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Gene transfer to skeletal muscle using adenoviral recombinants

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Erklärung

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Ehrenwörtliche Versicherung

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CHAPTER I

Background

Duchenne muscular dystrophy and dystrophin

Duchenne muscular dystrophy (DMD) is one of the most common and devastating neuromuscular disorders. DMD is a progressive skeletal muscle disease that is inherited as an X-linked trait and affects 1/3500 newborn boy worldwide. DMD is normally diagnosed before the sixth year of life. Affected boys are usually confined to a wheelchair before the age of 12 and die in their early twenties by respiratory or cardiac failure. The women and girls are carriers and some of them manifest limb weakness and cardiomyopathy. Diagnosis of DMD is suspected by the detection of elevated serum creatine kinase levels and by the muscle histology, which shows myopathic features: variation in diameter of muscle fibers, necrotic and regenerative fibers, and replacement of muscle tissue by adipose and connective tissue. DMD is confirmed by a complete absence of functional dystrophin (427 kDa) caused by point mutations or out-of-frame deletions in the 2.5 megabases spanning gene [1].

Dystrophin is a member of the α-actinin-β-spectrin family of proteins. Dystrophin is a rod-shaped protein divided into 4 structural domains: the N-terminal contains an actin-binding domain followed by a large rod-domain that is composed of spectrin-like repeats, followed by a cysteine-rich domain (CR) and C-terminal region (CT). The N-terminal domain shows sequence homology to the actin-binding domains (ABD) of α-actinin, β-spectrin and fimbrin; the crystal structure of the dystrophin ABD has been resolved recently [2]. The rod domain is composed of 24 repeating units and is interrupted by 4 proline-rich hinge regions (H1-H4) which may confer elasticity and flexibility[3]. The cysteine-rich and the C-terminal regions contain a multitude of protein binding sites: the WW domain, two putative Ca^{2+}-binding EF-hand motifs, a coiled-coil domain and a ZZ (zinc finger) domain [4-7]. The gene gives rise to several dystrophin isoforms through alternative splicing and are driven by different promoters. Isoforms of dystrophin sharing both the cysteine-rich and C-terminal domains are found in skeletal muscle, cardiac muscle, smooth muscle, brain, Purkinje neurons, retina, kidney, peripheral nerve and one isoform that is ubiquitously expressed [8].

Dystrophin was reported to constitute only 0,002% of total skeletal muscle protein, but it constitutes 2% of total sarcolemmal protein and 5% of subsarcolemmal cytoskeletal protein.

This suggested that dystrophin plays a major structural role in the cell membrane of skeletal muscle [9-11]. In muscle, dystrophin is located at the subsarcolemma and is associated with an oligomeric protein complex, the dystrophin-glycoprotein complex (DGC) which spans the plasma membrane and links the cytoskeleton to the extracellular matrix (Figure 1)[12-16]. The DGC can be divided into three sub-complexes: the dystroglycan, the sarcoglycan and the cytoplasmic complexes. The cysteine-rich and the C-terminal part of dystrophin binds to the transmembrane protein β-dystroglycan (43 kDa), which then binds to the extracellular protein, α-dystroglycan [17-20]. A detailed analysis of the dystrophin DBR (dystroglycan-binding region) demonstrated that β-dystroglycan binds to a region of dystrophin formed by the WW domain and the EF-hand-like domains [17;21]. The α-dystroglycan (156 kDa) makes the final link to the extracellular matrix upon its binding to the α2 subunit of laminin (merosin) [15;22]. The transmembrane sarcoglycan complex is composed of four glycoproteins, α–(50 kDa), β–(43 kDa), γ–(35 kDa) and δ–sarcoglycan (35 kDa) and the sarcospan (25 kDa) [23]. Although members of the sarcoglycan complex are severely reduced in dystrophin-deficient muscle, direct binding to dystrophin or to other proteins of the dystrophin associated complex have not been proven [18]. The C-terminus of dystrophin is also a binding site for the cytoplasmic complex, composed of α– and β1– syntrophins (60 kDa), dystrobrevin (90 kDa) which bind to dystrophin via the coiled-coil domain, and neuronal nitric oxide synthase (nNOS) [24-29]. Furthermore, α-syntrophin binds directly to the N-terminal part of nNOS [30;31].

Dystrophin plays an essential role in maintaining the integrity of skeletal and cardiac muscle cells via the dystrophin-glycoprotein complex. The dystrophin complex is thought to protect the muscle membrane from the mechanical stress of contraction and relaxation [32;33]. Immunohistochemical analysis revealed a drastic reduction in all of the dystrophin-associated proteins (DAPs)(sarcoglycans, dystroglycans, nNOS, syntrophins, dystrobrevins and sarcospans) in both DMD patients and the mouse model for DMD, the *mdx* mouse [15;23;26;27;31;34-42]. The absence of dystrophin causes the disruption of the linkage of the DAPs to the subsarcolemmal actin cytoskeleton, which leads to a drastic reduction in all of the DAPs[40]. DAPs mRNA levels are normal but the complex is not properly assembled and/or integrated into the sarcolemma or are degraded in the absence of dystrophin [15;43-45]. The resulting disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular

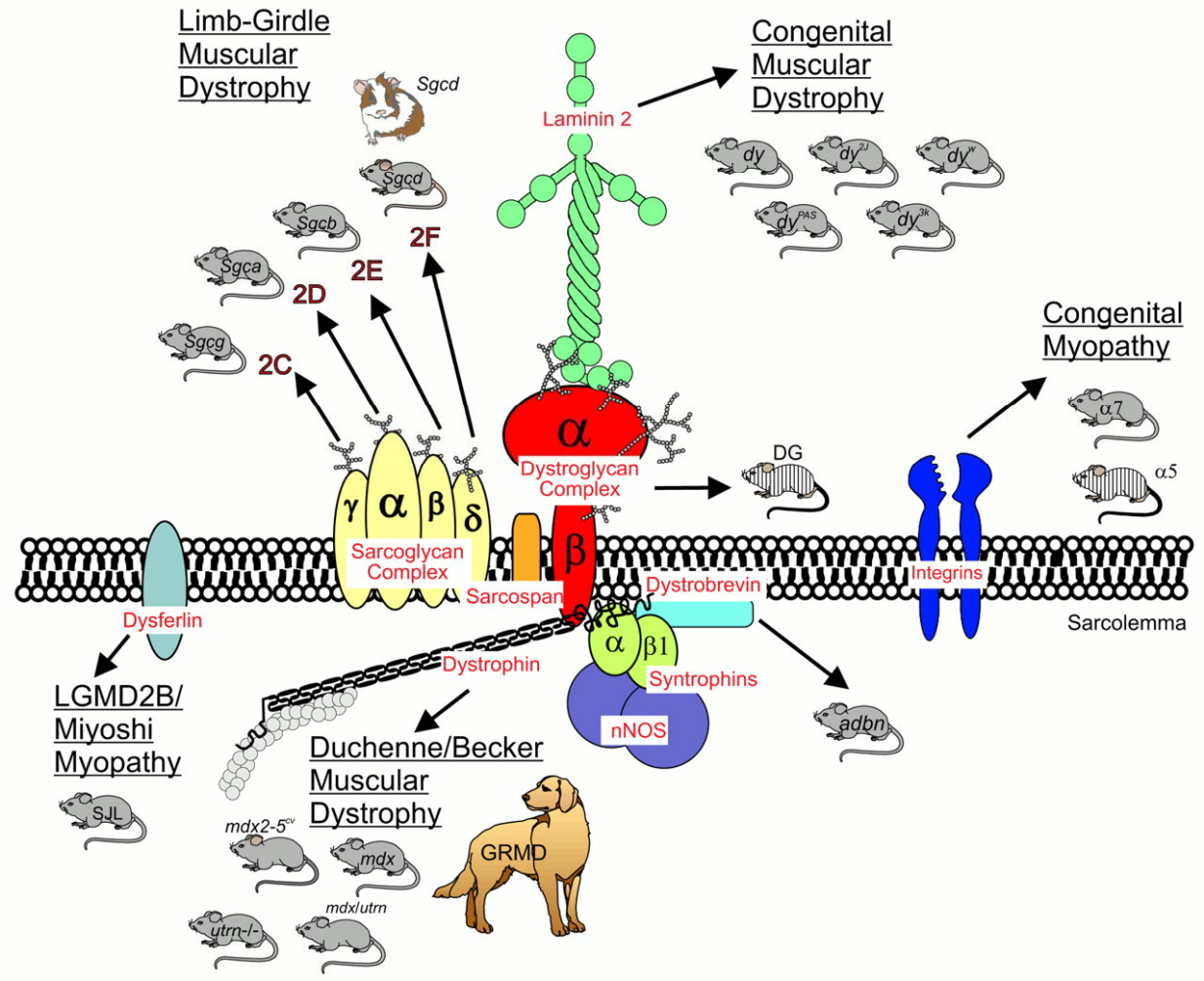


Figure 1. Organization of the dystrophin-glycoprotein complex (DGC) at the sarcolemma of muscle fibers. The dystrophin makes the link between the actin filaments and the extracellular matrix, upon its binding to the dystroglycan complex. Animal models of muscular dystrophies are also depicted (adapted from Allamand and Campbell 2000).

matrix may lead to sarcolemmal instability and eventually to muscle cell necrosis and muscle weakness [37;41]. This may be the case especially during muscle contraction, which may cause physical breaks of the sarcolemma [32;41]. The contribution of dystrophin to membrane structural integrity suggests that myofibers undergoing great physical stress may be the most affected by the absence of dystrophin [32;46]. The deficiency of dystrophin causes recurrent necrosis of muscle fiber segments [47]. While regeneration is vigorous, it cannot fully keep up with necrosis and as a result, progressive loss of muscle fibers occurs.

Becker muscular dystrophy (BMD) is an allelic disorder that is less severe than DMD, characterized by later onset and longer survival. Mutations that produce decreased amounts or abnormal molecular weight proteins are associated with BMD, while a complete absence of dystrophin correlates with the more severe form of muscular dystrophy, i.e. DMD [48-51]. Some patients with very large rod domain deletions showed only mild clinical phenotype. One patient who was still ambulant at age 61 was found to have exons 17-48 deleted corresponding to 46% of the protein, giving rise to a molecule of ≈ 200 kDa only [52]. Recently, patients with even larger intragenic dystrophin deletions (exons 17-51 and exons 13-48) corresponding to a loss of more than 50% of the molecule and having mild BMD phenotype were found [53;54].

DMD and animal models

The mouse model for DMD is the *mdx* mouse, which is dystrophin deficient [55;56]. DNA sequence analysis of normal and *mdx* dystrophin cDNAs revealed a nonsense mutation in *mdx* mice when compared to normal animals. *mdx* mice are viable, have normal body weight and are fertile [57]. Although they don't display severe progressive myopathy, *mdx* mice have biochemical and histological defects characteristic of dystrophin deficiency: elevated serum creatine kinase, cycles of necrosis followed by regeneration and persistence of central nuclei [55;57]. The necrosis-regeneration process of *mdx* skeletal muscle fibers starts at 15 days of age and still is present at 360-day-old animals, peaking between the ages of 45 and 60 days [58]. The mouse *mdx* diaphragm undergoes progressive degeneration and fibrosis comparable to that of DMD limb muscle, although aging mice show no overt respiratory compromise [59].

Golden retriever muscular dystrophy (GRMD) is a spontaneous and X-linked disease of dog that is a homologue of Duchenne muscular dystrophy. Analysis of the canine dystrophin gene reveals a splice site mutation that results in exon 7 skipping and premature termination of the reading frame [60]. These dogs share many similarities to Duchenne boys: elevated creatine kinase in serum, muscle atrophy, muscle necrosis, degeneration/regeneration, and cardiomyopathy. Clinical signs appear between the age of 6 to 8 weeks as shown by a progressive stiffness of limb muscles, mainly the hind limbs [61].

Utrophin is a cytoskeletal protein that is highly homologous to dystrophin (400 kDa, 4 structural domains) that is expressed in all normal tissues. In normal mature skeletal muscle fibers, utrophin is localized exclusively at the neuromuscular junction (NMJ). However, in regenerating fibers of adult muscles (DMD patients and *mdx* mice) or in embryonic muscle cells, utrophin is distributed throughout the sarcolemma [62]. Utrophin-deficient mice are healthy, have a normal life span and show no signs of weakness. They only display a very mild myasthenia [63;64]. However, when utrophin deficiency is combined with the dystrophin-deficient *mdx* mouse (dko or double knockout), a more severe progressive muscular dystrophy is produced. These dko mice display marked myopathy, leading to weight loss and breathing difficulties, resulting in premature death by 20 weeks of age [65;66].

Treatments for DMD

Because of the lack of effective treatments for Duchenne muscular dystrophy, novel approaches including gene therapy have been explored. Gene therapy may consist of providing a wild-type copy of a defective gene. For gene delivery so-called "vectors" such as plasmids or modified viruses are used. Most of the approaches using "naked" DNA expression vectors show poor delivery efficiency and only transient gene expression [67-69]. The use of viruses as vectors for human gene therapy is a powerful technique, since many of them have evolved a specific machinery to deliver DNA to host cells. The best-studied viruses include Adenoviruses, Retroviruses and Adeno-associated viruses.

Adeno-associated-viruses (AAV) are promising vectors for gene therapy since they permit efficient and long-term transgene expression in a variety of tissues *in vivo* without significant immune response [70]. Many reports have demonstrated the efficiency and the persistence of a

reporter gene (lacZ) expression for up to 19 months in skeletal muscle after a single injection of rAAV in immunocompetent animals [71-74]. There is an increasing interest in AAV-mediated gene therapy for genetic muscle disorders. Recent studies have shown a functional rescue in two dystrophic rodent models (delta-sarcoglycan deficient hamsters (Bio14.6) and dystrophin-deficient mouse (*mdx*)) by intramuscular injection of rAAV vectors containing sarcoglycan or dystrophin cDNAs [75-77]. One major drawback of using AAV vectors is the limited insert capacity of ≈ 4.6 kb [78], that preclude the use of the mini-dystrophin gene (cDNA = 6.3 kb). rAAV vectors also need a helper virus (adenovirus or herpesvirus) for optimal growth and multiplication, cannot be grown at high titers and the recombinant vectors lack site-specific integration [70;79].

Retroviral vectors are RNA viruses that can accommodate up to 8.0 kb of foreign genes and are mainly used for *ex vivo* gene delivery since they can infect only proliferating cells. They are able to integrate randomly into the host genome and thus should permit long-term transgene expression, but they tend to be silenced by either methylation or by incorporation into condensed chromatin. They are still difficult to grow at high titers and to retain their infectivity during concentration and storage. Moreover, the random integration may lead to insertional mutagenesis [80].

Adenoviral vectors

Recombinant adenoviruses (AdV) are well-defined vectors for *in vivo* gene transfer. The viral life cycle is well characterized and the AdV genome is easy to manipulate. E1/E3-deleted AdV can accommodate cassettes of ≈ 8 kb, while third generation AdV (gutted or gutless) can accommodate up to 36 kb of foreign DNA [81-86]. AdV can be produced at very high titers, have a broad host tropism and can infect both dividing and non-dividing cells. The transport of AdV to the nucleus is rapid in both dividing and non-dividing cells [87].

Adenovirus is composed of one copy of a double-stranded, linear DNA molecule of 36 kb, which contains in each terminus a 100 bp inverted terminal region (ITR). The E1A region, in the 5' part of the viral genome, encodes proteins that are involved in transactivation of the majority of other viral genes, and also repress the transcription of some cellular promoters [88].

