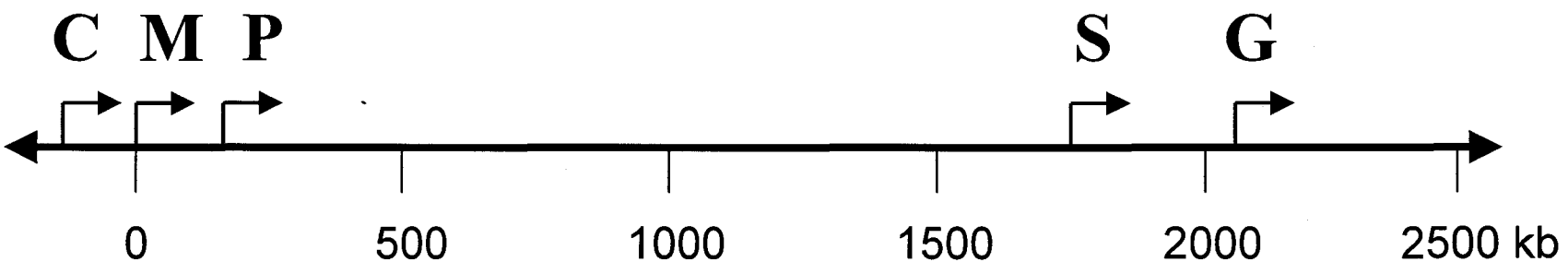
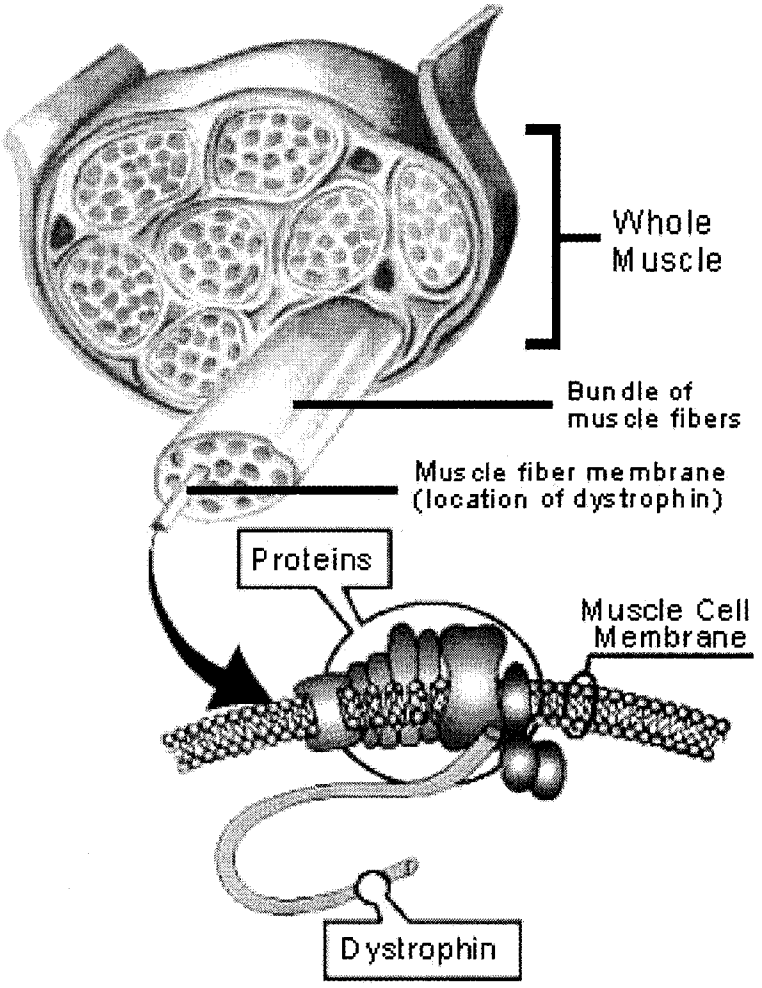

C M P
S G
0
500
1000
1500
2000
2500 kb

Figure 2. Localization of dystrophin within a muscle. Muscles are made up of bundles of fibers (cells). A group of independent proteins along the membrane surrounding each fiber helps to keep muscle cells working properly. When one of these proteins, dystrophin, is absent, the result is Duchenne muscular dystrophy; poor or inadequate dystrophin results in Becker muscular dystrophy (Muscular Dystrophy Association website, 2001).

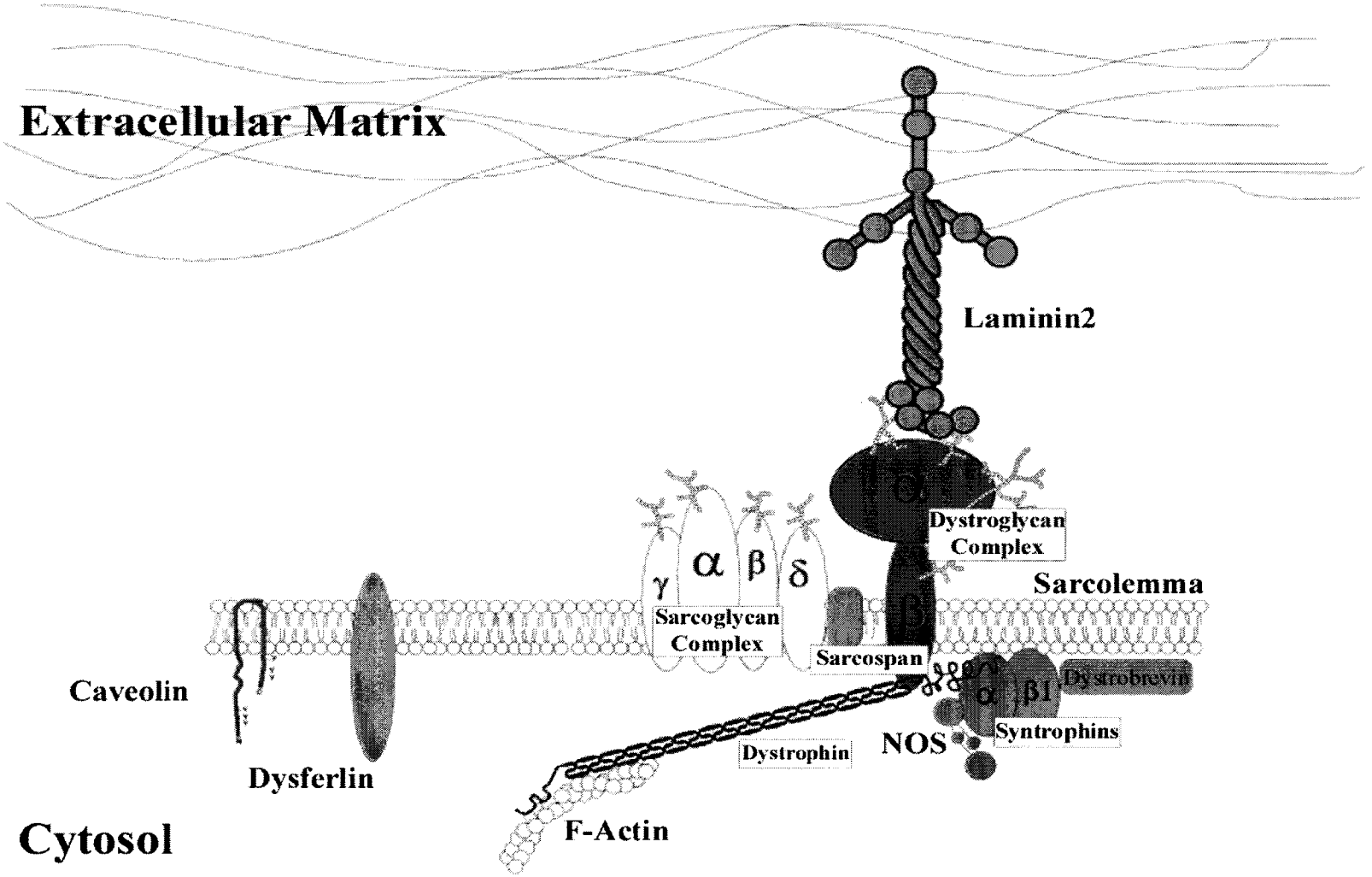

Whole Muscle
Bundle of muscle fibers
Muscle fiber membrane (location of dystrophin)
Proteins
Muscle Cell Membrane
Dystrophin

binds to β-dystroglycan. These proteins are collectively referred to as DAPs, dystrophin-associated proteins. Therefore, dystrophin acts as linker between the actin cytoskeleton and the extracellular matrix (Ervasti & Campbell, 1993).

The mutation in the dystrophin gene leads to the absence of the dystrophin protein. Mutations affecting the dystrophin gene can either be large DNA rearrangements (deletions or duplications) or small mutations (single base changes). Large deletions account for 60-65% of DMD cases. The large mutations are distributed unevenly, with hot spots occurring where the introns are particularly long (between the first 20 exons of the gene) and in the extremely large intron between exons 44 and 45 (Koenig et al., 1987; Koenig et al., 1989; Den Dunnen et al., 1989). A relationship has been found between the site of the deletion and the clinical syndrome. Deletions in the C-terminus result in more severe types while Becker phenotypes result from deletions within the rod domain or the N-terminal region (Koenig et al., 1989). Furthermore, frameshifting mutations result in a truncated dystrophin lacking a C-terminus. This leads to improper membrane attachment of dystrophin via DAPs, dystrophin deficiency and the Duchenne phenotype.