E1B region gene products drive the regulation of late gene expression and the transport of mRNA to the cytoplasm. This region is also required for replication of the viral DNA. The E2 region encodes different proteins involved in DNA replication: DNA binding protein, DNA polymerase and a terminal protein, while the E2B gene product serves as a primer for DNA synthesis. The E3 region is not necessary for viral replication but is involved directly in modulating the immune response of the host *in vivo* [88]. Finally, the protein products of the E4 region are required for efficient viral DNA replication as well as late gene expression. Adenovirus is able to package a maximum of 105% of its total genome length [87;88].

When an adenovirus particle enters a target cell, the fiber knob of the capsid binds first to the attachment receptor CAR on the cell surface. CAR is a high-affinity receptor shared by Coxsackie B virus and Adenovirus (CAR) and was recently cloned [89-92]. Then the penton base binds to low-affinity internalization receptors, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins, and the particles are rapidly internalized into clathrin-coated vesicles [93-95]. The adenovirus binding to the integrins is mediated by the Arg-Gly-Asp (RGD) sequences present in the penton base [96;97]. Recent analysis of the crystal structure of the CAR-Ad fiber knob complex has shown that three CAR monomers bind each knob trimer [90]. The cellular receptor CAR is used by different adenovirus serotypes from subgroups A, C, D, E and F [98].

CAR is encoded by a single gene located on chromosome 21 [99], giving rise to a 46 kDa transmembrane glycoprotein with 2 extracellular immunoglobulin-like domains, a transmembrane spanning domain and a cytoplasmic tail [89;92]. The cellular function of CAR is still unknown. Physiologically, CAR mRNA is detectable in liver, lung, heart and kidney in mouse and in brain, heart, pancreas and liver in human [89;92;100].

A major drawback of E1-deleted adenoviral vectors is the relatively short transgene expression after injection into adult animals. This is mainly due to immune clearance of transduced cells by cellular and humoral immune responses. The transgene antigenicity as well as a low level expression of AdV gene products have been shown to be responsible for triggering immune response [101-105].

Transgene to be used for gene therapy in DMD

It has been demonstrated that introducing a functional version of the dystrophin gene (full-length and mini-dystrophin cDNA) into the germ line of *mdx* mice, resulted in improvements in muscle histology (protection from necrosis, restoration of the DGC at the membrane) as well as strength of dystrophic muscles [106-111]. Also, first generation and high-capacity adenovirus vectors (AdV) have been used successfully to transfer human dystrophin genes to skeletal muscle of *mdx* mice and dystrophin-deficient golden retriever (GRMD) [33;82;84;85;112-119].

Studies using transgenic animals have demonstrated that expression of the truncated or full-length utrophin could also significantly improve the histology and function of *mdx* and dko muscles [120-123]. Utrophin is then localized at the entire sarcolemma, restores the expression of the DPC at the cell surface, reduces muscle necrosis, improves force generation and even dko animals have normal life span. First-generation adenovirus expressing truncated utrophin delivered to *mdx* or dystrophin/utrophin-deficient (dko) neonates improves the morphology of the dystrophic muscle. Utrophin is evenly distributed at the cell surface, protects muscle from subsequent dystrophic damage by restoration of members of the DPC, as evidenced by reduction in the number of centrally nucleated fibers and improvement in force generation [124-126].

Promoter/enhancer to be used for gene therapy in DMD

The ideal promoter and/or enhancer for driving expression of a therapeutic gene in skeletal muscle should be highly active and show specific activity in target cells to minimize possible toxicity and immunogenicity in non target-cells. In addition, for use in viral vectors, the regulatory sequences should be confined to a DNA fragment of small size.

The natural muscle promoter of dystrophin should theoretically be an ideal promoter but it was found that this promoter is very weak [127]. The constitutive viral promoters (Rous sarcoma virus late promoter, RSV-LTR, or the cytomegalovirus immediate early gene enhancer/promoter, CMV) are very active in muscle cells [67;69;128] and are relatively small (<1 kb), but their activity is not restricted to muscle cells [128;129].

Many muscle-specific genes have been cloned and their regulatory sequences are being mapped and characterized. These include α-skeletal actin [130-132], α-cardiac actin [133-135], troponin I [136], myosin light chain 2 [137;138], myosin heavy chain [139] and muscle creatine kinase (MCK) promoters [140]. The α-skeletal actin promoter is relatively weak [141], while myosin light chain, myosin heavy chain and troponin I promoters are fiber-type specific [142-147], which is a drawback for gene therapy in DMD. Muscle creatine kinase has high expression levels in muscle and its promoter/enhancer region is small enough to make it suitable for inclusion in viral vectors. The muscle creatine kinase promoter/enhancer is very active and may tend to "overproduce" dystrophin, but even a marked excess of the gene product did not seem to be deleterious for mature skeletal muscle fibers in transgenic mice [106].

High level of muscle creatine kinase (MCK) expression is restricted to differentiated skeletal and cardiac muscles [148]. In undifferentiated dividing myoblasts, MCK is not expressed, but early after myoblast fusion into myotubes MCK mRNA expression is induced and continues to increase until MCK becomes the predominant creatine kinase isoform [149;150].

The muscle creatine kinase gene is composed of 8 exons and the translation begins in exon 2, 3.2 kb downstream of the transcription initiation site [140]. The full length MCK promoter/enhancer (6.5 kb) consists of a muscle specific enhancer (E1)(-1256 to -1050 bp), a 358 bp proximal promoter (-358 to 0), and a second enhancer (E2)(+738 to +1599) in the first intron (Figure 2)[151-154]. While sequences between -4800 and -1800 bp do not significantly affect MCK gene expression [151;154], the E1 enhancer influences overall MCK expression and confers muscle specificity [151;152;154]. Although the E2 enhancer is not essential for MCK gene activity, it does increase expression levels of the basal promoter [152]. Transgenic mice carrying wild type or mutated 5'-flanking regions of the mouse MCK gene were generated [152]. Adult transgenic mice containing -3300 MCKCAT construct (CAT=Chloramphenicol acetlytransferase gene), showed high CAT activity only in skeletal and cardiac muscles. By deleting sequences between -3300 to -1256 nt, CAT expression in cardiac muscle decreased 200-fold compared to 10-fold in skeletal muscle, suggesting a cardiac regulatory element in this region [152]. -1256MCKCAT transgene exhibited lower CAT activity in skeletal and cardiac muscle compared to the –3300, but transgene activity detected in liver was extremely

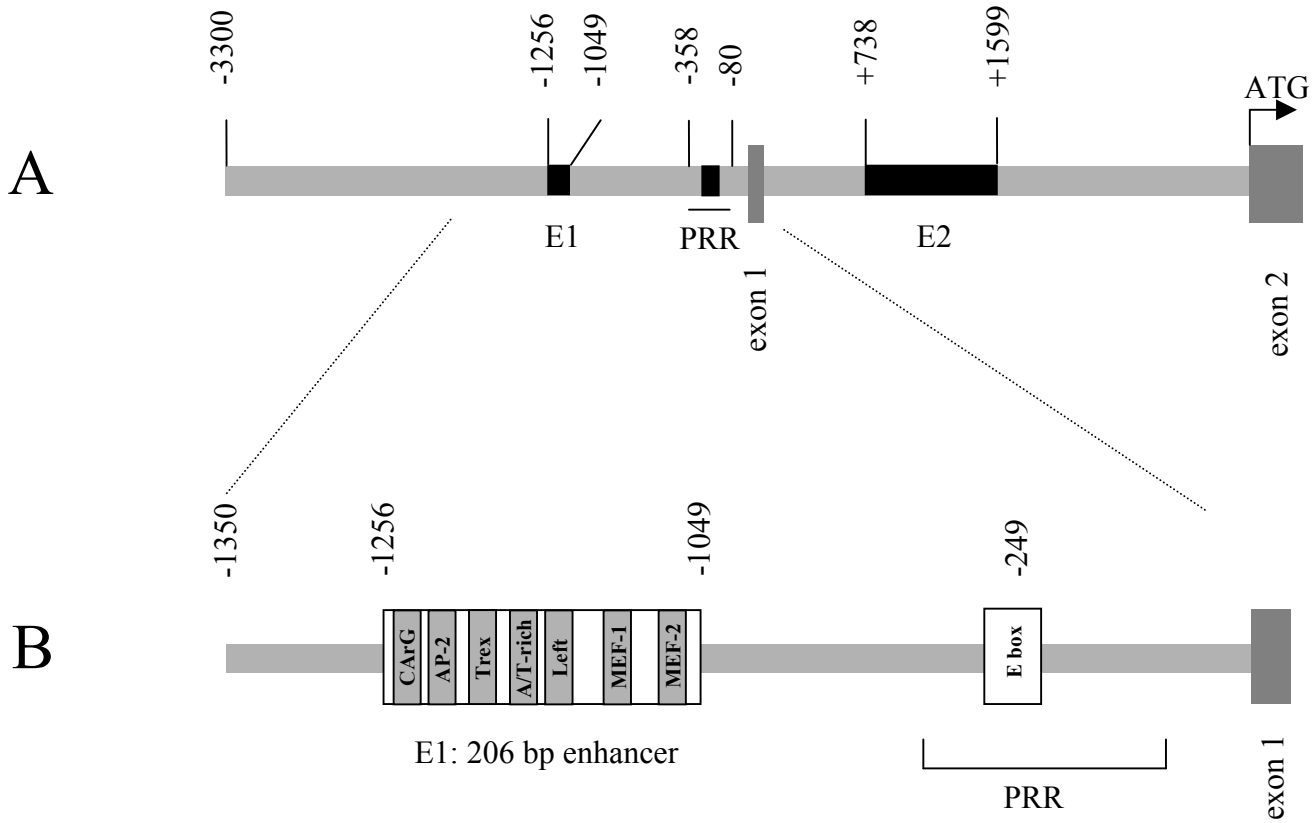


Figure 2. Depiction of the full-length mouse muscle creatine kinase regulatory upstream region **(A)**. E1 and E2 are the abbreviations for enhancer 1 and 2; ATG for the translation initiation site; PRR for proximal regulatory region. The 1.35 kb muscle creatine kinase promoter/enhancer used in these studies with a detailed view of the E1 enhancer and the E box **(B)**. The diagrams are not drawn to scale.

low [152;155]. Transgene expression by the intronic E2 enhancer was also evaluated: it does activate expression in skeletal muscle, but not in heart [152].

Several *cis*-elements in the first enhancer (E1) have been identified that regulate expression of MCK: CArG, AP2, AT-rich, left and right (MEF-1) E boxes, MEF-2 sites, and Trex (Transcriptional regulatory element x)(Figure 2)[154;156-160]. The MCK 5' (E1) enhancer contains many transcriptional regulatory elements that are target sites for DNA-binding factors. The CArG sequence is the binding site for serum response factors (SRF)[161], while the AT-rich element is thought to bind the homeoprotein MHox, MEF-2 and Oct-1 factors [162;163]. Left and right (MEF-1 site) E-boxes contain the consensus CAnnTG sequence and bind members of the MyoD family [157;164;165]. The MEF-2 region, rich in adenine and thymine, is the site for MEF2 proteins and BBF-1, a skeletal serum-inducible factor [159;161;166;167]. A Trex-specific binding factor (TrexBF) complex has been identified in skeletal but not in cardiac myocytes [158].

This 206 bp enhancer region has been delineated into several motifs which, when mutated, lead to diminished promoter activity in skeletal and cardiac muscle [156]. Mutations of the MCK right E box (MEF-1) site decreases dramatically enhancer activity in skeletal (30-90 fold) compared to cardiac muscle cells (up to 7 fold). On the other hand, mutations of the CArG or the MEF-2 sites seem more deleterious in cardiac (up to 20 fold) than in skeletal muscle cells (2-10 fold) [156]. In contrast to these differential effects, mutations of the AT-rich site or the left E box site decrease enhancer activity to about the same level in both skeletal and cardiac myocytes [156]. The presence of the AP2 site is thought to repress transcription in cultured skeletal and cardiac muscle cells since its mutation leads to an increased expression in both cell types [156]. When Trex is mutated in the -1256 MCKCAT construct, it decreases expression in skeletal muscle, but no significant effect is observed in cardiomyocytes. This suggests that the Trex element is required for skeletal muscle but not for cardiac muscle expression of the MCK gene [158].

In summary, expression levels driven by muscle creatine kinase promoter/enhancer are high and restricted to differentiated muscle cells. Furthermore, the E1 enhancer of MCK is sufficient to confer muscle specificity and this fragment is small enough to be included in

viral vectors

Rationale and objectives of the study (Summary)

At present, there are only few reports on successfully applying gene therapy to human diseases. This is in part due to our poor understanding of the various biochemical, genetical and immunological interactions of the host cell/organism with the transferred gene and transfer method/vector. Gene transfer to skeletal muscle of various animal models has been hampered in particular by relatively low efficiency and relatively high toxicity and immunogenicity. Therefore, the main objective of this study was to examine some of the above-mentioned interactions in murine models. Specifically, the attachment of adenoviral vectors to skeletal muscle fibers, the use of a muscle-specific promoter to regulate transgene expression, and the potential of germ line transmission through genomic integration of adenoviral sequences were studied in detail (see below). This may also allow for an improved design of gene therapy vectors and protocols to combat human muscular dystrophy in the future.

Adenoviral vectors are one of the best studied viral vectors for gene therapy. Their primary attachment receptor CAR has been cloned recently [89;92]. Highly efficient adenovirus-mediated gene transfer occurs in immature or in regenerating muscle, but not in adult muscle [112;168-171]. To evaluate how CAR expression may affect the transduction efficiency by AdV in skeletal muscle, transgenic mice expressing the CAR cDNA under the control of the MCK1350 promoter/enhancer were generated. Distribution and level of CAR expression were evaluated in different tissues of transgenic and non-transgenic animals. The susceptibility to AdV transduction was assessed in transgenic and non-transgenic littermates by intramuscular injection of AdVlacZ in adult animals. Transgene expression (lacZ) was quantified and visualized eight days post-injection using different adenoviral titers.

Gene therapy for Duchenne muscular dystrophy is envisaged to introduce an additional copy of the dystrophin gene/cDNA into skeletal muscle fibers to protect cells from necrosis, and to prevent their eventual loss that leads to muscle weakness. In most studies using adenoviral vectors, strong viral promoters such as the cytomegalovirus promoter/enhancer (CMV) or Rous Sarcoma Virus long terminal repeat (RSV-LTR) are used to drive dystrophin expression [33;112;115;118;119]. Since the tropism of adenovirus is non-selective, an ideal promoter and/or

enhancer for driving the expression of dystrophin for DMD gene therapy should be highly active in skeletal muscle cells and show muscle-specific activity. The muscle creatine kinase promoter/enhancer confers high level and restricted expression in differentiated muscle cells. It was previously shown that the MCK1350 promoter/enhancer confers muscle-specific expression of the luciferase transgene in newborn *scid* mice using AdV [172]. Moreover, MCK1350 gave strong transgene expression (luciferase), corresponding to 40% of the constitutive RSV promoter (RSV-LTR).

To determine the potential applicability of this short MCK promoter/enhancer for gene therapy of DMD, adenoviral recombinants containing the luciferase or the dystrophin gene under the control of the muscle-specific MCK1350 promoter/enhancer (AdVMCKlux and the AdVMCKdys) were used to determine the level and the pattern of transgene expression in dystrophic muscles. The AdV recombinants were directly injected into skeletal muscles of neonates and adult *mdx* mice, and transgene expression was assessed up to 60 days post-injection.