In addition to dystrophin, DMD patients show a remarkable reduction in DAPs. The absence of dystrophin causes the disruption of the linkage of the DAPs to the subsarcolemmal actin cytoskeleton, which leads to an important reduction in all of the DAPs. Sarcolemmal instability results due to the interruption between the subsarcolemmal cytoskeleton and the extracellular matrix. This leads to muscle cell necrosis and weakness of the overall muscle (Matsumura et al., 1993; Ohlendieck et al., 1993) resulting in sarcolemmal (physical) breaks during contraction (Ohlendieck et al., 1993). Thus, myofibers under great stress are the most affected (Watkins et al., 1988).

Figure 3. Schematic model of the sarcolemma depicting dystrophin and its associated proteins (Cohn & Campbell, 2000).


Extracellular Matrix
Laminin2
α
Dystroglycan Complex
γ
α
β
δ
Sarcoglycan Complex
Sarcolemma
β
Sarcospan
Caveolin
Dystrobrevin
α
β1
Syntrophins
NOS
Dystrophin
Dysferlin
F-Actin
Cytosol

Despite intense muscle regeneration, it cannot keep up with the ongoing muscle fiber necrosis. Furthermore, regenerating fibers do not always attain normal size (Carpenter & Karpati, 1979). Death usually occurs at an early age from muscle weakness due to continuous loss of muscle fibers.

Gene Therapy for Muscular Dystrophy

Necrosis and fiber loss in dystrophic muscle fibers can be avoided by establishing dystrophin expression in affected fibers (Deconinck et al., 1996; Phelps et al., 1995; Acsadi et al., 1996). As well, the presence of dystrophin can benefit neighboring fibers that did not receive the gene by reducing the overall mechanical stress (Deconinck et al., 1996). Currently, gene therapy is the most promising mode of action to deliver a functional dystrophin in the treatment of DMD. In gene therapy, a normal gene is delivered to target cells to provide a functional protein in order to overturn the harmful effects of a genetic mutation. In DMD, the most severely affected tissue is skeletal muscle, making skeletal muscle fibers the target cells (Karpati & Acsadi, 1994). A large and elongated cell, a muscle fiber is composed of thousands of myonuclei, each of which in turn contains a full genome (Grounds, 1991). Due to the fact that the dystrophin protein is located in close proximity to each myonucleus, a majority of myonuclei must possess a normal allele in order for the entire muscle fiber to collectively contain dystrophin (Karpati & Acsadi, 1993). Therefore, due to the existence of multiple nuclei and the elongated shape of the cell, it is possible for only a portion of a fiber to become necrotic. Unlike most other cells, the segmental necrosis observed in muscle cells creates

necrotic and non-necrotic portions that span continuously until a membrane produced by a non-necrotic segment provides a division (Carpenter & Karpati, 1979). For instance, in a female heterozygote carrier of a dystrophin gene mutation, a fiber segment under the control of any particular nucleus may have either the wild-type or mutant X activated. The result is a mosaic of dystrophin-positive and –negative nuclei and fiber segments (Anderson, 2002).

In developing a protocol to be used for gene therapy, one must take into account the gene of interest, the vector to transfer the gene and the promoter to drive its expression.

Alternative Vectors for Gene Therapy

Previously, many approaches have been taken to transfer genes into muscle fibers. They, on the most part, have been deemed unsuccessful. Plasmid expression vectors (Acsadi et al., 1991; Davies et al., 1983; Wolff et al., 1990), microprojectiles (Yang et al., 1990) and cationic liposomes containing the therapeutic gene (Curiel et al., 1991; Wagner et al., 1991) are examples of methods which have failed as they did not transfer the gene to a sufficient number of fibers. A procedure involving the transfer of myoblasts was also ineffective (Karpati et al., 1993). In this experiment, bicep muscles of DMD patients were injected with purified normal myoblasts derived from biopsy specimens of the fathers. The transfer efficiency was concluded to be poor based on, amongst others, the dystrophin content of the muscle and on the lack of donor derived dystrophin DNA and messenger RNA in the injected muscle. Viral vectors are another option in gene therapy.

However, retroviruses require replicating target cells for efficient transduction. Furthermore, their insert capacity of 7 kb is too small for dystrophin (Temin, 1989). The insert capacity of adeno-associated viruses (4.7 kb) poses the same problem (Suhr & Gage, 1993). On the other hand, the herpes virus has a large insert capacity (152 kb), but its tropism for muscle cells is poor (Suhr & Gage, 1993). Therefore, in the treatment of DMD, adenoviruses are currently the most promising vectors for gene transfer into muscle fibers (Quantin et al., 1992; Ragot et al., 1993).

Adenoviral Vectors

In gene therapy, adenoviruses are attractive vectors for the delivery of genes. Adenoviruses have a characteristic morphology (Stewart et al., 1993) with an icosahedral capsid consisting of three major proteins, hexon (II), penton base (III) and a knobbed fibre (IV), along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2. Adenoviruses have linear double stranded DNA genomes 30-35 kb in length with a terminal protein (TP) attached covalently to the 5' termini (Rekosh et al., 1977). As well, adenoviruses are flanked by inverted terminal repeats (ITRs) at both ends.

Adenoviruses infect a variety of post-mitotic cells, such as skeletal muscle, lung, brain and heart. Adenoviruses are very attractive vectors for the expression and delivery of therapeutic genes due to their ability to deliver their genome to the nucleus and to replicate very efficiently. The adenovirus infectious cycle can be divided into two phases (summarized in Russell, 2000). The first or "early" phase involves the entry of the virus into the host cell and the passage of the virus into the nucleus. The transcription and

translation of the early genes follow this. The early steps adjust the functions of the cell to allow the replication of the viral DNA and the subsequent transcription and translation of the late genes. Next, the assembly of the structural proteins and the maturation of the infectious virus occurs in the nucleus.

Adenovirus attachment to cells involves high affinity binding to cellular receptors via the knob portion of the fibre (Chroboczek et al., 1995). The same protein acts as a receptor for human adenovirus and coxsackie virus (Bergelson et al., 1997). Thus it has been named the coxsackie / adenovirus receptor (CAR). This receptor belongs to the immunoglobulin superfamily and has a weight of 46 kDa. It is a plasma membrane protein containing extracellular, transmembrane and cytoplasmic domains (Bergelson et al., 1997; Tomko et al., 1997). However, the extracellular domain is sufficient for attachment (Wang & Bergelson, 1999). After initial interaction at the cell surface, the virus enters the cell via clathrin-mediated endocytosis. During this process, an exposed RGD motif on the penton base (Stewart et al., 1997) interacts with cellular αv integrins (Wickham et al., 1993). As well, a number of signaling pathways can be initiated due to the interaction of the virus with the plasma membrane (Bruder & Kovesdi, 1997; Li et al., 1998; Rauma et al., 1999). The virus in the endosomes progresses into the cell cytoplasm where the virus capsid is disrupted by the proteolysis of the structural protein VI (Greber et al., 1996). The adenovirus is transported to the nuclear membrane where the viral genome enters through the nuclear pores allowing the initial transcription events to occur.

Adenovirus transcription can be separated in two stages, early and late, occurring before and after viral DNA replication (summarized in Leppard, 1997; Russell, 2000). There are four early regions of gene transcription termed E1, E2, E3 and E4. The E1

region can be further subdivided into E1A and E1B. The primary function of the E1A proteins is to adjust the cellular metabolism in order to make the cell more vulnerable to viral replication. The E1B 19K gene product prolongs cell survival by disposing of the apoptosis and necrosis inducing Bax proteins (Han et al., 1996). The E2 region is also divided into A and B. The E2A region gives rise to the DNA binding protein (DBP) while E2B produces the terminal protein (pTP) and polymerase (Pol). These proteins are responsible for the replication of viral DNA (Hay et al., 1995). The E3 genes mitigate host defenses but are not necessary for viral replication in tissue culture. The E4 region is made up of six open reading frames (ORFs). The major functions of E4 gene products are to facilitate virus messenger RNA metabolism (Goodrum & Ornelles, 1999; Weigel & Dobbelstein, 2000) and to promote viral DNA replication while arresting synthesis of host proteins (Halbert et al., 1985). DNA replication begins at both termini at origins of replication located within the ITRs (Hay et al., 1995). Late transcription follows which leads to the production of structural components and the encapsidation of the virus within the host nucleus. A packaging signal consisting of AT-rich sequences located at the left end of the viral DNA is responsible for the encapsidation (Hearing et al., 1987). Soon after, the nuclear membrane permeabilizes and the virus makes its way into the cytoplasm (Rao et al., 1996; Tollefson et al., 1996). This leads to the disintegration of the plasma membrane and the release of the adenovirus.

Adenoviruses are popular as gene vectors for a variety of reasons: they're easy to manipulate, they can be grown to high titers, they are capable of infecting many different cell types and as already mentioned, they transduce both replicating and nonreplicating cells such as muscle fibers and neurons (Leppard, 1997).

An adenoviral gene vector can incorporate only 2 kb of foreign DNA into its genome without affecting its stability or infectivity. Thus the addition of longer sequences requires the deletion of additional viral genes. Vectors can then be used to deliver genes that lead to tumor suppression and elimination, that increase or replace deficient genes in tissues and to withstand disease processes.

The first generation of adenoviral vectors was rendered replication deficient by deleting the E1 region. These vectors were also deleted for their E3 region. First generation vectors can be grown to high titers in complementing cell lines such as 293 cells (embryonic kidney cells) that provide the E1 functions in trans (Graham et al., 1977). These adenoviruses have an increased capacity for larger insertions, up to 6.5 kb of foreign DNA.

Although initial studies *in vitro* proved to be promising, these vectors have been associated with a transient transgene expression and cellular toxicity. A disadvantage of using adenoviruses is the interference of humoral and cellular immune responses with transduction efficiency. CD8+ T cells play an important role in this process (Acsadi et al., 1996; Dai et al., 1995; Yang et al., 1994a; Yang et al., 1994b; Yang et al., 1995a). In addition, a considerable role in eliminating adenoviral infections in vivo is played by the components of the innate immune response such as macrophages, complement and natural killer cells (Worgall et al., 1997). This innate antiviral response appears to be regulated, in part, by the transcription factor NF-κB due to its ability to activate the transcription and production of proinflammatory cytokines and to control signaling pathways (Ferreira et al., 1999). Activation of NF-κB occurs at early stages of infection, particularly at high multiplicities of adenoviral infection (Clesham et al., 1998; Lieber et

al., 1998). Repeated injections of first generation vectors were also found to be inefficient because of rapidly induced antibodies against the expressed transgene and adenoviral gene products such as viral capsid antigens which undergo low-grade synthesis (Yang et al., 1994a; Yang et al., 1994b; Tripathy et al., 1996; Yang et al., 1996; Michou et al., 1997). However, contrary to when inserted in adenoviruses, injection of naked dystrophin DNA into dystrophic muscle did not induce an immune response. It is assumed that the plasmid injection failed to produce a high level of expression necessary to evoke an immune response (Acsadi et al., 1991; Acsadi et al., 1996). Furthermore, the transgene expression can occur in many cells, including antigen presenting cells such as macrophages and dendritic cells if the promoter is not muscle specific (Acsadi et al., 1996; Gorman et al., 1982).

Attempts have been made to avoid the host immune response by administering immunosuppressive agents such as IL-12, CTLA4Ig and FK506 (Fang et al., 1995; Kay et al., 1995; Lochmuller et al., 1995; Vilquin et al., 1995; Yang et al., 1995b). A daily treatment of cyclosporin A, an inhibitor of activated T lymphocyte proliferation, was ineffective in increasing long term transgene expression after adenoviral injection into mouse diaphragm (Petrof et al., 1995). No prolonged expression was observed after IL-12 administration, perhaps due to only selective inhibition of certain humoral responses (Yang et al., 1995b). In the case of CTLA 4Ig, efficient secondary transduction was prevented due to low-level neutralizing antibodies despite administration of the immunosuppressive drug (Kay et al., 1995). However, a prolonged dystrophin expression of up to 2 months was observed in diseased muscles subjected to adenoviral-mediated dystrophin transfer followed by a thirty day treatment of FK506 (Lochmuller et al.,

1996). FK506 is an inhibitor in the signaling pathway from the T cell receptor to the nucleus, therefore halting cell proliferation, differentiation and cytokine production (Sawada et al., 1987; Schreiber & Crabtree, 1992). FK506 was also able to suppress the host humoral response against the vector following the first viral administration (Lochmuller et al., 1996).

In addition to their immunogenicity, another disadvantage for first-generation adenoviruses was that they were found to infect myoblasts, myotubes and regenerating fibers but were not highly infective for mature muscle fibers (Acsadi et al., 1994a; Acsadi et al., 1994b). The gene uptake efficiency was two fold lower in adult mice which contain mature muscle fibers (Acsadi et al., 1994b). The low initial efficiency can also be attributed to the severe downregulation of CAR receptors during muscle maturation (Nalbantoglu et al., 1999). These observations translated into a transgene expression that declined rapidly in adult mice compared to a more sustained expression in neonates (Acsadi et al., 1996; Ragot et al., 1993; Vincent et al., 1993).

To overcome some of the drawbacks of first-generation vectors such as limited capacity for foreign DNA and immunogenicity, the next generation of vectors was deleted in the E4 region in addition to E1 and E3 (Lusky et al., 1998; Moorhead et al., 1999). These adenoviruses demonstrated a decreased toxicity and caused less inflammation (Gao et al., 1996; Dedieu et al., 1997; Wang et al., 1997). However, the transgene expression dramatically decreased (Kaplan et al., 1997; Armentano et al., 1997) due possibly to the absence of E4 open reading frames 3 and 6 which increase late viral protein production by stabilizing the late viral mRNA (Ohman et al., 1993). The third generation of adenoviral vectors was fully deleted (also called "gutted" viruses);

that is, all the viral genes were removed. Termed helper-dependent adenovirus (Amalfitano et al., 1998; Hardy et al., 1997; Kumar-Singh & Chamberlain, 1996; Lieber et al., 1999; Morsy et al., 1998; Steinwaerder et al., 1999), these viruses retained only the ITRs and the packaging signal and required the aid of a helper virus in order to be propagated. Nonetheless, there were problems with helper virus contamination during the purification process. This problem was remedied with the development of the Cre-lox helper dependent system (Parks et al., 1996). The helper virus is an E1 deleted adenovirus in which *lox*P sites flank the packaging signals. The helper virus, along with the virus of interest are then used to infect 293 cells expressing the Cre recombinase (293Cre). Through the interaction between Cre and the *lox*P sites, the packaging signal is excised rendering the helper virus unpackageable. Thus the helper genome replicates and provides functions for replication and packaging in trans for the fully deleted adenovirus, without itself being packaged. As well, in the construction of these vectors, a "stuffer" DNA is necessary to maintain a certain vector size for efficient viral DNA packaging (Parks & Graham, 1997).

Theoretically, these adenoviruses would have represented the ideal vector; inducing the lowest immune response due to the deletion of all viral genes and enabling the insertion of larger amounts of DNA including the full-length dystrophin (12kb). These latest vectors proved to be promising yet it soon became clear that retention of some of the E4 genes would be necessary for efficient transgene expression in certain tissues such as the lung, liver and perhaps muscle. (Lusky et al., 1999; Yew et al., 1999; Gorziglia et al., 1999).

Promoters

For driving expression of therapeutic genes in skeletal muscle fibers, an ideal promoter and/or enhancer should be highly active in target cells and show specific activity in muscle cells to minimize toxicity and immunogenicity in non-target cells (Karpati & Acsadi, 1994).

An obvious selection would be the natural muscle promoter for dystrophin (Ahn & Kunkel, 1993; Klamut et al., 1990). However, this promoter was found to be very weak or even inactive (Klamut et al., 1990). The constitutive viral promoters (Rous sarcoma virus late promoter, RSV-LTR, or the cytomegalovirus promoter/enhancer, CMV) were found to be very active in muscle cells (Wolff et al., 1991). However, their activity is not restricted to the muscle (Gorman et al., 1982). Both promoters, when placed in first-generation vectors encoding dystrophin and injected into *mdx* muscle produced high expression levels after ten days in neonates (Acsadi et al., 1996). CMV, in particular, demonstrated increased sarcolemmal and cytoplasmic dystrophin staining in muscle fibers. Notably lower levels were observed in young adult and old mice. As well, levels expressed by both promoters dropped considerably by day sixty in neonates (Acsadi et al., 1996).

The muscle-specific muscle creatine kinase (MCK) promoter has also been extensively used in DMD therapy. MCK has been placed along with dystrophin in encapsidated adenovirus minichromosomes yielding a transient transgene expression for 3-4 weeks (Kumar-Singh & Chamberlain, 1996). Bacterial plasmids containing two fused inverted adenovirus origins of replication based in a circular genome, the adenovirus

packaging signals, a beta-galactosidase reporter gene and dystrophin cDNA regulated by MCK were used to generate the minichromosomes (Kumar-Singh & Chamberlain, 1996). MCK yields high expression levels in muscle; to the extent that it may overproduce dystrophin, although overexpression does not appear to be harmful for mature skeletal fibers in transgenic mice (Cox et al., 1993). Although transgene expression is high, MCK is limited to differentiated skeletal and cardiac muscles (Urdal et al., 1983). In the case of fully deleted viruses encoding MCK and dystrophin, dystrophin expression was noted in a large percentage of *mdx* muscle fibers (Kochanek et al., 1996; Clemens et al., 1996; Haecker et al., 1996; Chen et al., 1997). Research has also been conducted on different portions of the MCK promoter and levels of expression varied depending on the fragment of MCK promoter used. In our group, a 1.35 kb fragment of the MCK promoter, shown to be efficient in first-generation vectors (Larochelle et al., 1997), was not able to generate a high level of expression in any *mdx* age group at any time point when inserted in fully deleted viruses (unpublished data).