An important safety issue concerning the use of viral vectors as a vehicle for gene therapy is the integration of adenoviral DNA into the host genome and the possible germ-line transmission. *In vitro*, it has been shown that recombinant AdV may integrate into the cell genome at low frequency ($\approx 10^{-3}$ to 10^{-5} per cell)[173]. Administrated at high doses intravenously into mouse, AdV is distributed to both ovaries and testis of mice, but it does not lead to transmission to 578 offspring tested [174]. In contrast, transgenesis has been reported as a consequence of adenovirus-mediated gene transfer into mouse zona-free eggs [175]. Similarly, Kubisch and colleagues had evaluated the embryo survival rate and transgene expression after AdV microinjection into the perivitelline, but did not perform any studies in adult animals [176]. These studies showed that AdV DNA may integrate into the germ cells under certain circumstances. To assess the potential of integration of AdV, first generation and gutless adenoviral vectors containing different reporter genes (GFP, green fluorescent protein; lux, luciferase and lacZ, β-galactosidase) under the control of a constitutive promoter (CMV, cytomegalovirus promoter) or the muscle-specific promoter/enhancer MCK1350 were microinjected into early embryos. Different dilutions of AdV stocks were microinjected

under the zona pellucida of mouse embryos. Resulting mice were screened for the presence of the transgene and/or AdV sequences. Positive founders were mated and the offsprings were tested for the germ-line transmission and transgene expression.

CHAPTER II

Muscle-specific overexpression of the adenovirus primary receptor CAR overcomes low efficiency of gene transfer to mature skeletal muscle

Summary

Significant levels of adenovirus (AdV)-mediated gene transfer occur only in immature muscle or in regenerating muscle indicating that a developmentally regulated event plays a major role in limiting gene transfer into mature skeletal muscle. In developing mouse muscle, expression of the primary adenovirus receptor CAR is severely downregulated during muscle maturation. To evaluate how global expression of CAR throughout muscle affects AdV-mediated gene transfer into mature skeletal muscle, we produced transgenic mice that express the CAR cDNA under the control of the muscle-specific creatine kinase promoter. Five-month old transgenic mice were compared to their non-transgenic littermates for their susceptibility to AdV transduction. In CAR transgenics that had been injected in the tibialis anterior muscle with an AdVCMVlacZ, increased gene transfer was demonstrated by the increase in the number of transduced muscle fibers (433 ± 121 *versus* 8 ± 4 fibers) as well as the 25-fold increase in overall β-galactosidase activity. Even when the reporter gene was driven by a more efficient promoter (CMV enhancer/chicken β-actin gene promoter), differential transducibility was still evident (893 ± 149 fibers *versus* 153 ± 30 fibers, $p<0.001$). Furthermore, a five-fold decrease in the titer of injected AdV still resulted in significant transduction of muscle (253 ± 130 *versus* 14 ± 4 fibers). The dramatic enhancement in AdV-mediated gene transfer to mature skeletal muscle that is observed in the CAR transgenics indicates that prior modulation of the level of CAR expression can overcome the poor AdV transducibility of mature skeletal muscle and significant transduction can be obtained at low titers of AdV.

Introduction

Viral vectors used for gene transfer to skeletal muscle must be able to infect post-mitotic cells. In addition, replacement therapy of genetic diseases such as Duchenne muscular dystrophy requires a large viral insert capacity in order to accommodate large cDNAs (e.g., the 13.9 kb dystrophin cDNA). Adenovirus vectors (AdV) fulfill both criteria and have proven to be useful in gene therapy applications directed at muscle [177]. However, a major constraint in the use of AdV is that efficient gene transfer occurs only in immature muscle or in regenerating muscle [169-171;178]. The low level of transducibility of mature skeletal muscle may be partly due to inefficient binding of adenoviral particles to the cell surface.

The entry of AdV into cells involves two different types of receptors: a high affinity primary receptor, the Coxsackie and adenovirus receptor CAR [89;92]and lower affinity secondary receptors that consist of the α_V-containing integrins [$\alpha_V\beta_3$, $\alpha_V\beta_5$] [95;97] and perhaps also $\alpha_5\beta_1$ [93]. The structural features of adenovirus capsid that are implicated in AdV binding to the cell surface are the fiber protein and the penton base protein. AdV binds to its primary receptor through the knob domain at the tip of the fiber proteins projecting from the adenoviral capsid [90;91;179;180]. Analysis of the crystal structure of the CAR/adenovirus fiber knob complex has recently shown that three CAR monomers bind each knob trimer [90]. The N-terminal portion of CAR (amino acids 25-125, the Ig V domain) is sufficient for binding knob in solution and acts as a potent inhibitor of viral infection for cells in culture [181].

The AdV penton base consists of five identical RGD-containing subunits [182] that mediate binding to the heterodimeric cell surface receptors, integrins. Once bound, AdV is internalized via clathrin-coated pits [94]. Not only does recombinant penton base protein block internalization of AdV but RGD-containing peptides also inhibit AdV-mediated transduction[97]. However, prior incubation of cells with the penton base protein does not prevent AdV attachment to the cell surface[97]. Thus, the first stage of AdV infection has been viewed as consisting of two sequential steps involving an attachment receptor (CAR) and an internalization receptor (integrins). At high input doses of AdV (high multiplicity of infection), cells that do not express CAR can still be infected, albeit with a much lower

transduction efficiency. Under these conditions the low affinity binding of penton base to integrins may be sufficient for some CAR-independent binding and uptake of AdV. Conversely there is some evidence that CAR can mediate AdV uptake through an integrin-independent pathway, as AdV in which the RGD site in the penton base has been ablated can still be internalized, at a rate dependent upon the fiber receptor concentration[183].

The decrease in gene transfer that occurs with maturation of skeletal muscle suggests that a developmentally regulated event plays a major role in limiting transgene expression in mature skeletal muscle. Earlier studies had shown that although $\alpha_V\beta_3$ and $\alpha_V\beta_5$ levels decrease by about 70% during myogenesis and maturation of muscle fibers in the mouse[168], their lower levels could not account for the 95% decrease in AdV transduction between the neonate period and 4-6 weeks of age[178]. In developing mouse muscle, expression of the primary adenovirus receptor CAR is severely downregulated during muscle maturation, with CAR transcripts being barely detectable in the adult muscle[184]. Furthermore, it has been demonstrated that forced expression of CAR in mouse myoblasts, followed by transfer of these myoblasts to syngeneic host muscle, resulted in the formation of myofibers with increased susceptibility to AdV transduction[184]. These results suggested that CAR expression limits the susceptibility of myofibers to AdV transduction. The myoblast transfer experiments however did not permit to address certain issues. For example, a relatively small number of CAR-expressing myofibers were obtained by this approach, making it impossible to determine the level of CAR expression required to increase the susceptibility of individual myofibers to AdV transduction. In addition, the majority of CAR-expressing myofibers with increased susceptibility to AdV transduction were of lower diameter than non-transduced fibers. This raised the possibility that fibers with increased susceptibility to AdV transduction were forming predominantly from injected myoblasts and may not have been fully representative of myofibers in intact, mature skeletal muscle. Several factors, including the extensive basal lamina surrounding mature myofibers, may limit the access of exogenously introduced virus to the muscle fiber plasma membrane. To evaluate how global expression of CAR throughout muscle affects AdV-mediated gene transfer into mature skeletal muscle, we produced transgenic mice that express the CAR cDNA in a muscle-specific manner. In these

mice, we evaluated the transducibility of mature skeletal muscle by AdV. Indeed, the continued expression of CAR on muscle plasma membrane markedly improved the extent of AdV-mediated gene transfer to skeletal muscle of 5-6 month old transgenic mice.

Material and Methods

Production and genotyping of transgenic mice

A cDNA containing the full-length coding sequence for Mus musculus CAR mRNA (GenBank accession number Y10320) that was generated by reverse transcriptase-polymerase chain reaction was described previously[184]. The full-length CAR cDNA (nucleotides 1 to 1098) was cloned downstream of regulatory sequences of the muscle specific creatine kinase (MCK) gene (GenBank accession number AF188002). This fragment which spans the region from -1354 to +1 base pairs from the transcription initiation site has previously been described[172], and contains the MCK E1 enhancer and promoter, but not the E2 enhancer found in the first intron of the gene. Transgenic mice were generated by pronuclear microinjection. Founder mice were identified by genotyping of tail DNA for the presence of MCK/CAR fusion sequences. The founders were bred with B6C3F1 mice to verify transgene expression by Northern and Western blot analyses. A single founder expressed CAR mRNA and protein. Subsequently, all mice were genotyped for the presence of CAR cDNA by Southern blot analysis of tail DNA following digestion with *Bam*H1 which yielded an ~ 1kb fragment (Figure 3). Hemizygous CAR transgenic mice were used for AdV transduction experiments between the ages of 5 and 6 months. These animals were normal in development, growth and behaviour.

Analysis of CAR transgene expression

To determine the level of CAR expression, skeletal muscle was homogenized in Trizol (Life Technologies, Burlington, ON, Canada) to extract total RNA according to the manufacturer's instructions. Northern blot analysis was performed as described previously [184]using total RNA (10 μg per sample) that was electrophoresed on a formaldehyde agarose gel, transferred to nitrocellulose membrane and followed by hybridization to the CAR cDNA probe. Equal loading of samples was verified by hybridization with a cDNA probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For Western blot analysis, muscles were excised from mice and homogenized in sample buffer as described [184]. Protein samples (10 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS) using 10% (w/v) acrylamide gels, followed by

electrotransfer to nitrocellulose as described previously [184]. The production, purification and characterization of the polyclonal antibody against CAR has been described in detail previously[184].

In vivo AdV transduction

The recombinant AdVCMVlacZ has been described previously[178]. The AdV recombinant containing an *E.coli* lacZ reporter gene driven by the CMV enhancer/chicken β-actin gene promoter was a kind gift of Dr. James Wilson (Philadelphia)[103]. The AdV preparations were purified by two centrifugations over discontinuous cesium chloride gradients[178]. The viral band was collected, diluted in phosphate buffered saline and desalted on a Sephadex G25 column (Pharmacia). All injections were performed with freshly purified AdV and within each experiment, all groups of animals received the same preparation of AdV at the indicated titer. For all preparations of AdV, the ratio of total particles (as determined by optical density) to infectious titer (as determined by total cytopathic effect on 293 A cells) was between 50:1 to 100:1. Immunosuppression was carried out as described previously [185]. All mice received daily subcutaneous injections of FK506 (5 mg/kg body weight) starting two days prior to AdV administration and continuing until they were euthanized. Hindlimbs (tibialis anterior) of adult mice (5-6 months old) were injected percutaneously with a single deposit of 25 μl of recombinant AdV at a titer of $1X10^{12}$ particles/ml. In some animals, the contralateral tibialis anterior was injected with AdV at a titer of $2X10^{11}$ particles/ml. Eight days after AdV injection, the animals were euthanized and AdV-transduced muscle fibers were identified by histochemistry for β-galactosidase activity. The number of β-galactosidase-positive fibers in the entire tibialis anterior and extensor digitorum longus (EDL) muscles were quantitated by counting the positive fibers under light microscopy. For quantitation of β-galactosidase activity, sixty sections of 10 μm thickness were prepared from the region immediately adjacent to the one that had been sampled for histochemistry. After homogenizing the frozen muscle sections in 100 mM phosphate buffer, pH, 7.8, containing 0.2% Triton X-100, chemiluminescent detection of β-galactosidase was performed according to the manufacturer's instructions (Galactolight, Tropix Inc., Bedford, MA). A BioOrbit luminometer (Turku, Finland) was used to measure light emission. A standard curve was generated by serial dilutions of pure β-galactosidase (Boehringer-Mannheim, Laval, Quebec) and the muscle β-galactosidase activity was converted to ng of enzyme. Differences between

groups were determined either by t-test (unpaired) for the AdVCMVlacZ-injected groups or by analysis of variance (ANOVA) for all other groups, with statistical significances being defined as $p < 0.05$.

Results

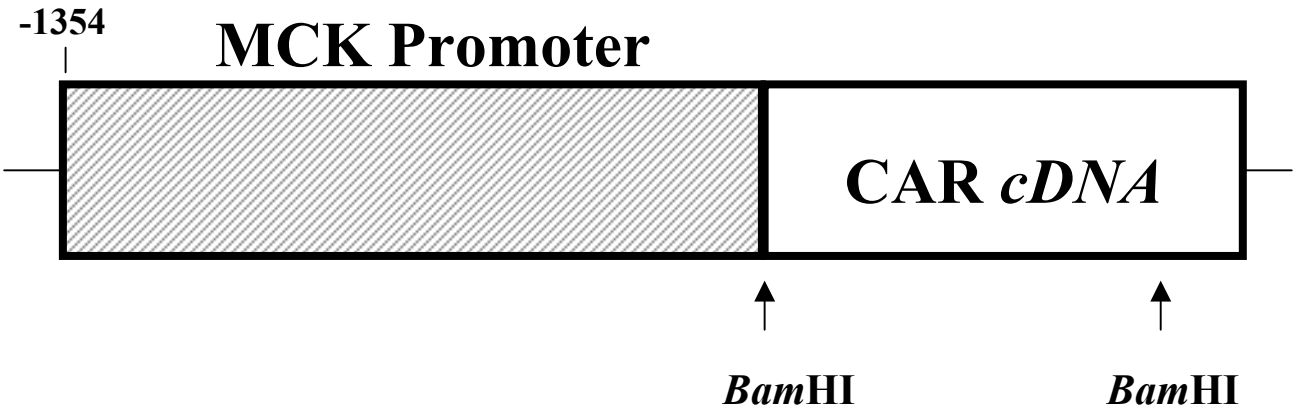
We had previously demonstrated that high level, muscle-specific transgene expression can be obtained using 5' regulatory sequences from the muscle-specific creatine kinase (MCK) gene [172]. In this study we used the same 1354 base pair fragment from the MCK gene to regulate the expression of a full-length CAR cDNA in transgenic mice (Figure 3). Transgenic mice were produced that expressed moderately high levels of CAR in skeletal muscle as assessed by Northern and Western blotting. As shown in Figure 4, little or no expression of the endogenous CAR transcript or protein was detected in the adult control, non-transgenic mouse littermates, confirming previous results [100;184;186]. In contrast, the CAR transgene that was under the control of the MCK1354 promoter had sustained expression even in adult tissue. Furthermore, of several tissues tested, significant expression of CAR was only detectable in skeletal muscle (Figure 5A). To some extent this is a question of antibody sensitivity, as on prolonged exposure, traces of CAR expression could be seen in liver and heart tissue (data not shown). Failure to detect more substantial expression of CAR protein in lung, liver, and heart is somewhat surprising as CAR transcripts are readily detectable in these tissues in nontransgenic mice [92;100], and some protein accumulation would be expected from the endogenous gene. Clearly, CAR expression in muscles of adult transgenic mice far exceeds levels of endogenous expression seen in any adult tissue tested. However, expression of CAR in muscles of the transgenic mice is of the same order of magnitude as expression from the endogenous gene in 10-day postnatal mouse brain (Figure 5B).

The transducibility by recombinant AdV of adult mouse skeletal muscle is much lower than that of skeletal muscle of neonates [169,170;178;187;188]. This decrease in transducibility is evident as early as two weeks of age. To determine the susceptibility of the CAR transgenic mice to transduction by AdV, 5-6 month old transgenic mice were compared to their non-transgenic littermates that served as controls. Animals were injected with a recombinant (E1 and E3 deleted) AdV carrying a CMVlacZ expression cassette. To minimize immune reaction to transgene expression, animals were treated with FK506. Euthanasia was performed eight days later; the injected muscles were sectioned and stained histochemically for β-galactosidase activity. Muscle samples from CAR transgenic mice had a significantly higher

number of β-galactosidase-positive fibers than those of nontransgenic controls (Figure 6A). In accordance with previous data, nontransgenic adult mice had 8 ± 4 positive fibers; in contrast, the CAR transgenics had 433 ± 121 positive fibers (p=0.0015, unpaired t-test). The increase in the number of transduced fibers was accompanied by a similar increase in overall β-galactosidase activity (Figure 6B).