Thus, for the purpose of this project, two constitutive viral promoters, the cytomegalovirus (CMV) promoter/enhancer (Wolff et al., 1991) and the chicken β-actin promoter with the CMV enhancer (CB) (Ishii et al., 1999), shown to be very active in muscle cells, were placed in partially deleted helper-dependent adenoviruses. The CB promoter, in particular, seemed promising for gene transduction in muscle. An adenoviral vector, in which CB was present, was shown to efficiently transfer genes (66% of muscle fibers expressed the lacZ gene at seven days post-injection) and maintain expression (for up to 180 days) in mature skeletal muscle, an outcome that had only been observed in neonates previously. Furthermore, the dose of adenovirus used was relatively low,

suggesting the relative strength of this promoter. However, the recombinant viruses used were first-generation, thus inducing serious immune responses (Ishii et al., 1999). It was thought that by combining the effects of the CB promoter and a fully deleted or partially deleted (containing portions of E4) adenovirus would be ideal for use in gene therapy for DMD in all age groups. This would then allow for the insertion of the full-length dystrophin gene, a lower dosage of adenovirus if desired and subsequently, a lower immune response against the vector and improved transduction efficiency.

Animal Models

During the course of this study, the animal model for dystrophin deficiency used was the *mdx* mouse (Bulfield et al., 1984). The dystrophic phenotype arises due to a mutation on the X chromosome in C57BL/10ScSn inbred mice. The molecular basis of the mutation is a C to T nucleotide change at position 3185 that converts a CAA glutamine codon to TAA stop codon. This results in premature termination of translation at 27% of the length of the dystrophin polypeptide (Sicinski et al., 1989). This truncated and unstable dystrophin is unable to attach to the sarcolemma. A marked secondary DAP deficiency is a consequence of the absence of the sarcolemmal dystrophin (Ohlendieck & Campbell, 1991). *Mdx* mice have similar biochemical and histological defects compared with DMD patients. However, unlike in humans, affected mice show little if any disability in addition to having a normal life span. Furthermore, muscle regeneration does not seem to be compromised which may explain the lack of muscle weakness. At birth, few muscle fibers are affected but necrosis increases suddenly at day 20. However, the

intensity of the fiber necrosis decreases after day 60 and keeps occurring at low frequencies during the life of the animal (Tanabe et al., 1986). There is excessive atrophy with loss of normal muscle fibers. Instead of connective tissue, regenerating fibers continuously replace the necrotic fibers. During regeneration, debris is removed by macrophages and proliferation with the formation and fusion of myoblast proceeds. As an indicator of previous damage, the new fibers contain central nuclei. Both of these traits are in contrast to the observations made in humans. The mild consequences of the dystrophin deficiency in *mdx* mice are still not understood. Possible reasons include a greater replicative potential of murine satellite cells (Grounds & McGeachie, 1992) or the small-caliber muscle fibers of the mouse uphold less mechanical stress than larger fibers in humans.

It should also be mentioned that there are canine and feline animal models for muscular dystrophy as well (Valentine et al., 1990; Valentine et al., 1986; Carpenter et al., 1989). These models were not used in this study due the increased costs attributed to the acquisition and maintenance of these animals.

Current Therapies for Muscular Dystrophies

Much work has been done on muscular dystrophy using adenoviral vectors. In mouse models, dystrophin expression increases in muscle fibers transduced with adenoviruses but the transgene expression is only transient due to an immune response against the vector and transgene (Yang et al., 1998; Yuasa et al., 1998). The potency of the immune response has also been shown in the canine model system. Increased

dystrophin expression was observed in dogs that had been treated with cyclosporin, an immunosuppressive drug, after viral injection (Howell et al., 1998). There are attempts to evade this problem by using a homologue of dystrophin, utrophin, which has improved maintenance of gene expression (Gilbert et al., 1999). Another obstacle for researchers to overcome is the lack of viral receptors for the vectors being used (Acsadi et al., 1994b; Huard et al., 1995; Feero et al., 1997).

Previous research conducted by our group has shown transgene expression with fully deleted adenoviruses to be extremely low. In the case of dystrophin, expression and signal intensity was significantly lower than first generation vectors in vitro and in vivo (720 to 3 dystrophic fibers in neonates) (Gilbert et al., 2001). Despite this, a substantial increase in dystrophin expression was observed when a first generation (E1/E3 deleted) adenovirus without dystrophin, was used in trans (when viral products produced by a vector aid the expression from an adjoining but independent second vector) with AdCMVDysFl, a fully deleted vector encoding dystrophin. This finding, suggesting the importance of adenoviral gene products, was observed in vitro and in two animal models for DMD (Gilbert et al., 2001). Furthermore, no increase in dystrophin expression from the helper-dependent AdCMVDysFl was observed when it was co-administered with an E1/E4 deleted adenovirus revealing the requirement of the E4 region in gene expression (Gilbert et al., 2001). Thus, it was imperative to find the gene products within E4 responsible for these results.

Objective

Realizing the role of E4 in toxicity and expression, researchers had already begun testing individual and/or combinations of the seven E4 open reading frames (ORFs). The results obtained varied between groups but one similarity seen was that E4 ORF3 played a pivotal role. Both in combination with other ORFs or by itself, ORF3 was found at times to enhance and most of the times to maintain transgene expression (Yew et al., 1999; Lusky et al., 1999; Gorziglia et al., 1999).

Thus, the aim of this project was to create a fully deleted adenovirus encoding ORF3 capable of high dystrophin expression.

In terms of therapeutic solutions for DMD, the ideal vector would combine low toxicity with strong and stable transgene expression. Thus, an adenovirus encoding E4 ORF3 and dystrophin was constructed.

MATERIALS & METHODS

Cell culture

Cells were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Burlington, Ontario, Canada) supplemented with 30 μg/ml gentamicin, 20m*M* L-glutamine and 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at 37^0C under an atmosphere of 5% CO_2.

Construction of AdCBDysORF3

To construct an adenovirus expressing the full-length dystrophin cDNA and E4 ORF3, plasmids pCBDysFl and pUCλ-ORF3 were first produced using regular methods of molecular biology (Sambrook et al., 1989). pUCλ-ORF3 was generated using two plasmids pUCλI and pORF3. The construction of pUCλI has been described in detail previously (Gilbert et al., 2001). pUCλ-ORF3 was created by replacing the 3 kb NcoI/XbaI fragment of pUCλI with the 2.9 kb NruI/DraIII fragment of pORF3 (Ohman et al., 1993). pCBDysFl was created using two previously constructed plasmids, pCMVDysFl (Gilbert et al., 2001) and pCAGGS (Niwa et al., 1991). The CMV promoter was removed from pCMVDysFl by partial digestion with SpeI and SalI and replaced with a MluI site. pCBDysFl was created by extracting the 1.7 kb SalI/ApaI fragment from pCAGGS, blunting it, adding MluI linkers and inserting it into the MluI site of pCMVDysFl. AdCBDysORF3 was generated by ligating the 17.1 kb XhoI/HindIII fragment of pUCλ-ORF3 with the 14.3 kb SalI/NotI fragment of pCBDysFl at 16°C overnight. Prior to ligation, all ends except the XhoI were dephosphorylated using calf

intestinal alkaline phosphatase (New England Biolabs Inc, Mississauga, Ontario, Canada). After phenol/chloroform extraction and ethanol precipitation, the ligation product was used to transfect 293Cre4 cells in a 60 mm plate using TransIT-LT1 (Mirus Corporation, Madison, Wisconsin, U.S.A.). The following day, the cells were infected with a helper virus (AdLC8cluc) (Parks et al., 1996) and harvested 48 hours later. As described previously (Parks et al., 1996), the adenovirus was amplified by several passages through 293Cre4 cells. After amplification, dot blot hybridization (Jani et al., 1997) using a probe specific for lambda DNA was used to confirm the presence of AdCBDysORF3 in the cell lysate. Two cycles of CsCl buoyant density centrifugation (Hitt et al., 1995) were used to purify the adenoviral vector. The titer (virus particles/ml) was determined from the optical density at 260 nm (Mittereder et al., 1996). The infectious titer of AdCBDysORF3 was confirmed by in situ hybridization (see below). Agarose gel electrophoresis preceded by restriction analysis established the structure of the purified viral DNA (Graham & Prevec, 1991). SYBR® Gold (Molecular Probes, Inc. Eugene, OR) was used to stain the gel which was subsequently analyzed on a phosphoimager (STORM, Molecular Dynamics Inc).

In situ hybridization

In situ hybridization was conducted to determine the infectious titer of fully deleted adenoviruses. The ratio of virus particles to infectious particles was found to be 100:1. The following is an abbreviated description, a more detailed version has been published previously (Gilbert et al., 2001). Each well of an eight-well Permanox® Chamber Slide™ (Nalge Nunc International, Naperville, IL) was coated with 0.01%

collagen (Roche Molecular biochemicals, Laval, Quebec) and plated with 3.0 x 10^4 293A cells. The following day, the cells were infected with 100µl of various virus dilutions in growth medium containing helper virus (AdLC8cluc) at an MOI of 3 infectious particles. 60 minutes post-infection, 200µl of growth medium was added and the Chamber Slide™ was placed at 37°C overnight. All ensuing steps involving glutaraldehyde, Triton and proteinase K were conducted at room temperature. Cells were then treated with prehybridization buffer for 2-4hours at 37°C. Diluted denatured probes were added directly to the prehybridization buffer and incubated overnight at 37°C. Made from the purified DNA fragments of dystrophin cDNA or phage lambda stuffer, the probes were labeled with a random primer fluorescein labeling kit (NEN™ Life Science Products, Boston, MA). The following day, cells were incubated with a biotin conjugated monoclonal anti-FITC antibody (clone FL-D6, Sigma, St. Louis, MO) followed by horseradish peroxidase conjugated to streptavidin (Vector Laboratories, Inc., Burlingame, CA). By immersing the wells in AEC Turbo Reagent (DAKO Diagnostics, Mississauga, Ontario, Canada), the peroxidase activity was verified. The infectious titer was calculated by determining the percentage of positively labeled cells multiplied by the dilution factor.

Western blot analysis

293A cells were plated at 2 x 10^5 cells in 30 mm plates and infected the next day with adenoviral vectors at a multiplicity of infection (MOI) of 100 (based on particle number). The cells were incubated for 2 days, lysed and harvested in sample buffer consisting of 62*mM* 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 . The protein concentration was determined and the samples were processed for western blot analysis, using a monoclonal antibody specific for the carboxyl terminus of dystrophin (NCL-DYS2, Novocastra, Newcastle upon Tyne, UK) (Gilbert et al., 1998).

Complementation experiment

Five replicates of 293A cells were plated at 2.5 x 10^5 cells per 30 mm plate and grown overnight. The following day, the confluent plates were all infected with Ad5/δE1(β-gal)δE4, an E1/E4 deleted adenovirus that encodes β-galactosidase, directed by the phosphoglycerate kinase promoter (Wang et al., 1995). Subsequently, four of the plates were each infected by a different adenovirus: AdCMV-dys, AdMCKDysE4, AdCMVDysFl and AdCBDysORF3. A first generation adenovirus, AdCMV-dys, encodes the minidystrophin cDNA while the AdCMVDysFl is a fully deleted adenovirus encoding only the dystrophin gene (Jani et al., 1997; Gilbert et al., 2001). All infections were carried out at a MOI of 100 (based on particle number). 48 hours post-infection, the cells were harvested and lysed. Concurrently, five groups of 293A cells were plated as described above. Each group was then infected with a specific lysate from the previous group. 24 hours post-infection, the cells were stained with X-gal and the number of blue cells were counted to determine blue forming units (BFU).

Intramuscular injection and histochemistry

Experiments performed on animals were carried out according to McGill University's guidelines for animal care. As described previously (Acsadi et al., 1996), 10 or 30 μl of

adenoviral vectors, at a titer of 1 x 10^{12} virus particles/mL were injected into the right and left tibialis anterior muscles (TA) of neonatal (2 to 4 day old) or young adult (4 week old) *mdx* mice respectively (C57BL/10ScSn-*mdx*/J; Jackson Laboratory, Bar harbor, ME). The transgenic mice expressing CAR in a muscle-specific manner under the control of the muscle-specific creatine kinase promoter have been described previously(Nalbantoglu et al., 2001). Hemizygous CAR transgenics were crossed with homozygous *mdx* mice to obtain the *mdx*/CAR transgenic line. Four-five month old *mdx*/CAR mice were injected in the TA as described above. The animals were euthanized 10 days post-injection, the TA muscles were removed and stored in liquid nitrogen-cooled isopentane. Cryostat sections were stained for dystrophin by immunochemistry as described previously (Acsadi et al., 1994b; Acsadi et al., 1996; Gilbert et al., 2001) using a monoclonal antibody specific for the amino terminus of human dystrophin (NCL-DYS3, Novocastra, Newcastle upon Tyne, UK).

RESULTS

Before undertaking the production of a virus, the legitimacy of ORF3 needed to be verified. An experiment was conducted in which a plasmid containing ORF3 (pORF3) was transfected followed by a fully deleted adenovirus encoding dystrophin. The expression was later analyzed by western blot (Figure 4). Compared to a fully deleted adenovirus, the combination of ORF3 and fully-deleted adenovirus provided a higher transgene expression. Taking this result into account, it was assumed that an ORF3 expression cassette inserted into a virus would be more efficiently transduced into the cell than through transfection of one contained in a plasmid. We therefore attempted to create a virus in which expression cassettes for both ORF3 and dystrophin were simultaneously present.

As described in the Materials and Methods section, a three-step cloning protocol was used to construct the adenoviral recombinant that contained both the E4 ORF3 sequence and the full-length human dystrophin gene (Figure 5). Briefly, a plasmid that contained the E4 ORF3 under the control of the cytomegalovirus (CMV) promoter/enhancer was inserted into a second plasmid that included the 3' inverted terminal repeat (ITR) to produce pucλ-ORF3. The final ligation was performed with a third plasmid, pCBDysFl, which incorporated the second ITR and the dystrophin gene under the control of the hybrid chicken β-actin promoter/CMV enhancer to generate the adenoviral recombinant AdCBDysORF3. Since this adenovirus is fully deleted and thus replication deficient, its production requires the presence of a helper virus. Therefore, AdCBDysORF3 is recognized as a helper-dependent adenovirus and is amplified by

Figure 4. pORF3 increases dystrophin expression of AdCMVDysFl. Increased transgene expression is obtained when a helper dependent adenovirus encoding dystrophin is used to infect HeLa cells that had previously been transfected with an expression plasmid encoding E4 ORF3 under the control of the cytomegalovirus promoter/enhancer. Lane 1 represents a mock infection while lanes 2 and 3 were infected with AdCMVDysFl with only lane 3 being transfected with pORF3. The low molecular weight bands result from non-specific binding. The position of full-length dystrophin is depicted with an arrow.

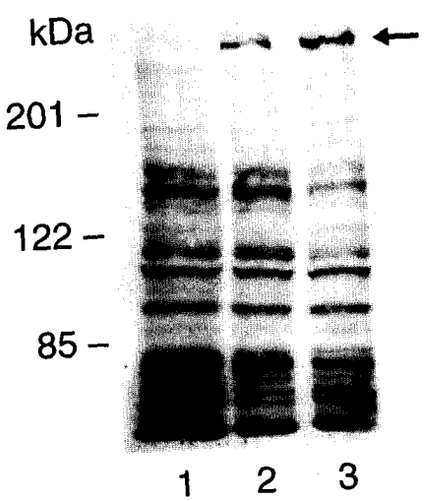
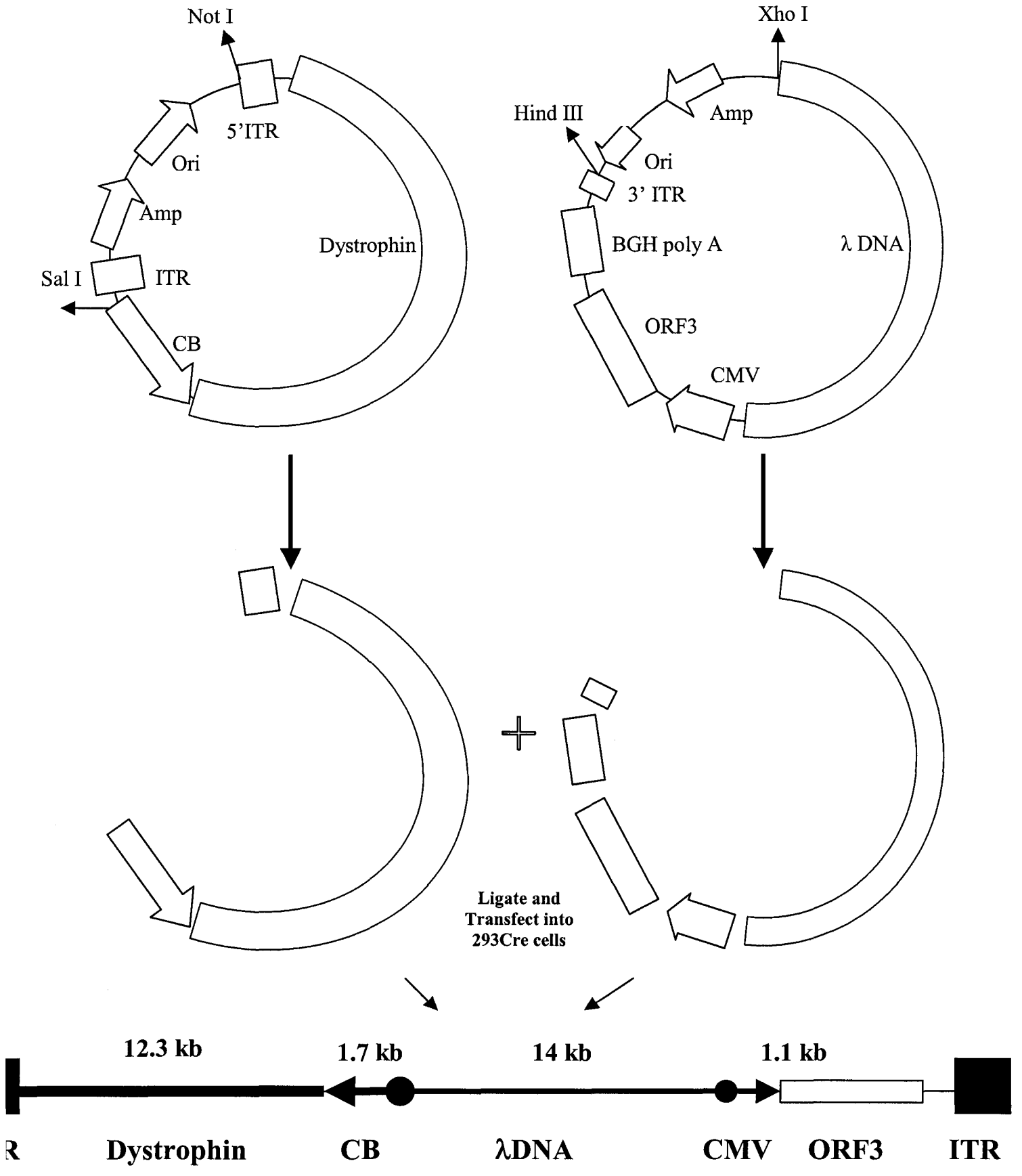