Adenovirus-mediated gene expression in adult muscle is affected by the nature of the promoter that is used to regulate the transferred gene. It had previously been shown that the use of a hybrid promoter comprised of the chicken β-actin promoter and the CMV enhancer resulted in better gene expression in adult skeletal muscle than that obtained with the CMV promoter [189]. To test whether sustained expression of CAR in adult skeletal muscle influences the transducibility by AdV carrying the lacZ gene under the control of this alternative promoter, 5-month old animals were injected in the tibialis anterior muscle with an AdV recombinant containing an *E.coli* lacZ reporter gene driven by the CMV enhancer/chicken β-actin gene promoter. Even with this more efficient promoter there was differential transducibility of skeletal muscle that depended on CAR expression: although nontransgenic littermates had an average of 153 ± 30 positive fibers, CAR transgenics had 893 ± 149 positive fibers ($p<0.001$, ANOVA) (Figure 7). In one animal, a single 25 μl injection of the AdV resulted in the transduction of the entire tibialis anterior and EDL muscles (3000 muscle fibers) (Figure 8A).

We hypothesized that the presence of increased numbers of AdV attachment receptors might obviate the need to inject large doses of AdV to obtain a meaningful number of transduced muscle fibers. The mice that had been injected in the above experiment with AdV at $1X10^{12}$ particles/ml also received AdV in the contralateral tibialis anterior muscle at the lower titer of $2X10^{11}$ particles/ml (injectate containing a total of $5X10^{9}$ particles). Increased transduction due to expression of CAR was obtained even at these lower titers (Figures 7 and 8B) with an average of 253 ± 130 fibers being positive in the CAR transgenics as opposed to 14 ± 4 fibers in the nontransgenic littermates.



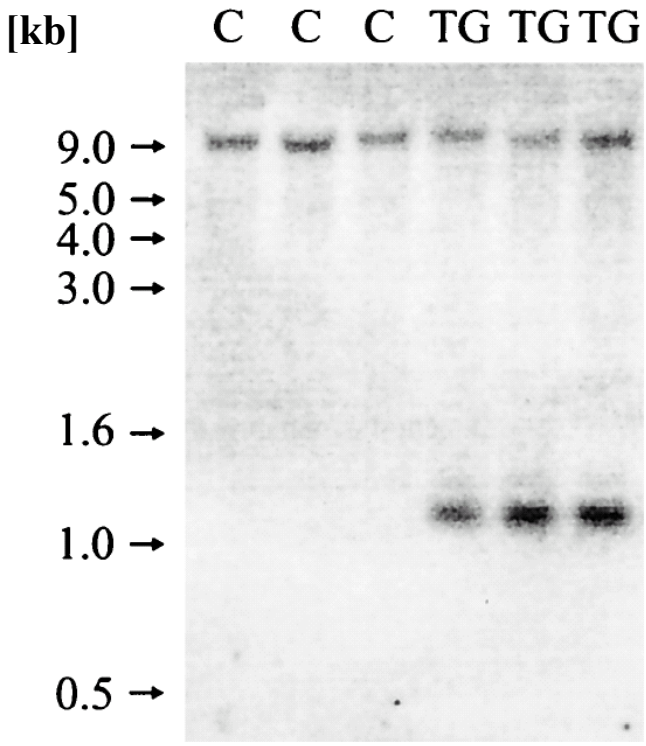


Figure 3. Depiction of the construct used to generate the transgenic mice **(A)** and results of genotyping of the mice by Southern blot analysis **(B)**. **(B)** Genomic DNA was digested with the restriction enzyme *Bam*HI, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with CAR cDNA. A *Bam*HI fragment of ~1 kb is present in transgenic mice (TG) but absent in control, nontransgenic littermates (C). The high-molecular-weight fragment (~9 kb) that is visible in all lanes represents the endogenous CAR gene that cross-hybridizes with the CAR cDNA. Numbers on the left indicate the position of molecular weight markers (1-kb ladder) included in a flanking lane.

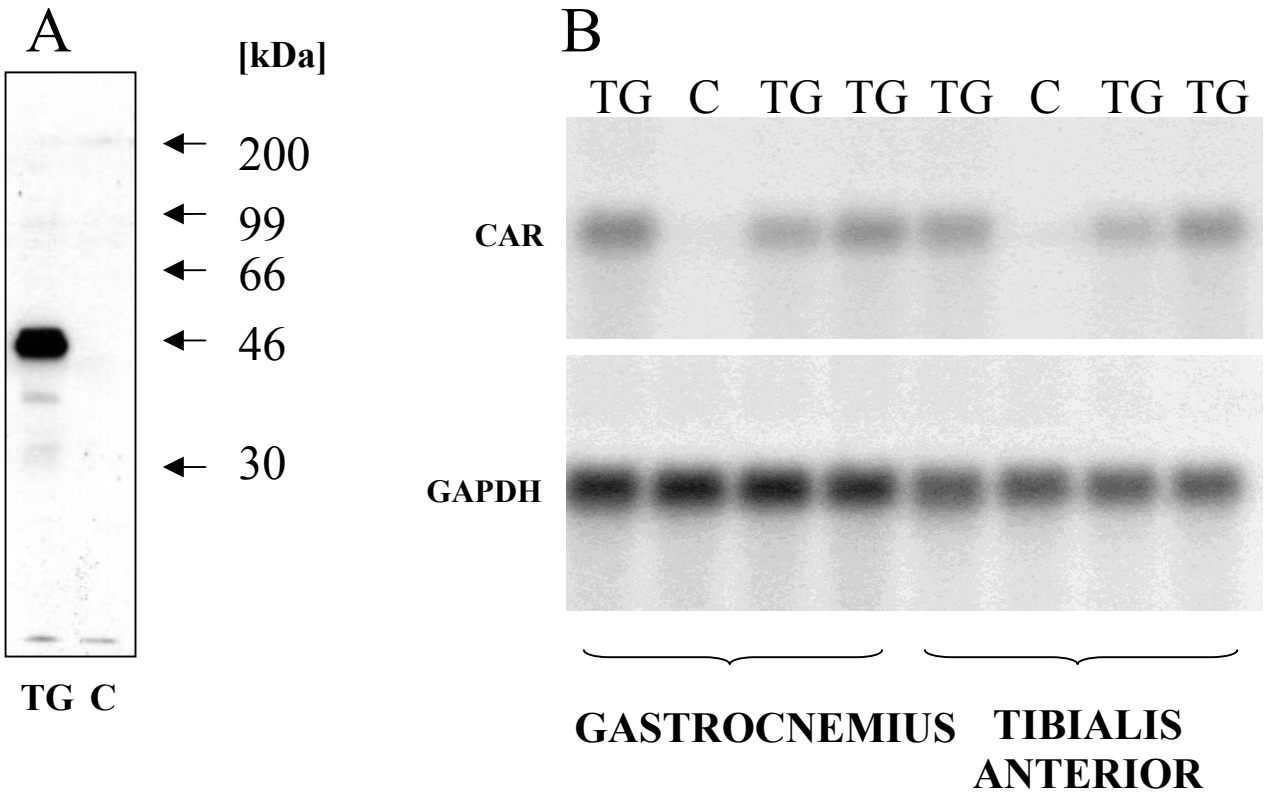


Figure 4. Expression of CAR in transgenic mice and control, nontransgenic littermates as analyzed by Western **(A)** and Northern **(B)** blotting. (A) Samples (10 μg of protein each) of gastrocnemius muscle from an adult mouse (TG) and a control nontransgenic littermate (C) were run on an SDS-10% polyacrylamide gel. Proteins in the gel were transferred to nitrocellulose and incubated with antiserum to the extracellular domain of mouse CAR. A major band corresponding to CAR is visible at ~46 kDa in the transgenic muscle but absent in control muscle. **(B)** Samples (10 μg of total RNA each) from the gastrocnemius and tibialis anterior muscles of three transgenic mice (TG) and one control nontransgenic littermate (C) were electrophoresed on a formaldehyde agarose gel and hybridized with CAR cDNA and glyceraldehyde-3-phosphate dehydrogenase cDNA probes as indicated.

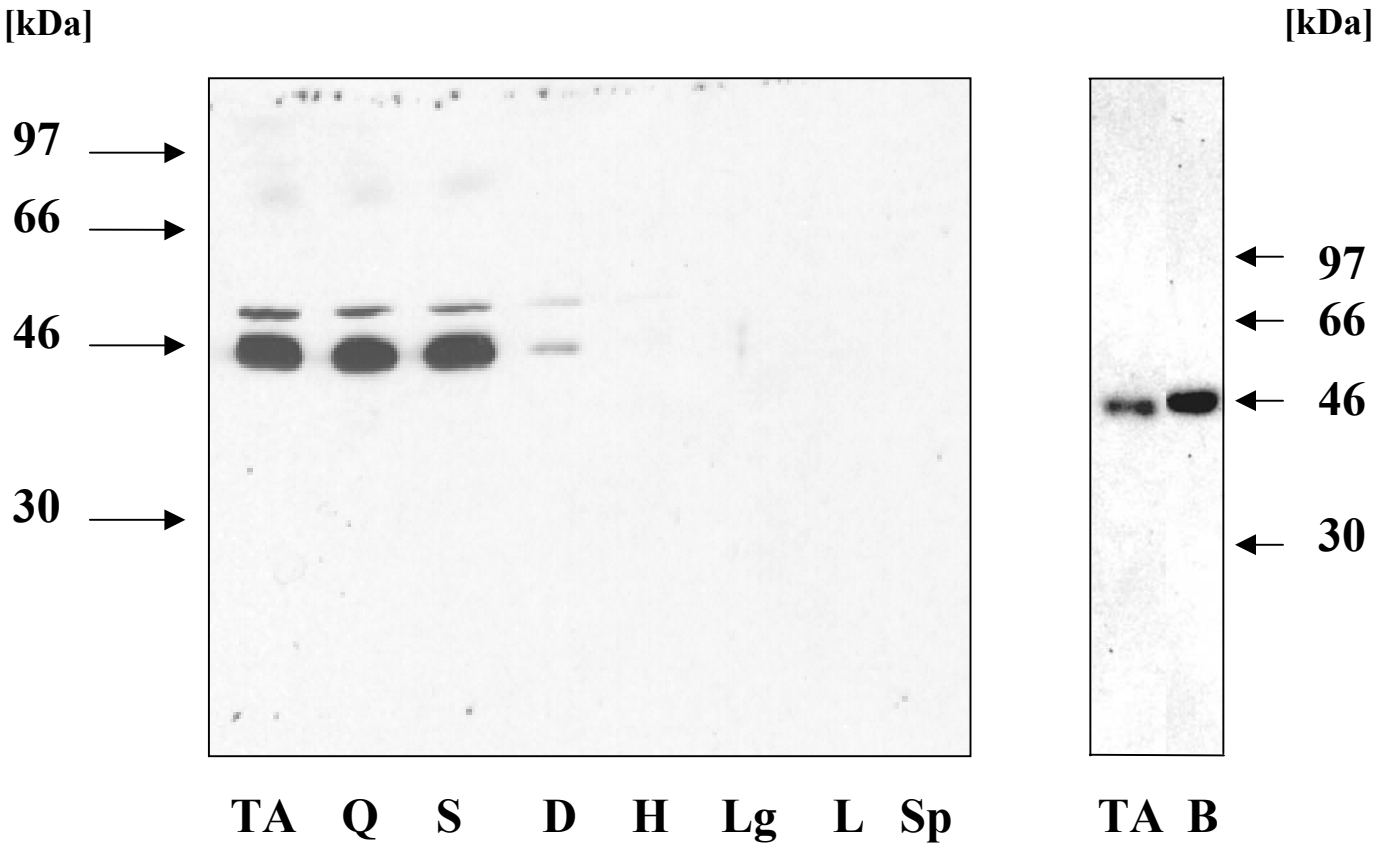


Figure 5. **(A)** Expression of CAR in various tissues of adult (5-month-old) transgenic mice. Samples (10 μg of protein each) of tissue dissolved in Laemmli sample buffer were loaded in each lane of an SDS-10% PAGE gel. Proteins in the gel were transferred to nitrocellulose and incubated with antiserum to the extracellular domain of mouse CAR. From left to right, the samples were from tibialis anterior (TA), quadriceps (Q), soleus (S), diaphragm (D), heart (H), lung (Lg), liver (L), and spleen (Sp) tissue. Numbers on the left indicate the position of molecular weight markers included in a flanking lane. **(B)** Comparison of the level of expression of CAR in tibialis anterior muscle from a 5-month-old transgenic mouse (lane TA) with expression in brain of a 10-day-old control, nontransgenic littermate (lane B). Samples (5 μg of protein each) of tissue dissolved in Laemmli sample buffer were loaded in each lane of an SDS-10% PAGE gel. Proteins in the gel were transferred to nitrocellulose and incubated with antiserum to the extracellular domain of mouse CAR. Numbers on the right panel indicate the position of molecular weight markers included in a flanking lane.

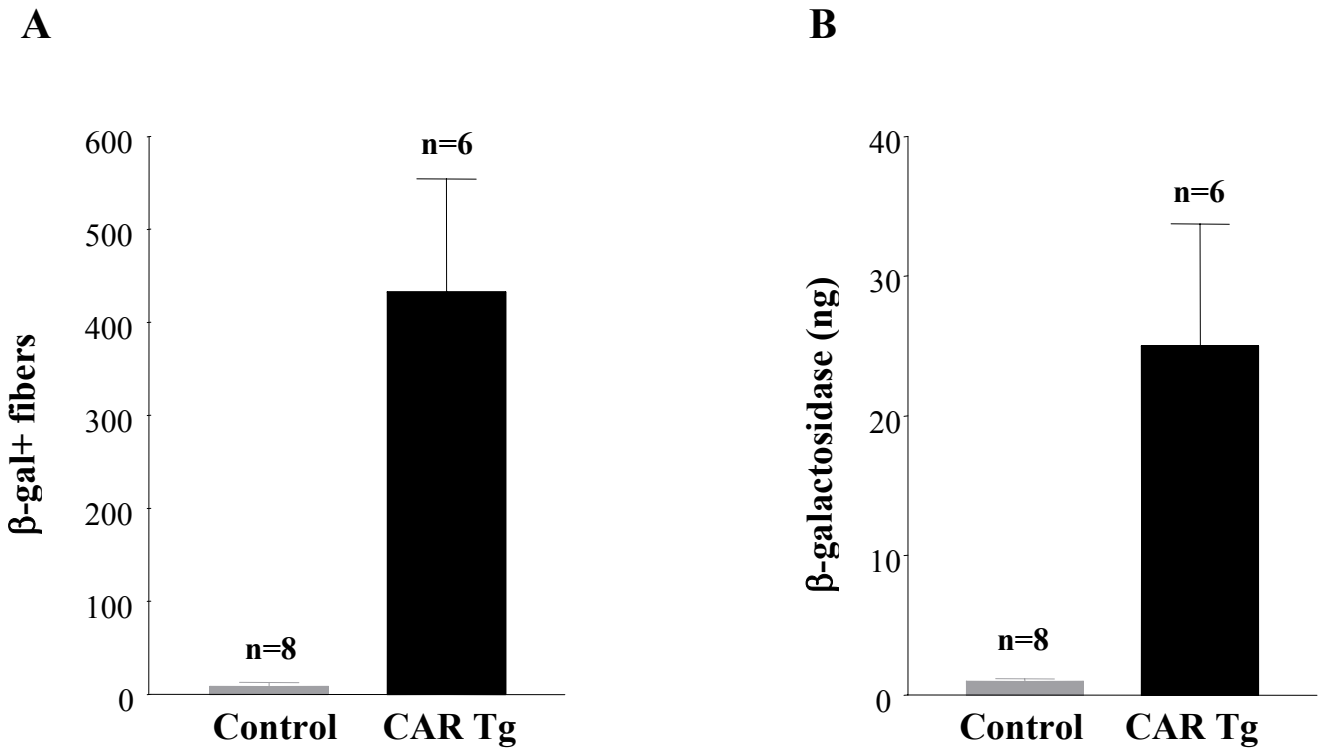


Figure 6. AdV transducibility of CAR transgenics by AdVCMVlacz**. (A)** Quantitation of the number of β–galactosidase-positive fibers, following a single injection of AdVCMVlacz (10^{12} particles/ml) into the tibialis anterior muscle of 5- to 6-month-old hemizygous CAR transgenic mice compared to control, nontransgenic littermates (*P*= 0.0015; unpaired t test). **(B)** The muscles that had been examined in the results shown in panel A were sectioned, and β-galactosidase activity was determined as described in Material and Methods. Results are means ± standard error.