kDa
201 –
122 –
85 –
1
2
3

Figure 5. Construction of AdCBDysORF3 from pCBDysFl and pucλ-ORF3. pCBDysFl contains dystrophin, two inverted terminal repeats (ITR), Ampicilin (Amp), an origin of replication (Ori) and the CB promoter. In addition to an ITR, Ori and Amp, pucλ-ORF3 contains lambda DNA (λ DNA), the cytomegalovirus (CMV) promoter/enhancer, early region 4 open reading frame 3 (ORF3) and the bovine growth hormone polyadenylation site (BGH poly A). pCBDysFl was treated with SalI and NotI while XhoI and HindIII were used to digest pucλ-ORF3. The resulting DNA fragments were ligated together, transfected and amplified in 293 Cre cells to generate AdCBDysORF3.


Not I
5'ITR
Ori
Amp
Sal I
ITR
CB
Dystrophin
Xho I
Hind III
Amp
Ori
3' ITR
BGH poly A
λ DNA
ORF3
CMV
Ligate and
Transfect into
293Cre cells
12.3 kb
1.7 kb
14 kb
1.1 kb
R
Dystrophin
CB
λDNA
CMV
ORF3
ITR

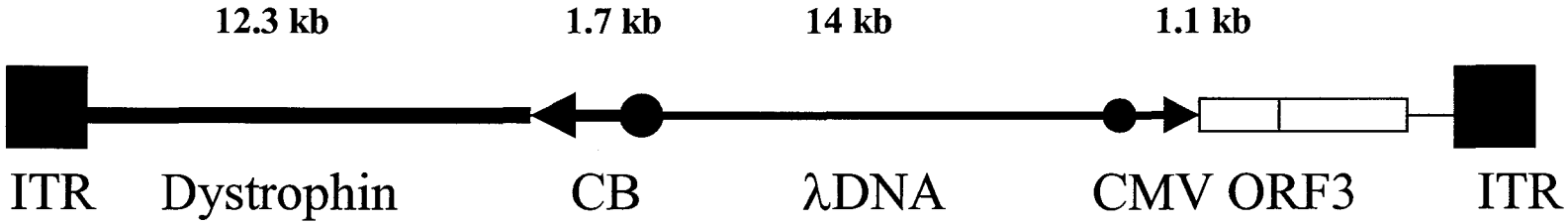
passaging it through 293 cells expressing the Cre recombinase (293Cre) according to already established methods (Parks et al., 1996).

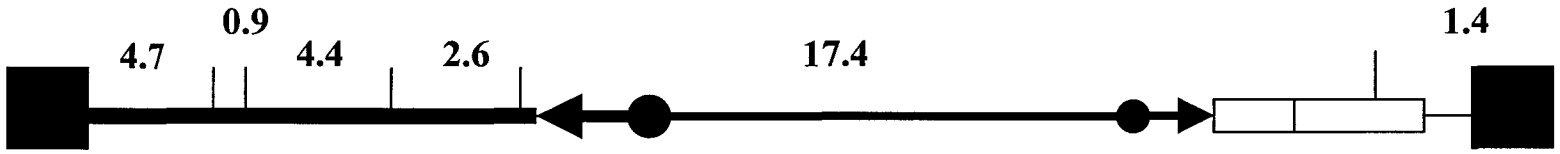
After amplification, AdCBDysORF3 was found to have a titer of 1.15×10^{12} virus particles/mL. Viral DNA was isolated and the presence of both dystrophin and ORF3 genes were confirmed by restriction analysis (Figure 6). Expression of dystrophin was verified by Western blot analysis of 293A cells infected with AdCBDysORF3. As shown in Figure 7, moderate levels of expression were observed consistently in these experiments, demonstrating that AdCBDysORF3 transduced cells efficiently and proper translation of dystrophin occurred.

In order to verify the functional activity of ORF3, a rescue experiment was conducted in which five different groups of 293A cells were first infected with a ΔE4 virus containing LacZ, AdPGKΔE4. Subsequently, four of these groups were each infected by a virus that differed in the E4 region. The viral titer of AdPGKΔE4 was then estimated by subsequent infection of 293A cultures and counting of the number of blue cells produced from the original AdPGKΔE4 – infected cell lysates. As shown in Figure 8, AdCMVDysFL (a fully gutted virus) rescued very minimally. However, in comparison to the ΔE4 adenovirus, AdCBDysORF3 contributed to a five thousand-fold increase in the number of blue cells. Remarkably, this was found to be equivalent to an adenovirus containing the entire intact E4 region but deleted of all other viral genes (AdMCKDysE4). However, in comparison to a first generation adenovirus (AdCMV-dys) that is deleted only in the E1 and E3 regions, the ability of AdCBDysORF3 to rescue the ΔE4 virus was four fold lower (Figure 8).

Figure 6. Characterization of AdCBDysORF3 by restriction analysis. (A) Position of the cleavage sites for Eco RI and Not I on the genome of AdCBDysORF3. The numbers above the drawings correspond to the size of fragments in kilobases after digestion with the restriction enzymes. (B) Agarose gel of digested AdCBDysORF3 DNA with Eco RI and Not I. A DNA size marker in kilobases is depicted on the right hand side.

A





Enzyme: Eco RI

Bands expected: 17.4, 4.7, 4.4, 2.6, 1.4, 0.9 kb

Enzyme: Not I

Bands expected : 28.4, 2.9 kb

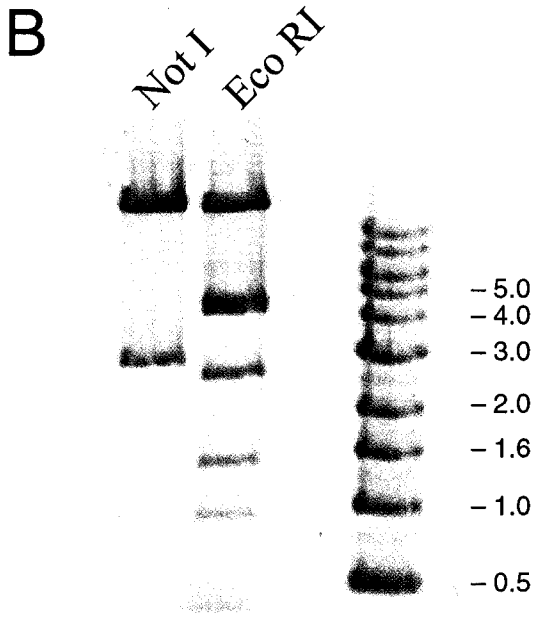
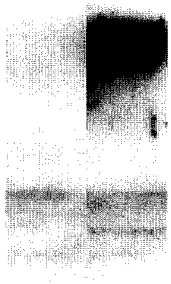

B
Not I
Eco RI
– 5.0
– 4.0
– 3.0
– 2.0
– 1.6
– 1.0
– 0.5

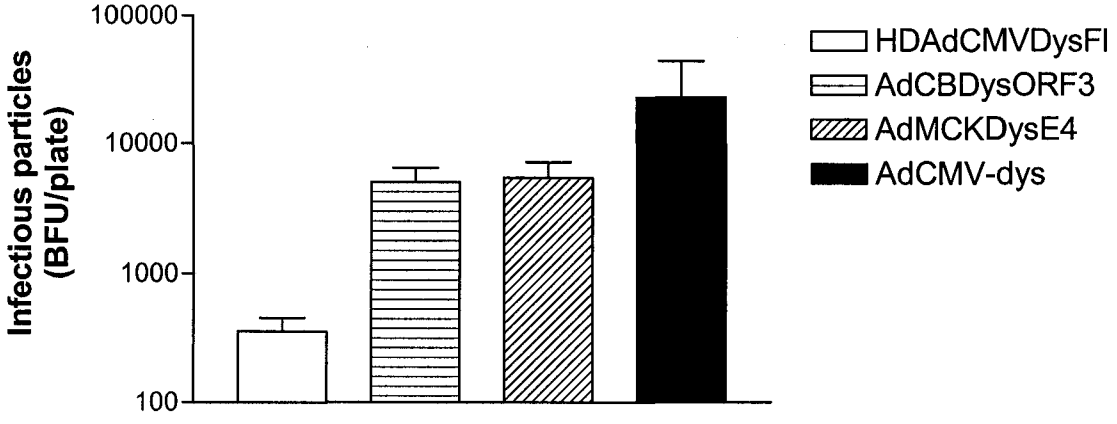
Figure 7. AdCBDysORF3 expresses moderate levels of dystrophin as analyzed by western blotting. 293A cells were mock infected (lane 1) or infected with AdCBDysORF3 (lane 2) at an MOI of 3 infectious particles per cell. At 2 days post-infection, the cells were lysed and 30 μg of total protein were run on a 4% SDS gel. Proteins in the gel were transferred to nitrocellulose membrane and incubated with an anti-dystrophin antibody.

427 kDa -



1 2

Figure 8. The rescuing ability of ORF3 is equivalent to that of the intact E4. It was found by means of a rescue experiment that both AdCBDysORF3 and AdMCKE4 produced approximately equivalent amounts of blue forming units (BFU), 5000 and 5350 respectively when they were used to transduce 293A cells that had previously been infected with a ΔE4 virus that contained an expression cassette for the *E. coli* β-galactosidase gene (AdPGKΔE4). In comparison, a fully deleted vector, AdCMVDysFl produced a much lower amount of 257. As expected, a first generation adenovirus, AdCMV-dys was able to rescue to the greatest extent, 22750 BFU.


Infectious particles (BFU/plate)
100000
10000
1000
100
HDAdCMVDysFl
AdCBDysORF3
AdMCKDysE4
AdCMV-dys

Since these analyses indicated that expression of both the dystrophin and ORF3 occurred at good levels, AdCBDysORF3 was tested in a group of dystrophic *mdx* mice to determine the extent of transduction of muscle fibers. Sixteen neonate and four adult mice were injected in the TA muscles with 5ul or 50ul respectively of AdCBDysORF3. All adult mice were euthanized ten days post-injection while neonates were analysed at three time points of 10, 30 and 90 days. In the neonates, quantification of the dystrophin-stained cryostat muscle sections revealed an average of 120 positive dystrophic fibers at ten days. The number of dystrophin-positive fibers peaked at thirty days with 180 positive fibers before decreasing to 40 fibers at ninety days (Figure 9). In adult *mdx* mice, injection of AdCBDysORF3 resulted in approximately 50 dystrophin-positive fibers. However, when the same virus was injected at the same titer in adult *mdx* mice transgenic for the coxsackie and adenoviral receptor (CAR), the number of positive fibers rose to 150 (Figure 10), indicating that even with the gutted AdCBDysORF3 increased internalization through CAR leads to increased transduction, as observed for first-generation adenovirus (Nalbantoglu et al., 1999).

Inclusion of ORF3 did not produce an initial high level of expression. However, when AdCBDysORF3 was injected into neonate *mdx* muscle in conjunction with another partially deleted adenovirus, AdRP1001 which contains intact E1B and E4 regions, the number of dystrophin positive fibers rose ten-fold as compared to AdCBDysORF3 alone (Figure 11). This result suggests that the E1B region may be beneficial for increasing initial expression levels from a gutted adenovirus.

Figure 9. Dystophin expression after gene transfer with AdCBDysORF3 peaks at day 30. The tibialis anterior (TA) muscles of neonatal *mdx* mice were injected with 10 μl of AdCBDysORF3 at a titer of 1 x 10^{12} virus particles/mL. At 10, 30 and 90 days post-injection, the mice were sacrificed (N=8, 7 and 7 respectively) and the TA muscles were stained for dystrophin using a dystrophin antibody specific for the amino terminus.


Dystrophin positive fibers
200
150
100
50
0
10
30
90
Days post-injection

Figure 10. Presence of coxsackie-adenoviral receptor (CAR) increases the amount of dystrophin positive fibers. Cryostat sections of (A, B) *mdx* and (C, D) *mdx*-CAR muscles injected with AdCBDysORF3. Adult *mdx* and *mdx*-CAR mice were injected with 30 µl of AdCBDysORF3 at a titer of 1 x 10^{12} virus particles/mL. The mice were euthanized 10 days post-injection and stained with a dystrophin antibody. Panels B & D are at a higher magnification.

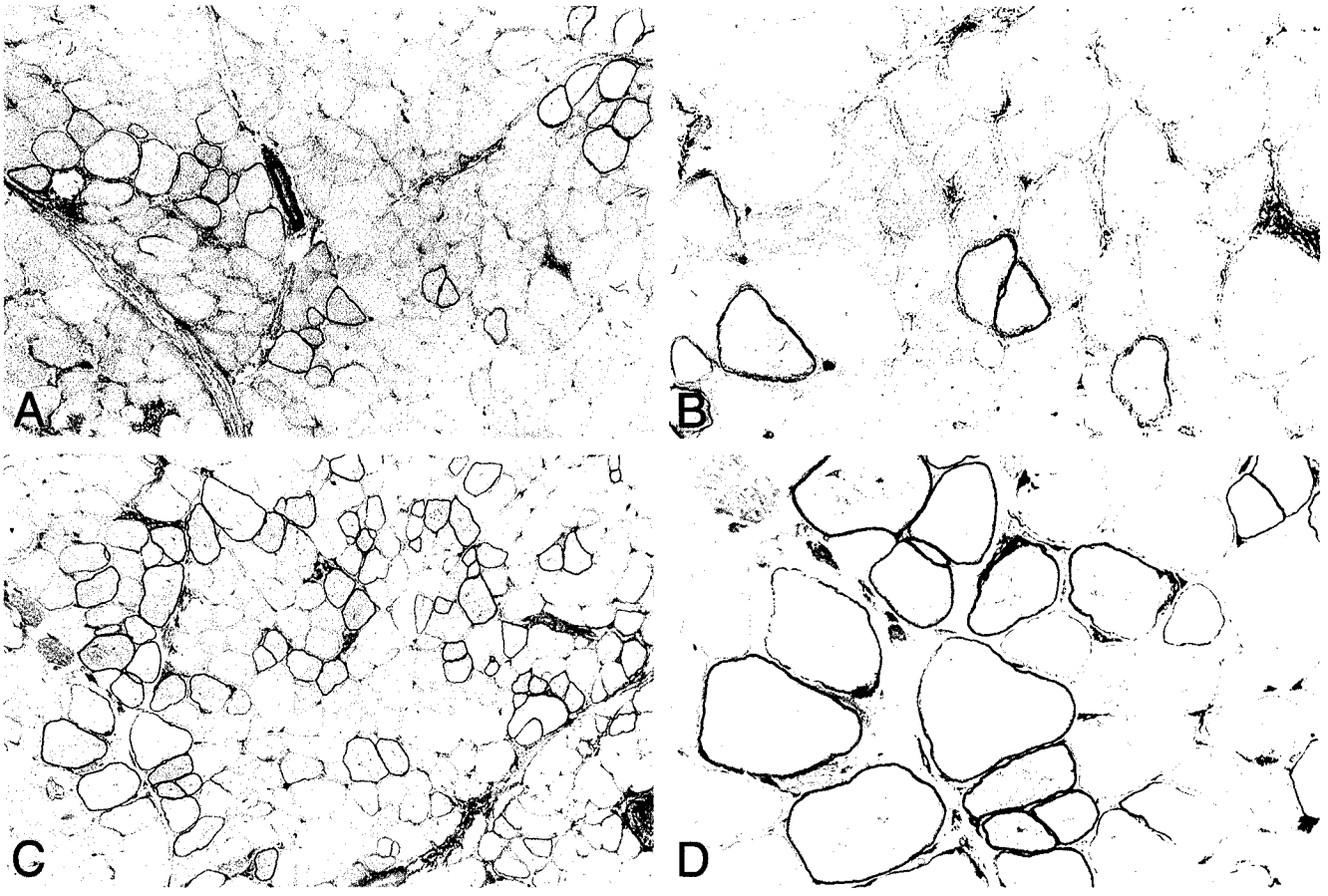
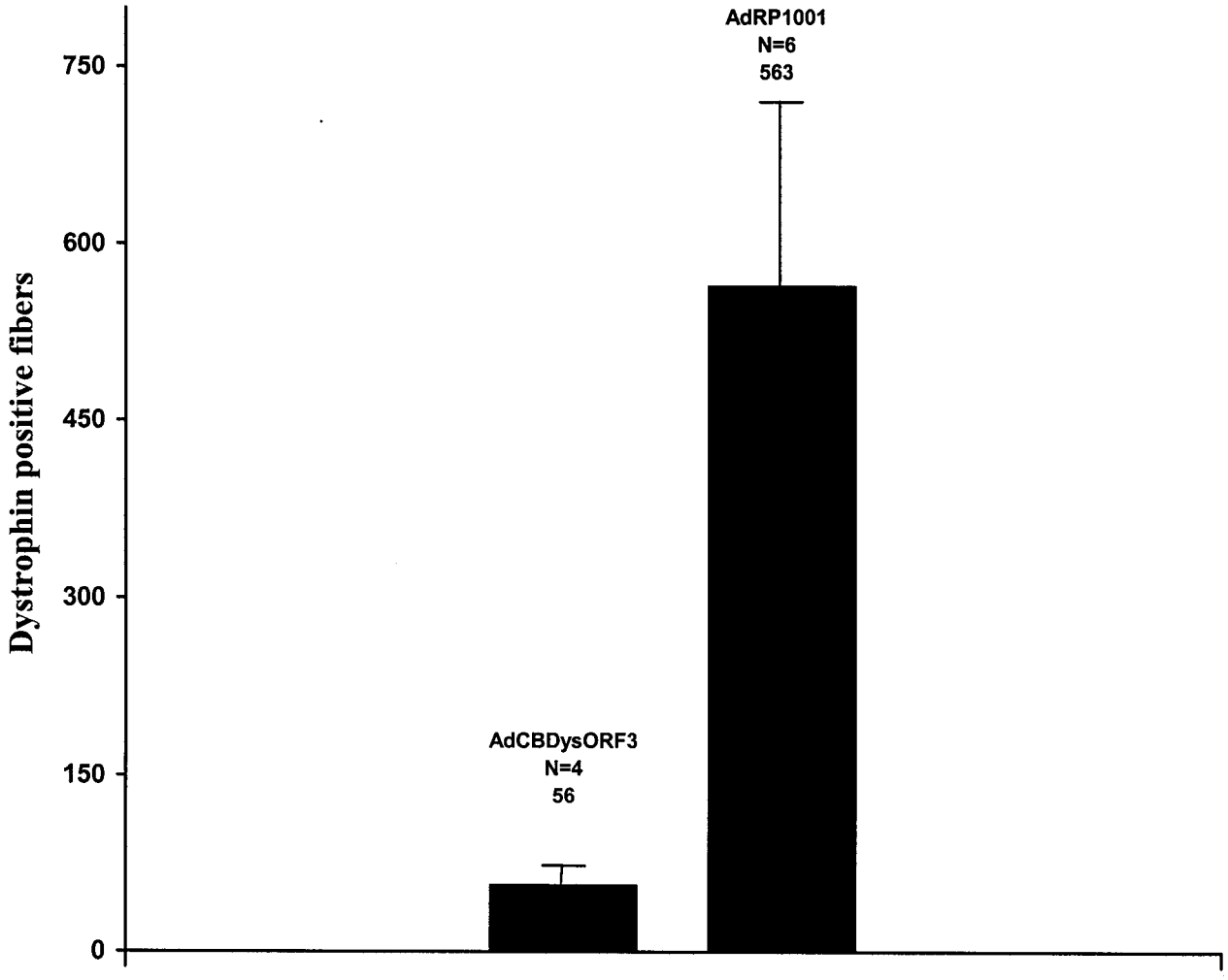