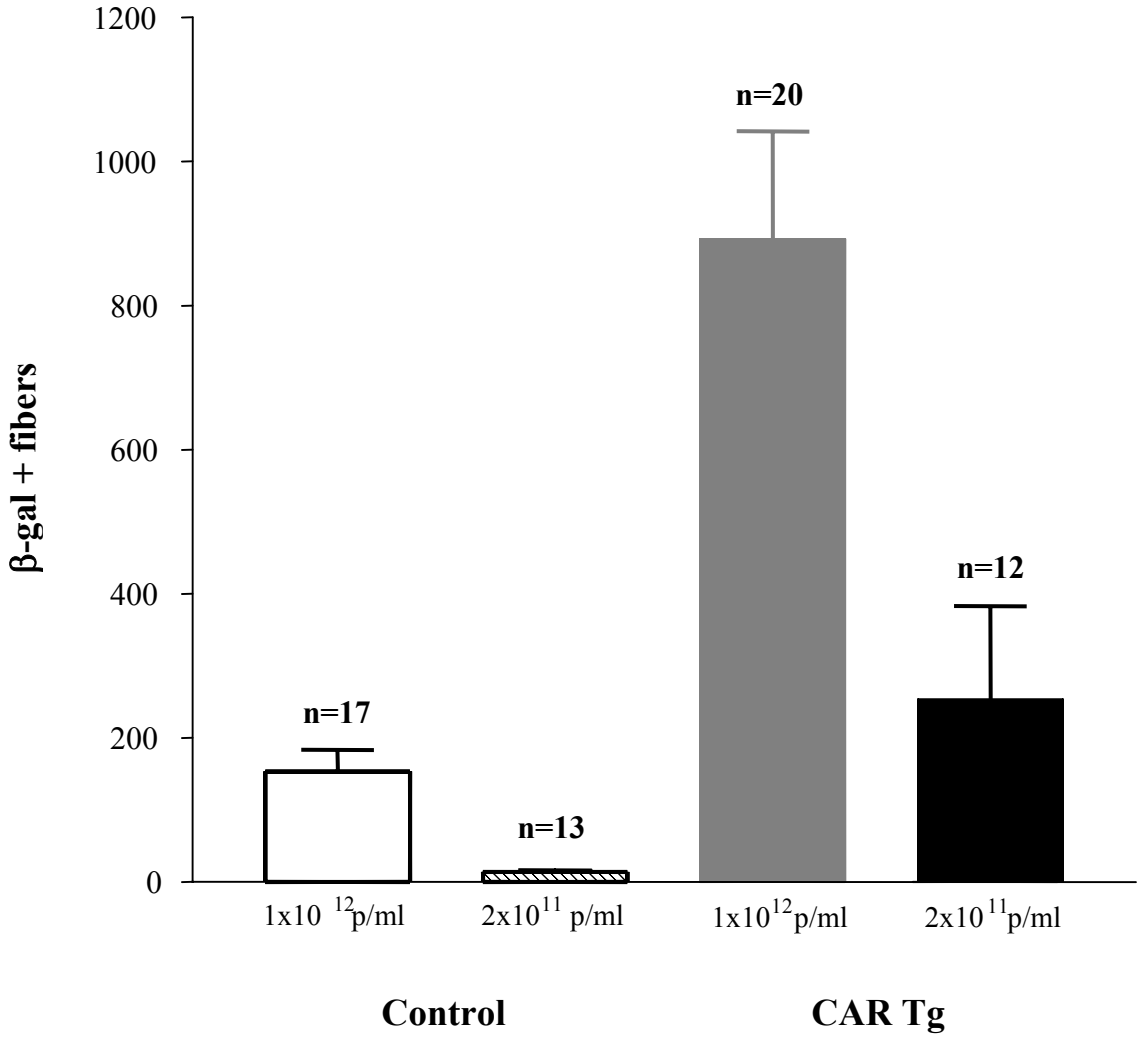


Figure 7. AdV transducibility of CAR transgenics by AdV expressing *lacz* under the control of the CMV enhancer-β-actin promoter. Quantification of the number of β−galactosidase-positive fibers following a single 25-µl injection of either $1X10^{12}$ or $2X10^{11}$ particles/ml into the tibialis anterior muscle of 5- to 6-month-old hemizygous CAR transgenic mice compared to control, nontransgenic littermates. At the higher dose, differences between the control group and the CAR transgenics were significant ($P< 0.001$; ANOVA). Note that the transducibility obtained with the CAR transgenics injected with the lower dose is similar to that with controls receiving the higher dose of AdV (differences between these two groups are not significant by ANOVA), followed by the Bonferroni posttest). Results are means ± standard error.

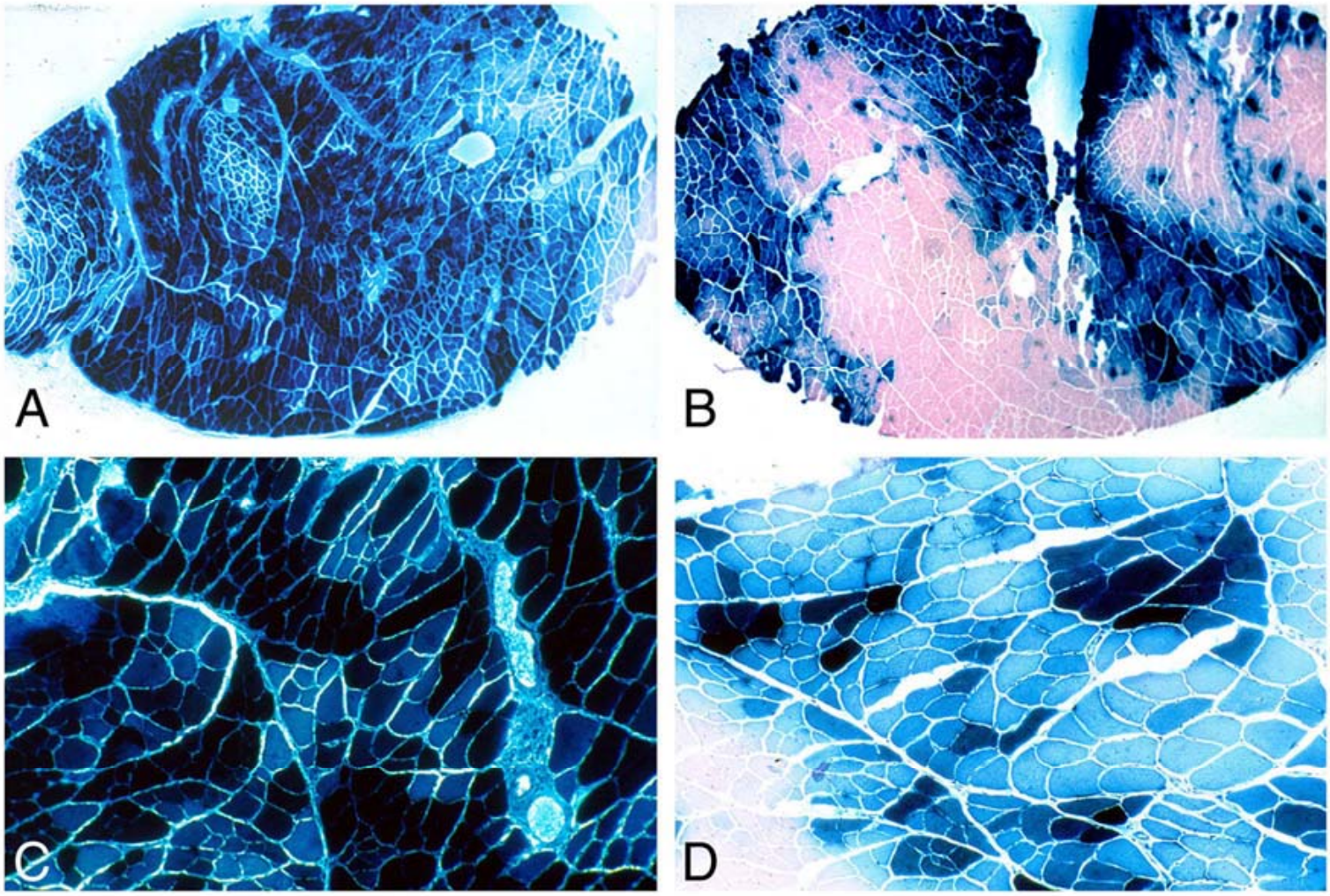


Figure 8. AdV transducibility of CAR transgenics by AdV expressing *lacZ* under control of CMV enhancer/β–actin promoter. Photomicrographs of mouse tibialis anterior muscle showing histochemical staining of muscle fibers for β-galactosidase activity subsequent to a single injection of AdV at a titer of $1x10^{12}$ **(A and C)** or $2x10^{11}$ **(B and D)** particles/ml in the contralateral muscle are shown. Note that at the higher dose of AdV, all fibers are expressing β-galactosidase.

Discussion

A major issue in gene therapy is efficient and widespread delivery of the therapeutic gene to the target tissue. AdV-mediated gene transfer occurs inefficiently in adult skeletal muscle. The transcript for the primary AdV receptor CAR is undetectable by Northern blot analysis of skeletal muscle tissue of human [89;92;190], mouse [92;100;184;191;192] and rat [193] origin. Even when sensitive detection methods are used such as reverse transcriptase/polymerase chain reaction (RT-PCR) [184] and competitive RT-PCR [193]to estimate the abundance of the CAR mRNA, the levels of CAR transcript are extremely low in adult mouse skeletal muscle. Thus, the lack of CAR may be a major impediment to efficient gene transfer to mature skeletal muscle.

Forced expression of exogenous CAR has recently been used to facilitate the entry of AdV serotype 5 into a number of cell types that are not generally susceptible to AdV such as primary fibroblasts [194], lymphocytes [195], myoblasts [184] and tumor cells [196]. However, it was unclear whether CAR could effect an increase in gene transfer *in vivo* where presumably greater barriers exist that can hinder the interaction between AdV and the cell surface. In this regard, mature skeletal muscle fibers are surrounded by a well-developed basal lamina that could theoretically limit the access of AdV to the muscle fiber plasmalemma. In order to address the question of whether the absence of CAR is a major limiting factor in AdV-mediated gene transfer *in vivo* to mature skeletal muscle, we produced transgenic mice that maintained a relatively high level of CAR expression in their skeletal muscle. In the present study, when AdVCMVlacZ was injected intramuscularly at a single site into the tibialis anterior of 5-6 month old animals, the number of β-galactosidase positive fibers was consistently higher in the CAR-expressing transgenics as compared to their non-transgenic littermates (Figure 6A). The dramatic enhancement in AdV transducibility indicates that upregulation of CAR can overcome local constraints to tissue penetration by the AdV in these relatively old animals.

The experimental results obtained *in vivo* measure the transducibility of the tissue: the final readout is a consequence of both the entry of AdV and the subsequent expression of the expression cassette contained within the viral vector. Thus, a low level of transducibility as

determined by low transgene expression may result from lack of virus attachment/internalization and/or transcriptional inefficiency within specific tissues of the transgene promoter that is used. We specifically examined this issue by comparing in CAR transgenic mice and control littermates the transduction efficiency that was obtained when the *E. coli* lacZ reporter gene was placed under the control of the hybrid β-actin/CMV promoter. As expected, this transcriptional unit is expressed at higher levels in mature skeletal muscle [189], resulting in a higher number of β-galactosidase positive fibers in the control mice (153 ± 30) than in muscles of those injected with AdVCMVlacZ (8 ± 4) [Figures 6 and 7]. Remarkably, the presence of CAR also influenced the levels of transducibility that was attained in this experiment with an average of six-fold increase in the number of β-galactosidase positive fibers in the CAR transgenics. In addition, in one transgenic mouse, a single injection of $2.5X10^{10}$ viral particles resulted in the transduction of the entire tibialis and EDL muscle, achieving the same extent of transduction that is usually only observed in neonate mice (Figure 8). In this context, the efficiency of AdV-mediated gene transfer has been ascribed to the presence of myoblasts in the developing muscle [197]. Moreover, it was suggested that multinucleated myofibers were incapable of being transduced by AdV [186]. However, our results clearly demonstrate that under appropriate biological conditions, extremely efficient AdV transduction of mature skeletal muscle can be achieved. Furthermore, these results also suggest that modulation of CAR levels can significantly decrease the dose of administered vector needed to obtain acceptable levels of gene transfer for therapeutic purposes.

A potential means of circumventing the poor transducibility of adult skeletal muscle would be through the use of adenoviral vectors engineered to have modified tissue tropism. One such vector is the AdZ.F(pK7) in which lysine moieties have been incorporated into the AdV fiber protein to target surface receptors containing heparan sulfate [198;199;200]. Although no significant difference was observed in transduction efficiency between AdV with wild-type fiber protein (AdZ) and AdZ.F(pK7) in neonatal mice injected in the hind limb, there was a four-fold-increase in the adult mice (4 to 5 month of age) that were injected in the EDL muscle [201]. Curiously, in the neonates, unlike the relatively even distribution of AdZ, the fibers that were transduced with AdZ.F(pK7) were those at the periphery of muscle fascicles

and the perimysial connective tissue [201]. In the adult, only a proportion of muscle fibers were transduced perhaps as a consequence of the occupancy of the receptors by endogenous ligands and components of the extracellular matrix. In a separate study by van Deutekom and colleagues, the efficiency of transduction of adult normal and dystrophic muscle with AdZ.F(pK7) was shown to be significantly lower than what is commonly obtained with wild-type fiber-containing AdV in neonate skeletal muscle [186]. In this regard, our results clearly show that upregulation of CAR can lead to complete transduction of the tibialis anterior and EDL of adult 5-to 6-month-old mice (Figure 8A).

We produced transgenic mice that express CAR in order to address specific issues in AdV-mediated gene transfer to adult skeletal muscle (lack of AdV receptors, presence of physical barriers). The dramatic enhancement in AdV-mediated gene transfer to mature skeletal muscle that is observed in these CAR transgenics indicates that prior modulation of the level of CAR expression results in extremely efficient AdV transducibility of mature skeletal muscle. In the context of gene therapy directed to human muscle, a transient increase in CAR expression could be achieved either through activation of the transcription of endogenous CAR gene, or as part of a two-step gene therapy protocol, by regulatable expression of CAR delivered through a different viral vector such as the adeno-associated virus.