A
B
C
D

Figure 11. E1B increases dystrophin expression of a helper-dependent adenovirus. The tibialis antierior (TA) muscles of neonatal *mdx* mice were injected with AdCBDysORF3 alone or a mixture of AdCBDysORF3 with AdRP1001. Ten days post-injection, the mice were sacrificed and the TA muscles were stained for dystrophin using histochemical procedures.


AdRP1001
N=6
563
750
600
450
300
150
0
Dystrophin positive fibers
AdCBDysORF3
N=4
56

DISCUSSION

In dealing with DMD, a viable treatment must be attained where a sufficient amount of dystrophin is produced in order to reverse the deleterious effects of the disease. However, as in the case of first generation adenoviruses, transgene expression is accompanied by high levels of immunogenicity resulting in high but temporary expression. The introduction of fully deleted adenoviruses brought about reduced immunogenicity and in some cases a prolongation of transgene expression. In the case of a helper dependent vector versus a first generation adenovirus encoding leptin, efficient gene delivery, higher serum leptin levels, lower liver toxicity, decreased inflammation and cellular infiltration was observed when using the fully deleted virus (Morsy et al., 1998). Erythropoietin has also been cloned into helper-dependent viruses. A one hundred fold increase in expression lasting six months was observed compared to a first generation adenovirus containing the same expression cassette (Maione et al., 2000). Furthermore, helper-dependent adenoviruses containing the human alpha-1 antitrypsin gene were found to demonstrate a sustained expression for up to 10 months compared to 3-5 months for first generation vectors (Morral et al., 1999).

In the case of dystrophin, expression levels in muscle were over two magnitude lower with a fully deleted adenovirus (AdCMVDysFl) compared to the first generation (FGAdCMV-dys). AdCMVDysFl was found to be completely inefficient in muscle. Initial expression of dystrophic positive fibers in the tibialis anterior of *mdx* mice were very low (2 and 9 fibers at day 10 in neonatal and adult mice respectively). By day 90, levels had dropped off to background levels (revertant fibers) (Gilbert et al., 2001).

The failure of AdCMVDysFl in combination with the emerging importance of the E4 ORF3 region led us to believe that an adenovirus containing solely the E4 ORF3 and the dystrophin gene would represent the ideal vector in treating DMD. In addition to being credited with late RNA accumulation leading to increased late viral gene expression and their subsequent transport to the cytoplasm (Ohman et al., 1993; Imperiale et al., 1995), research surrounding ORF3 has demonstrated its ability to compensate for the entire E4 region in terms of transgene expression in certain tissues other than muscle. For instance, when ORF3 was inserted into plasmid DNA vectors, and mixed with a chloroamphenicoltransferase (CAT) reporter plasmid, CAT expression in murine lung, over the course of the experiment, was similar to that observed with an E4 containing plasmid (Yew et al., 1999). Furthermore, other studies have shown that the E4 ORF3 is absolutely required for long-term gene expression with the CMV promoter. However, expression is dependent on the target tissue, requiring either ORF3 alone or together with additional E4 products. For example, it has been found that in the lung, ORF3 in combination with either ORF4 or ORF6 and ORF6/7 is necessary for the sustained expression of the cystic fibrosis transmembrane conductance regulator (CFTR). However, in the liver, ORF3 alone is sufficient for the initial and long-term expression of CFTR (Lusky et al., 1999). The inconsistency observed in previous research conducted on ORF3 can be attributed to the variation within the factors used in the experiments. For example, the promoters (CMV, RSV, human mucin I (MUC-1), human interleukin 8 (IL-8), adenovirus E1a) and mouse models (BALB, SCID, C57BL/6, C3H) used, the transgenes tested (CAT, human α-galactosidase A (HAGA), CFTR) and the tissues (lung, liver) in which the experiments were conducted all varied. Despite these variables, the

results suggested that in terms of transgene expression, the E4 region could be replaced by ORF3 (Gorziglia et al., 1999). This feature is attractive for researchers for it enables a greater deletion of adenoviral genes and thus a larger insert, such as dystrophin to be added. As well, the almost complete removal of the E4 region aids in decreasing the cytotoxicity attributed to it (Gorziglia et al., 1999).

Previous studies had shown that loss of persistent expression occurred only when E4 ORF3 had been mutated within adenovirus vectors (Yew et al., 1999). ORF3 alone has been found to be sufficient in the lung for expression (up to 20-30 days post injection) (Yew et al., 1999). Other research involving the lung suggested that ORF3 in combination with ORF6/7 resulted in expression enduring approximately 80 days. However, expression in the liver lasting approximately 90 days required only ORF3 (Lusky et al., 1999).

Initially, an adenovirus containing dystrophin and ORF3 under the control of CMV promoters was constructed. This virus, AdCMVDysORF3 generated feeble amounts of dystrophic positive fibers in *mdx* mice (data not shown). As a result, the ability of ORF3 to at least sustain transgene expression could not be verified due to the virus' inability to generate an initial elevated expression of dystrophin. Therefore, it was thought that a more powerful promoter was needed for the dystrophin expression cassette. Concurrently, a team in Japan had constructed a powerful promoter shown to be very effective in muscle fibers (Ishii et al., 1999). Termed CB, it uses the β-actin promoter with the CMV enhancer. It was hoped that the CB promoter would provide the enhancement in gene expression that CMV lacked, thus enabling ORF3 to perform its function in maintaining expression. Despite outperforming its predecessor,

AdCBDysORF3 failed to live up to expectations. In an attempt to increase gene expression, AdCBDysORF3 infected cells were transfected with a plasmid encoding the E4 ORF6 gene. However, no difference in dystrophin expression was seen (data not shown). The failure of AdCBDysORF3 can be attributed to a few factors. The initiation of an immune response may be partly responsible in addition to loss of viral DNA in the host. As well, the inefficiency of the CB and CMV promoters could also be contributing factors.

The inability of an adenovirus containing E4 ORF3 to sustain gene expression does not necessarily rule out any involvement of ORF3 in this process. It may simply require the presence of an additional viral protein, such as E1B. For instance, the E1B 19K gene product is known to be involved in prolonging cell survival (Han et al., 1996) which in turn would help maintain transgene expression. As well, the other E1B product, 55K has been found to interact with p53. This interaction appears to be essential for viral replication (Ridgway et al., 1997) and for the transport of viral RNAs (Horridge & Leppard, 1998). Furthermore, E1B 55K has an effect on late viral mRNA transcription (Harada & Berk, 1999). Likewise, an increased number of CAR receptors on the surface of muscle cells would also benefit the uptake of virions (Nalbantoglu et al., 1999). Subsequently, a better understanding of gene expression in muscles is required. In addition, the conditions for viral entry and gene expression still need to be optimized. Once attained, an enhanced and sustained dystrophin expression, comparable to therapeutic levels may be obtained.

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