CHAPTER III

The short MCK1350 promoter/enhancer allows for sufficient dystrophin expression in skeletal muscles of *mdx* mice

Summary

First generation adenovirus vectors (AdV) have been used successfully to transfer a human dystrophin minigene to skeletal muscle of *mdx* mice. In most studies, strong viral promoters such as the cytomegalovirus promoter/enhancer were used to drive dystrophin expression. More recently, a short version of the muscle creatine kinase promoter (MCK1350) has been shown to provide muscle-specific reporter gene expression after AdV-mediated gene delivery. Therefore, we generated an AdV where dystrophin expression is controlled by MCK1350 (AdVMCKdys). AdVMCKdys was injected by the intramuscular route into anterior tibialis muscle of *mdx* mice shortly after birth. Dystrophin expression was assessed at 20, 30 and 60 days after AdV-injection. At 20 days, muscles of AdVMCKdys injected *mdx* mice showed a high number of dystrophin-positive fibers (mean: 365). At 60 days, the number of dystrophin-positive fibers was not only maintained, but increased significantly (mean: 600). In conclusion, MCK1350 allows for sustained dystrophin-expression after AdV-mediated gene transfer to skeletal muscle of newborn *mdx* mice. In contrast to previous studies, where strong viral promoters were used, dystrophin expression driven by MCK1350 peaks at later time points. This may have implications for the future use of muscle-specific promoters for gene therapy of Duchenne muscular dystrophy.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive skeletal muscle disease that is inherited as an X-linked trait and affects 1/3500 newborn boys. Affected boys are usually confined to a wheelchair before the age of 12 and die in their early twenties by respiratory or cardiac failure. DMD is characterized by a complete absence of functional dystrophin (427 kDa) caused by various mutations of the dystrophin gene [202]. As in DMD patients, *mdx* mice lack dystrophin in their muscle fibers due to a stop codon. Although *mdx* mice do not show a severe clinic phenotype, their skeletal muscles exhibit degeneration and necrosis throughout life which makes them a good animal model for DMD [56]. Becker muscular dystrophy (BMD) is an allelic disorder that is less severe with later onset and longer survival. A "mini-dystrophin" has been isolated from a benign BMD patient lacking a large part of the central region including exons 17 to 48 [52]. Studies in transgenic mice have shown that the dystrophic phenotype improves dramatically in *mdx* mice expressing the 6.3 kb mini-dystrophin gene [110].

First generation and high-capacity adenovirus vectors (AdV) have been used successfully to transfer human dystrophin genes to skeletal muscle of *mdx* mice [82;112;118;119]. In most studies, strong viral promoters such as the cytomegalovirus promoter/enhancer (CMV) or Rous Sarcoma Virus long terminal repeat (RSV-LTR) were used to drive dystrophin expression. Since the tropism of adenovirus is non-selective, an ideal promoter and/or enhancer for driving the expression of dystrophin for DMD gene therapy should be sufficiently active in all skeletal muscle cells, but not in other inadvertently transduced cells.

We have shown that a short version of the muscle creatine kinase promoter/enhancer (MCK1350) provides muscle-specific reporter gene expression after adenovirus-mediated gene transfer in newborn *scid* mice [172]. Moreover, MCK1350 gave strong transgene expression (luciferase), corresponding to 40% of the constitutive RSV promoter (RSV-LTR). We next tested this reporter construct (AdVMCKlux) in *mdx* animals in order to assess efficacy of expression in dystrophic muscle. We also generated an adenoviral recombinant in which the mini-dystrophin gene was placed under the control of MCK1350 (AdVMCKdys). Our results indicate that the short MCK promoter/enhancer functions very efficiently in the extended first-generation adenoviral backbone.

Material and methods

Construction of recombinant adenoviruses

The MCK fragment contains the region from -1354 to +1 bp from the transcription initiation site, as described previously [172]. We have previously described the adenoviral recombinants AdVMCKlux and AdVRSVlux that express luciferase [172]. The 6.3 kb mini-dystrophin was cloned under the control of the MCK promoter/enhancer fragment as an expression cassette into a plasmid containing sequences from human adenovirus type 5. The homologous recombination of the replication-defective human adenovirus type 5 with the cassetted gene was performed using an adenovirus in which the entire E1- and E3-regions were deleted to accommodate the 8.5 kb expression cassette [203]. Plaque-purified AdVMCKdys was used to infect 293 cells grown in spinner cultures at a density of 10^6 cells/ml by using a multiplicity of infection (moi) of 3. The large-scale production, purification and titration of the recombinant AdV have been described in detail previously [178]. The absence of E1-containing replication-competent AdV was confirmed using a sensitive PCR screening assay as previously described [204].

Injection of adenoviral recombinants into *mdx* mice

Experiments were performed on groups of *mdx* mice (Jackson Laboratories, Bar Harbour, ME, USA). The animal studies were conducted in accordance with the guidelines of the Canadian and the German Council of Animal Care. *mdx* mice were anesthetized by intraperitoneal injection with 0.3-0.4 ml of avertin (2.5% tribromoethyl alcohol and 2.5% amyl alcohol) in normal saline. Bilateral tibialis anterior muscles (TA) were injected percutaneously with AdV suspension ($1X10^{12}$ particles per ml) in a volume of either 5 µl (newborn 3-5 day old mice) or 25 µl (adult 6 week old mice). Animals were euthanized at 20, 30 and 60 days post-injection.

Immunohistochemistry

The muscles were embedded on blocks, then frozen in liquid nitrogen-cooled isopentane. Six-micrometer serial cross sections were obtained in a cryostat (Zeiss), placed on gelatinized slides and fixed with acetone for 1 minute. Immunohistochemical procedures were carried out for dystrophin using a rabbit anti-dystrophin (carboxy-terminus) polyclonal antibody (kind

gift of Dr. Paul Holland, Montreal Neurological Institute, Canada). Biotinylated anti-rabbit secondary antibody was applied and visualized by horseradish peroxidase. In animals injected with adenoviral recombinants, the total number of dystrophin-positive fibers was counted for each TA. The extent of central nucleation was quantified in dystrophin-positive fibers by lightly counterstaining immunostained sections with hematoxylin to visualize all skeletal muscle nuclei. The data were then statistically analyzed by ANOVA (Analysis of variance) tests.

Luciferase assay

For luciferase quantification, the dissected muscles were homogenized and centrifuged as described [178]. For each individual sample the luciferase activity (given in mV equals the integrated light emission for 20 sec.) was calculated for the total sample volume of 200 µl and converted to picograms of pure luciferase protein (ALL) using a standard curve for luciferase. The data were then statistically analyzed by ANOVA (Analysis of variance) tests.

Results

The MCK1350 promoter/enhancer used in this study contains the MCK E1 enhancer and promoter, but not the E2 enhancer that is present within intron 1 of the MCK gene [154]. The MCK1350 regulatory sequences were shown previously to drive the expression of the luciferase reporter gene in a muscle-specific manner in newborn *scid* mice [172].

Injection of AdVMCKlux into adult *mdx* skeletal muscle was undertaken to determine levels of expression that could be achieved in the *mdx* model through the use of the MCK1350 promoter /enhancer in an adenoviral recombinant. Similar levels of luciferase activity were detected after injection of either AdVRSVlux or AdVMCKlux into anterior tibialis muscles (Figure 9). However, in comparison to the pattern of expression obtained with AdVRSVlux, luciferase expression that is regulated by the MCK1350 promoter/enhancer peaked later, being very low at the 10 day time point, and reaching a maximum at the 30 day time point, comparable to the levels attained at 10 days after injection of AdVRSVlux.

We then generated adenoviral recombinants in which MCK1350 was used to regulate the expression of the 6.3 kb human mini-dystrophin gene. To compare the distribution and level of expression of the mini-dystrophin, *in vivo* experiments were carried out in newborn 3-5 day old *mdx* mice. AdV recombinants were injected directly into tibialis anterior muscles, which were sampled at 20, 30 and 60 days after injection. Young mice were chosen to enhance transduction efficiency of muscle by AdV [178], and to minimize immune reaction that may compromise long-term transgene expression, as demonstrated previously [112]. As shown in Figure 10, a high number of dystrophin-positive muscle fibers was detected at 20 days post-injection (mean=365). At 30 days, the number of dystrophin-positive fibers was significantly increased ($p<0.05$) and sustained up to 60 days (mean=600). These results parallel the same temporal pattern of expression that had previously been observed with AdVMCKlux (Figure 9).

Dystrophin staining after AdVMCKdys injection was confined to the subsarcolemmal region of muscle fibers (Figure 11a). In contrast, dystrophin staining after AdVdys injection using a strong viral promoter (CMV) was detected in the cytoplasm in addition to the subsarcolemmal

region (Figure 11b). Such cytoplasmic staining, which is visualized best in smaller size fibers in which the accumulation yields more intense signal, may result from an overexpression of the transgene by the viral promoter as has been reported previously [112;118].

In *mdx* muscle, multiple cycles of necrosis and regeneration result in the presence of centralized myonuclei which are characteristics of regenerated fibers. To determine whether the level of dystrophin expression obtained by injection of AdVMCKdys is sufficient to restore the dystrophin-glycoprotein complex and to avoid multiple cycles of regeneration/degeneration of the muscle fibers, the number of central nuclei was quantified in dystrophin-positive fibers (Table 1). The low percentage of central nuclei in the AdVMCKdys-transduced fibers, which reflects the lower prevalence of regenerated fibers, indicates that the dystrophin levels produced by this adenoviral recombinant can efficiently protect muscle fibers from necrosis.

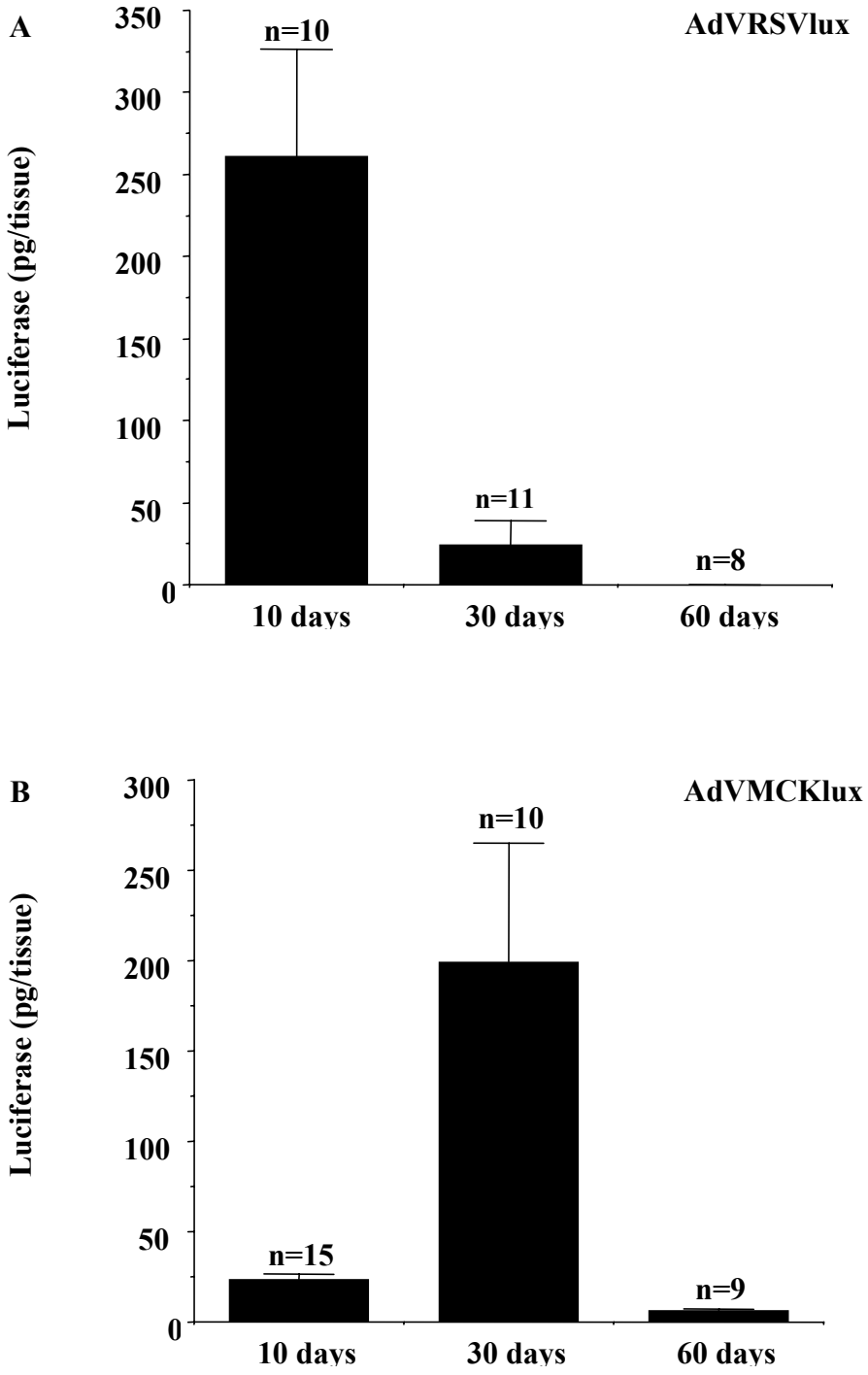


Figure 9. Expression of luciferase in TA of adult *mdx* mice analyzed 10, 30 and 60 days after direct injection of AdVRSVlux **(A)** or AdVMCKlux **(B)**. Total luciferase activity (mean ± standard error) was calculated as described in Material and Methods.

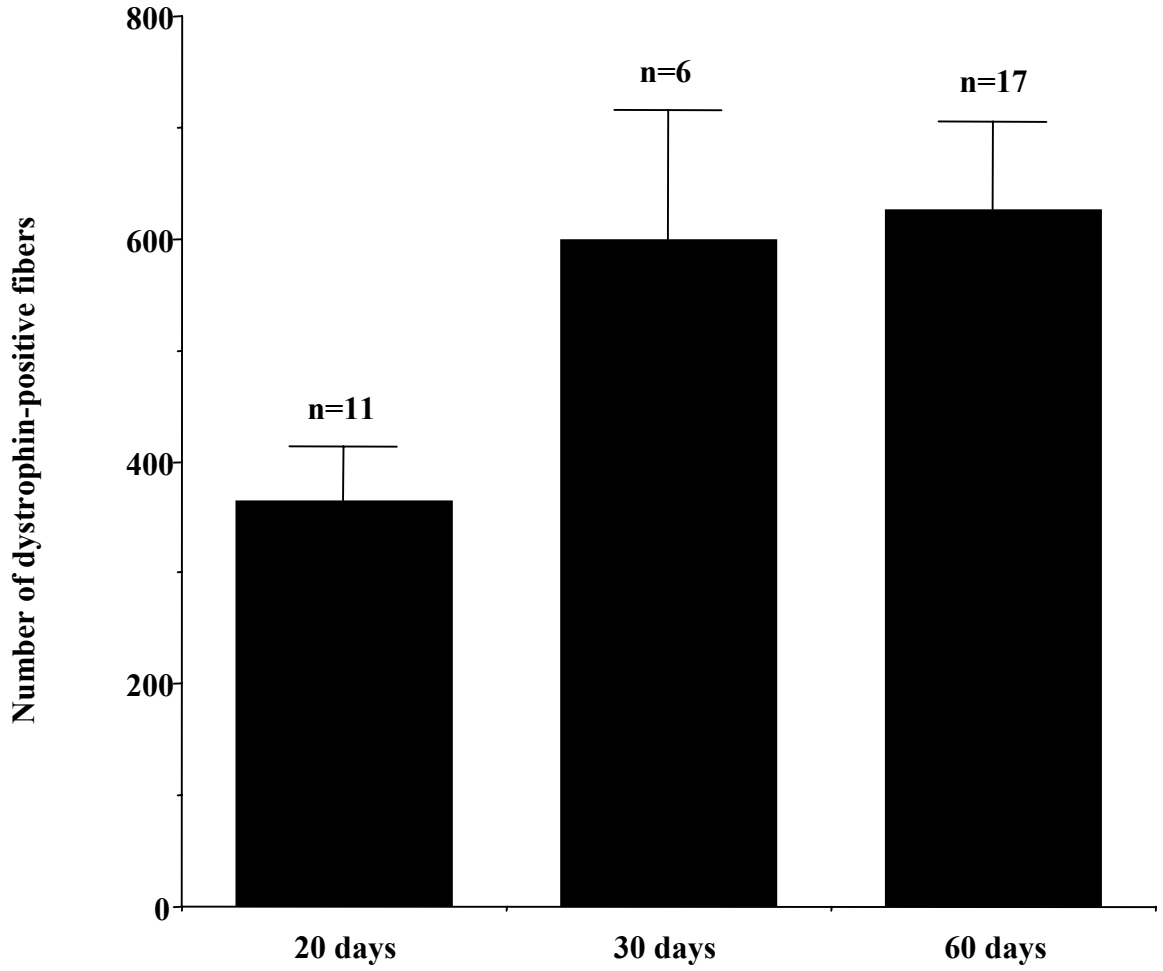


Figure 10. Number of dystrophin-positive fibers in tibialis anterior of newborn *mdx* mice 20, 30 and 60 days post-injection with a viral suspension of AdVMCKdys. The number of dystrophin-positive fibers is high in animals analyzed at 30 and 60 days after AdV injection if compared to animals analyzed at 20 days ($P<0.05$). Results are means ± standard error.

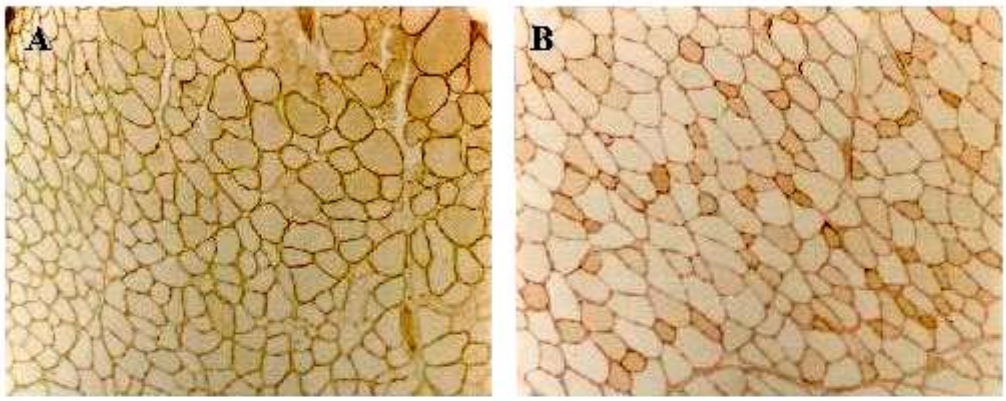


Figure 11. Dystrophin staining of tibialis anterior muscle of *mdx* mice injected at the age of 3-5 days with **A)** AdVMCKdys or **B)** AdVCMVdys analyzed at 60 days post-injection.

Table 1. Quantitative analysis of central nuclei (cn) in dystrophin-positive and dystrophin-negative fibers after AdVMCKDys injection into *mdx* muscles (numbers of cn in dystrophin-positive fibers were counted in the great majority of the positive fibers for dystrophin). Numbers of central nuclei in dystrophin-negative fibers (approximately 500 fibers in uninjected controls) were as previously published [126] and are similar to Torres and Duchen, 1987 [57].

End point measurement	# of dystrophin-positive fibers counted for cn	# of cn in dystrophin-positive fibers	Central nuclei in dystrophin-positive fibers	Central nuclei in dystrophin-negative fibers
20 days	277 +/- 36	6.3 +/- 1.3	2.3 %+/- 0.4	~45 % [(57)]
30 days	585 +/- 111	11.3 +/- 4.4	2.0 %+/- 0.5	~48-56 % (57;126)
60 days	433 +/- 43	21.9 +/- 3.5	5.4 % +/- 0.7	~56-60 % (57;126)

Discussion

First generation AdV are widely used as vectors for gene therapy. Since the tropism of AdV is not restricted to muscle, a muscle-specific promoter/enhancer to drive dystrophin expression is desirable for future gene therapy for DMD. Previously, we had shown that the MCK1350 promoter/enhancer confers muscle-specific expression of the luciferase transgene in newborn *scid* mice using AdV [172]. This conclusion was reached after direct injection of AdVMCKlux into various organs, followed by a sensitive luminometric assay for the reporter gene (luciferase). These studies indicated that while AdVMCKlux had transduced the various organs (as assessed by *in situ* PCR assays for detection of AdV genomes), minimal or no luciferase activity was present in tissues other than skeletal muscle. In this model, MCK1350 also showed strong transgene expression, corresponding to 40% of the constitutive RSV promoter [172].

The present results also confirm the robustness of the MCK1350 regulatory sequences in driving the expression of the mini-dystrophin gene (Figures 10 and 11). It has previously been demonstrated that the full-length MCK promoter/enhancer (6.5 kb) or upstream regulatory elements of 3.3 kb containing the E1 enhancer driving dystrophin genes rescued the dystrophic phenotype in transgenic *mdx* mice [106;107;109;111]. However, larger deletions of the MCK promoter/enhancer resulted in a severe diminution of transgene expression and loss of muscle specificity as ascertained in mice transgenic for reporter genes driven by various truncated promoter constructs. It was thus concluded that the E1 enhancer (positioned between –1256 and –1049) plays an important role in muscle-specific expression. Interestingly, MCK1256 conferred only low levels of expression in transgenic mice [152;155] or within the context of a first generation AdV [205]. In transgenic mice expressing reporter constructs, depending on the site of integration, MCK1256 provided 10 to 100-fold lower expression than MCK3300 [152]. In contrast, as demonstrated in this study, MCK1350 is extremely efficient. Although the additional ~ 100 bp fragment does not contain additional positive transcriptional activity [151], we hypothesize that the presence of this fragment may allow for a better interaction of *trans*-acting factors with the E1 enhancer in these situations.

The effectiveness of MCK1350 was further demonstrated when we produced a first generation AdV in which expression of the mini-dystrophin gene was regulated by these sequences (AdVMCKdys). Dystrophin staining was confined to the subsarcolemmal region of muscle fibers after injection of AdVMCKDys. In contrast to previous experiments using AdVDys containing constitutive promoters [112], dystrophin was not observed in the cytoplasm when using MCK1350. This suggests that overexpression of dystrophin did not occur in skeletal muscle after AdVMCKdys transduction. Nevertheless, the sustained low percentage of central nuclei in AdVMCKdys-transduced muscle fibers (2-5%) shows that these fibers are protected against the ongoing dystrophic process of dystrophin-deficient muscle (Table 1). In comparison, *mdx* mice have ~56% central nucleation at 2 months of age and ~88% central nucleation at 3 months of age [109;110;112;126]. Importantly, these results indicate that the level of dystrophin expression provided by the MCK promoter in the context of the AdV is sufficiently high to protect dystrophic fibers from necrosis.

When newborn *mdx* mice were administered a single injection of AdVMCKdys, the number of dystrophin-positive fibers increased significantly with time, from 20 to 30 days and remained high at 60 days post-injection ($p<0.05$). In previous studies, the use of strong constitutive promoters such as CMV or RSV led to an earlier expression of the transgene, with the number of dystrophin-positive fibers reaching a maximum at 10-20 days post-injection, followed by a decrease at later time points [112;118]. The observed delay in peak expression of dystrophin under the regulation of MCK1350 is neither due to the dystrophin transgene nor due to the developmental stage of the mice at the time of injection since similar findings were obtained using MCK1350 driving the luciferase reporter gene in older *mdx* mice (Figure 9). Furthermore, when Hauser and colleagues [205] examined the time course of AdV-mediated β-galactosidase expression in adult *mdx* using a short MCK promoter/enhancer which was comprised of a fusion of the E1 enhancer (-1256 to -1050) with the proximal promoter region extending from –358 to +7 (CK6), they observed a similar delay in transgene expression, being low at 3 to 14 days and reaching a maximum at the 30 day time point. Moreover, a similar delay in expression may also occur with the longer MCK promoter fragment which includes the first intron [82]. Thus, this delay in expression may be a characteristic of the MCK E1 enhancer or the proximal MCK promoter.

In adult *mdx* animals, expression of the reporter luciferase peaked at 30 days but was not maintained after this period, decreasing by 95 % at 60 days. A similar decrease of 90% of the β-galactosidase activity was observed 60 days after intramuscular injection of the CK6 driven transgene by Hauser and colleagues [205]. This suggests that the use of a muscle-specific promoter in adult skeletal muscle may not be sufficient if the transgene itself is immunogenic. In the present study, we observed a high number of dystrophin-positive muscle fibers for up to 60 days after injection of AdVMCKdys in skeletal muscles of very young *mdx* mice. Most likely, the high stability of transgene expression is due to the immature state of immune system at the time of injection [112;118;119].

In conclusion, the muscle-specific MCK1350 promoter/enhancer allows for high level and sustained expression for up to 60 days of dystrophin in dystrophic (*mdx*) muscles using adenoviral vectors. In addition, the transgene expression peaked at later time points when compared to strong viral promoters, and overexpression of dystrophin was not observed. Moreover, the MCK1350 promoter/enhancer used in this study allows for a sufficiently high level of gene expression in dystrophic muscles as well as normal muscles as demonstrated in a line of transgenic mice produced using MCK1350 driving the expression of the AdV primary receptor CAR [206].

CHAPTER IV

Genomic integration of adenoviral gene transfer vectors following infection of fertilized mouse oocytes

Summary

Adenoviral vectors (AdV) are popular tools to deliver foreign genes into a wide range of cells. More recently, AdV have also been used in clinical gene therapy trials. The rate of integration of AdV sequences into the host cell genome appears to be low. This – together with triggering an adverse immune response - explains the short duration of transgene expression after AdV-mediated gene transfer *in vivo*. However, lack of chromosomal integration is regarded as a positive safety characteristic, in particular to prevent cancerogenesis and longitudinal transmission of genes. Recently, AdV-mediated gene transfer to mammalian oocytes and transmission through the germ-line has been reported controversially. Therefore, we wanted to investigate the integration of AdV sequences into the mouse genome by microinjecting AdV into the perivitelline space of fertilized oocytes. We demonstrate that under optimized conditions fertilized mouse oocytes are infected efficiently by AdV and give rise to founder animals that are transgenic for AdV sequences. Also, the great majority of these founders transmitted the transgene to their offspring. Southern blot analysis demonstrated that only one AdV copy was integrated into the mouse genome. Using first-generation AdV, none of the transgenic mice expressed the transgene (GFP or luciferase). In contrast, third-generation AdV devoid of all viral genes resulted in a low rate of transgenic founders, but in expression of the transgene.

Introduction

Most gene therapy protocols of recessive disorders require the delivery of a gene or DNA. Approaches using "naked" DNA frequently suffer from low gene transfer efficiency and transient gene expression [67;68;69]. Recombinant viruses (vectors) are powerful tools for gene delivery due to a sophisticated machinery of inherent molecular mechanisms. A major safety concern using viral vectors is the possibility of DNA integration into the host genome that may cause heritable alterations in offspring or the activation of oncogenes and inactivation of tumor-suppressor genes.

Recombinant adenoviruses (AdV) are commonly used vectors for *in vivo* gene transfer. First-generation (E1/E3-deleted) AdV may accommodate transgene cassettes of ≈ 8-10 kb, while third-generation (gutted or gutless) AdV is deleted of most viral genes and may accommodate up to 36 kb of foreign DNA [85;86;207]. Frequently, transgene expression is only transient after AdV-mediated gene transfer. This is due to the lack of viral DNA integration into the host cell genome, as well as the induction of host immune reactions against viral gene products and the transgene product [101;104;105;112].

In vitro, it has been shown that AdV may integrate into the cell genome at low frequency of approximately 10^{-3} to 10^{-5} [173]. Exposure of oocytes with intact zona pellucida and sperm with AdV did neither result in genomic integration of AdV sequences or in transgenic animals [208;209]. Chromosomal integration, transgene expression and germ-line transmission has been reported after AdV-mediated gene transfer into zona-free oocytes of mice [175]. In contrast, Kubisch and colleagues were not able to demonstrate transgenesis in different mammalian species in a similar experimental approach [176].

The goal of this study was to determine the probability and characteristics of genomic integration of AdV by infecting fertilized mouse oocytes at the one-cell stage. Our results show that first-generation AdV may integrate efficiently into the mouse genome and AdV sequences may be inherited to the progeny. Interestingly, expression of the transgene was not detected using AdV constructs with different promoters and transgenes. In contrast, third-

generation AdV resulted in a low rate of transgenic founders, but in expression of the transgene.

Material and methods

Construction of recombinant AdV

The homologous recombination of the replication-defective human adenovirus type 5 with reporter gene cassettes, the large-scale production, purification and titration of the first-generation AdV have been described in detail [178]. The CMV promoter driving the expression of the green fluorescent gene or the muscle creatine kinase enhancer/promoter (MCK1350) driving the expression of the luciferase gene (AdVCMVGFP and AdVMCKlux) were cloned as expression cassettes into plasmid containing sequences from human adenovirus type 5 [172]. First-generation AdV were produced and titrated in 293 cells as described elsewhere [178]. The third-generation AdV (AdGS46) has been described previously [210]. Briefly, AdGS46 contains the Ad5 left terminus, HPRT stuffer (nt. 17853-1799), the CMV promoter driving the lacZ expression cassette, C346 stuffer (nt. 21484-12421), and the Ad5 right terminus. AdGS46 was produced in 293-based cre66 (Schiedner et al., manuscript in preparation) cells which were coinfected with the loxP helper virus AdLC8cluc [86]. Subsequent amplification steps, large scale production and purification were performed as described [211]. The infectious titer of AdGS46 was determined by slot blot.

Generation of fertilized mouse oocytes

All animal procedures have been carried out according to local and provincial animal care regulations. B6D2F1 females (C57BL/6 x DBA/2 hybrid mice) were hormonally superovulated and mated with B6D2F1 males. The next morning, fertilized one-cell eggs were flushed from the oviduct with M2 medium [212]. The eggs were then kept in M2 culture medium droplets under mineral oil for microinjection.

Titration of AdV for infection of fertilized oocytes

AdVCMVGFP was diluted in PBS to concentrations ranging from 1 plaque forming unit (pfu)/100 pl to $1x10^3$ pfu/100 pl. Fertilized oocytes were visualized under a microscope and microinjected with approximately 100 pl into the perivitelline space. The plasmalemma was not penetrated by the needle. The embryos were kept in culture overnight in M16 culture medium [212] at 37°C and 5% CO_2. Embryos were then assessed for GFP expression using a fluorescent microscope with a blue filter and for viability and developmental rate under white light.

Dilution of the different adenoviral vectors used for microinjection

First-generation vectors AdVCMVGFP and AdVMCKlux were diluted in PBS (without calcium and magnesium) to a concentration of 5-100 pfu/100 pl. Furthermore, third-generation AdVCMVlacZ was diluted in PBS to a concentration of $2x10^{1}$-$1x10^{3}$ infectious units (IU)/100 pl. Fertilized oocytes were microinjected into the perivitelline space and transferred to the oviduct of pseudopregnant females at the same day.

Identification of transgenic animals

Mice derived from AdV-infected oocytes were ear tagged at 4 weeks of age, and tail tips were cut. The genomic DNA was extracted and the optical density was done at 260 and 280 nm to determine the concentration and the purity of the genomic DNA. PCR analysis was performed on 500 ng DNA using primers specific for the E4 region of AdV, the luciferase gene or the human *HPRT* gene (E4-sense: 5'-GTAGAGTCATAATCGTGCATCAGG-3' and E4-antisense: 5'-TTTATATGGTACCGGGAGGTGGTG-3'; Lux-sense: 5'-CCGACCGCGCCCGGTTTA-3' and Lux-antisense: 5'-GGGTTACCTAAGGGTGTG-3'; HPRT sense: 5'-GCTGGCCTCCCTCCTCAACCG-3' and HPRT antisense: 5'-CCCCGCCAGGGGCCATGCAAG-3'). The 50-µl polymerase chain reaction (PCR) contained 50 pmol of each primer, 5 µl of 2.5 mM dNTPs, 5 µl DMSO and 5 µl 10x Taq polymerase buffer containing 15 mM magnesium chloride. PCR was carried out for 40 cycles (annealing at 54°C for 1 minute and extension at 72°C for 90 seconds) using a Perkin Elmer 2400 thermocycler. The PCR products were then separated on a 2% agarose gel containing EtBr and visualized with ultraviolet light.

Study of germ-line transmission of the transgene (F1 generation)

Three transgenic founders for AdVCMVGFP, 14 founders for AdVMCKlux and 1 founder for AdVCMVlacZ were mated with C57BL/6 males or females. One transgenic founder for each AdVCMVGFP and AdVMCKlux did not generate offspring. F1 animals from the various transgenic lines were analyzed by PCR.

Analysis of transgene expression

Transgenic (AdVCMVGFP) and non-transgenic littermates were sacrificed, various organs were dissected, fixed on cork blocks and frozen in liquid nitrogen-cooled isopentane. 10 μm thick tissue sections were prepared and assessed for GFP expression under a fluorescent microscope using a blue filter. Similarly, various organs of AdVMCKlux transgenic mice were dissected and analyzed for luciferase expression using the Promega Luciferase Assay System (Promega, cat.# E4030) as described previously [178]. The light emission was measured for a period of 10 seconds using a luminometer Lumat LB 9501. For each individual sample the luciferase activity (given in relative light units, RLU) was calculated for the total sample volume of 200 μl. Tibialis anterior (TA), quadriceps, soleus, diaphragm, heart, lung, liver, kidney, spleen, brain, stomach, intercostal muscles and spinal cord from AdVCMVlacZ transgenic and non-transgenic littermates were extracted and analyzed for β-galactosidase expression as described earlier [172]. Light emission was measured for a period of 10 seconds using a Lumat LB 9501 luminometer. For each individual sample the β-galactosidase activity (given in relative light units, RLU) was converted into picograms of lacZ using a standard curve. The data were then statistically analyzed by ANOVA (analysis of variance). The β-galactosidase staining on embedded tissues was done as described earlier [178].

Southern blot analysis

Genomic DNA isolated from the liver of transgenic and nontransgenic mice was digested with *Eco*RV, electrophoresed on a 1% agarose gel and transferred onto nylon membrane. The membrane was subsequently hybridized with radiolabeled GFP probe, using (α-^{32}P)dATP and (α-^{32}P)dCTP by random hexamer priming. *Eco*RV cuts the linear AdVCMVGFP genome at the following nucleotide positions: 2349, 9266, 10504, 18143, 22689, 24741, 27364 and 30695. Only the 5' fragment (2349 bp) hybridizes with the GFP probe (data not shown).

Validation of the AdVlux stocks

Newborn nontransgenic mice (3-5 day-old) were directly injected into both tibialis anterior (TA) with 10-15 µl/muscle of AdVMCKlux ($1x10^7$ pfu/µl). Animals were sacrificed 11 days post-injection, TAs were extracted and luciferase assay was done as described above.

Preparation of satellite cells from AdVMCKlux transgenic mice

Muscle satellite cells from TA of transgenic mice were extracted as described [213]. Briefly, muscles were cut into small pieces using a sterile scalpel and trypsinized in a Wheaton trypsinizing flask. After 3 rounds of trypsinization, the cells were spun at 500 g and kept in culture at 37°C, 5% CO_2 in Skeletal Muscle Cell Growth Medium (PromoCell, cat.# C-23060) supplemented with 15% FBS.

Exposure of cell cultures to 5-aza-cytidine and trichostatin A

Three primary cell cultures derived from different AdVMCKlux transgenic mice were used for demethylation and deacetylation experiments. $1x10^3$ cells were plated in each well of a 12-well plate and kept in culture for 13 days. At day 2 and day 10, 5-aza-cytidine (2.5 and 5 µM) or trichostatin A (75 nM-3 µM) was added to the medium and incubated for a 5-hour period [214;215]. After the 5-hour incubation period medium containing 5-aza-cytidine or trichostatin A was replaced with fresh medium. The cells were harvested at day 13 in 200 µl luciferase lysis solution and 20 µl of supernatant was used to measure luciferase activity as described above.

Results

In a first experiment we wanted to determine the conditions under which fertilized mouse oocytes could be efficiently infected with AdV. Fertilized mouse oocytes with intact zona pellucida were exposed to AdV in droplets containing increasing titers of AdVCMVGFP under mineral oil. The green fluorescent protein gene (GFP) was used, since transgene expression can be detected in viable embryos. None of the eggs were infected or showed GFP expression (data not shown). This is in accordance with previous reports demonstrating that for efficient AdV infection the zona pellucida needs to surmounted by various methods such as acidic Tyrode solution, pronase or microinjection [176;209]. All 3 methods allowed for AdV infection and GFP expression, but the microinjection method showed higher rates for development and survival of embryos regardless of AdV titer if compared to the other methods (data not shown).

To determine the infectability of fertilized mouse oocytes by subzonal AdV-microinjection, different AdV titers varying from 1 pfu/100 pl to $1x10^3$ pfu/100 pl of AdVCMVGFP were microinjected into the perivitelline space. The microinjected embryos were kept in culture and transgene expression was visualized (Figure 12). The rate of embryos expressing GFP increased with increasing AdV titer. However, very high titers led to developmental arrest and degeneration of embryos (data not shown). Control embryos that received PBS only did not show any fluorescence.

Second, we wished to determine whether AdV sequences would remain episomally located or integrate chromosomally in the mouse genome. In the first case offspring derived from infected mouse embryos would have undetectable or mosaic AdV sequences, while in the second case offspring and following generations would be transgenic for AdV sequences. Therefore, the AdV-microinjected eggs (5 to 10 pfu/100 pl of AdVCMVGFP) were transferred into pseudopregnant foster mothers. Of 21 live-born animals, 3 were positive for AdV-sequences as shown by PCR analysis of genomic DNA purified from the tail. The 3 AdV-transgenic mice (founders) were mated with C57BL/6 mice and the F1 mice were also tested for AdV-sequences by PCR to show germ-line transmission (Table 2). One animal was bred twice and all 16 progenies were found negative for AdV sequences. The 2 other

founders transmitted the transgene to the F1 generation according to a Mendelian distribution (11 positive offspring out of 20). To further confirm chromosomal integration of AdV-sequences into the genome of AdV-transgenic mice, Southern blot on genomic DNA isolated from liver was performed (Figure 13). We show that approximately one AdV-copy per genome of AdV-transgenic animals is integrated. A 15-16 kb band hybridizing with GFP-sequences is found that cannot be generated from linear or circular AdV genomes. To test whether the transgene was expressed, different tissues of transgenic and non-transgenic control mice were frozen, sectioned and visualized under fluorescent microscope. None of the animals tested (3 different lines) showed GFP expression (data not shown).

We reasoned that failure to detect transgene expression in AdV-transgenic animals despite demonstration of embryonal transgene expression could be due to one or a combination of the following factors: 1) low detection sensitivity for the transgene (GFP) in adult mouse tissues; 2) toxicity of widespread transgene expression (GFP) during development; 3) shut-off of the viral promoter (CMV) during development; 4) other biological factors independent of the transgene and promoter used. To exclude 1)-3) AdVMCKlux was used in the same experimental paradigm since the MCK1350 promoter/enhancer restricts the expression of the transgene to skeletal muscle [172], and the luciferase expression can be easily detected using a sensitive chemiluminescence assay. To validate whether AdVMCKlux would result in robust and detectable luciferase expression in skeletal muscle, non-transgenic control mice received i.m. injections of AdVMCKlux into both tibialis anterior (TA). As expected, the luciferase transgene was highly expressed in skeletal muscle after direct AdV-mediated gene transfer (Figure 15). Consecutively, AdVMCKlux (10 to 100 pfu/100 pl) was microinjected into the perivitelline space of fertilized mouse oocytes. Of 24 live-born animals derived from AdVMCKlux-infected oocytes, 19 animals were shown to be transgenic for luciferase sequence by PCR. Thirteen positive founders were bred with C57BL/6 mice and resulted in a total of 113 offspring. All 13 mouse lines showed transmission of luciferase sequences to the F1-generation in a Mendelian distribution (Figure 14 and Table 2). To test whether the luciferase reporter gene was expressed, 9 different tissues of 13 AdV-transgenic lines were extracted (total of 19 PCR-positive and 7 PCR-negative animals), lysed and assayed for luciferase activity. Surprisingly, there was no detectable luciferase expression for any of the animals/tissues tested (Figure 15 and data not shown). Therefore, we concluded that other

factors than the promoter and the transgene used were responsible for the lack of transgene expression. We hypothesized that the transgene may be methylated and/or integrated in a chromosomal region where the histones are deacetylated and/or methylated causing gene silencing [216;217]. Primary muscle cells of 3 AdVMCKlux-transgenic animals were treated by incubation with 5-aza-cytidine and with the histone deacetylase inhibitor trichostatin A [214;215]. However, luciferase activity was not detected even after extended treatment (data not shown).

We hypothesized that AdV genes present in first-generation AdV may be responsible for the lack of transgene expression in AdV-transgenic animals. To verify this hypothesis, we used third-generation AdVCMVlacZ that does not contain any viral sequences, except for the inverted terminal repeat (ITR) and the packaging signal, in the same experimental paradigm. One AdV-transgenic animal was detected (Table 2) and found to express the transgene in various tissues such as skeletal muscles (tibialis anterior, quadriceps, soleus), heart, lung, diaphragm, intercostal muscles and tail (Figure 16). Quantification showed an 80000-fold increase of β-galactosidase activity in skeletal muscles of the transgenic animal if compared to nontransgenic controls. Other organs such as lung, intercostal muscles and heart showed a 100 to 500-fold increase of activity. However, no increase of β-galactosidase activity was seen in liver, spleen and brain. The AdV-transgenic animal was bred three times, but none of the offsprings (21 totally) was found positive for the *HPRT* sequences by PCR analysis and for the β–galactosidase activity. We concluded that the integrated sequence in the founder animal was mosaic.

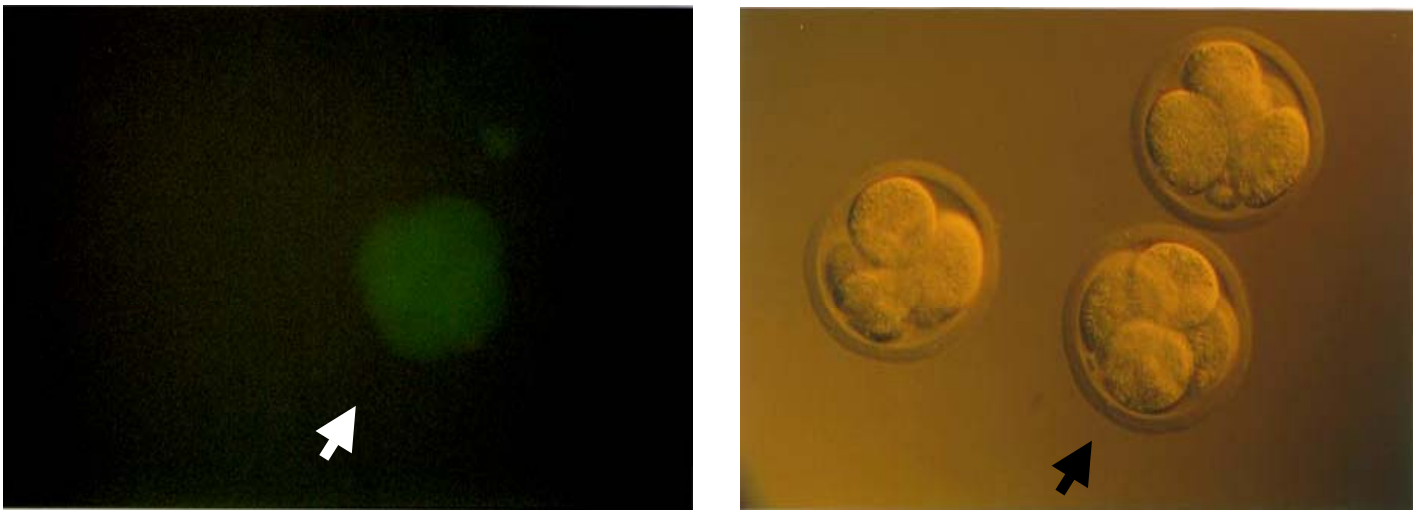


Figure 12. Mouse embryos microinjected with AdVCMVGFP (10 pfu/100 pl) into the perivitelline space at one-cell stage. Embryos were kept in culture and GFP expression was assessed 48 hours post-injection using a fluorescent microscope. Photomicrographs were taken on the same field with fluorescence light (left panel) or with bright light (right panel) at a magnification of 400X. The arrows point to the corresponding embryo.

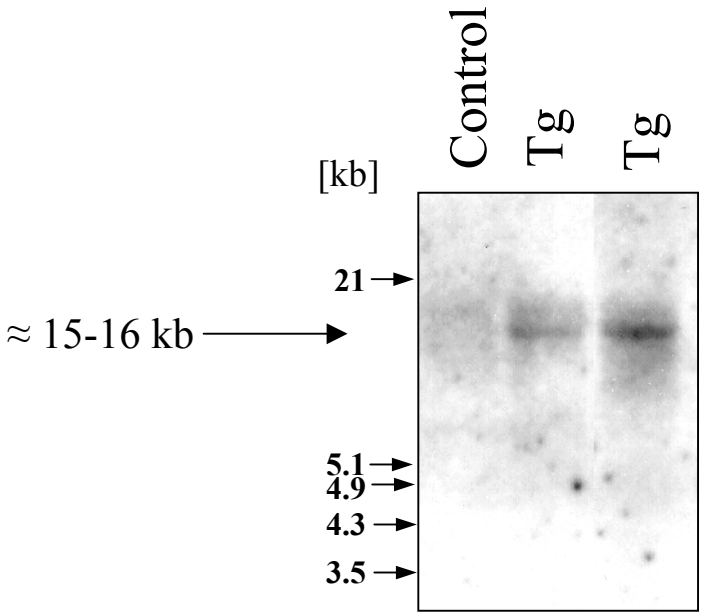


Figure 13. Southern blot analysis using genomic DNA from liver of normal and transgenic animals for the AdVCMVGFP. Genomic DNA was digested overnight with the restriction enzyme *Eco*RV, electrophoresed on a 1% agarose gel, transferred to a nylon membrane and hybridized with a probe for the GFP transgene. A single fragment of ≈ 15-16 kb is present in 2 transgenic mice (Tg) and absent in control, nontransgenic animal.

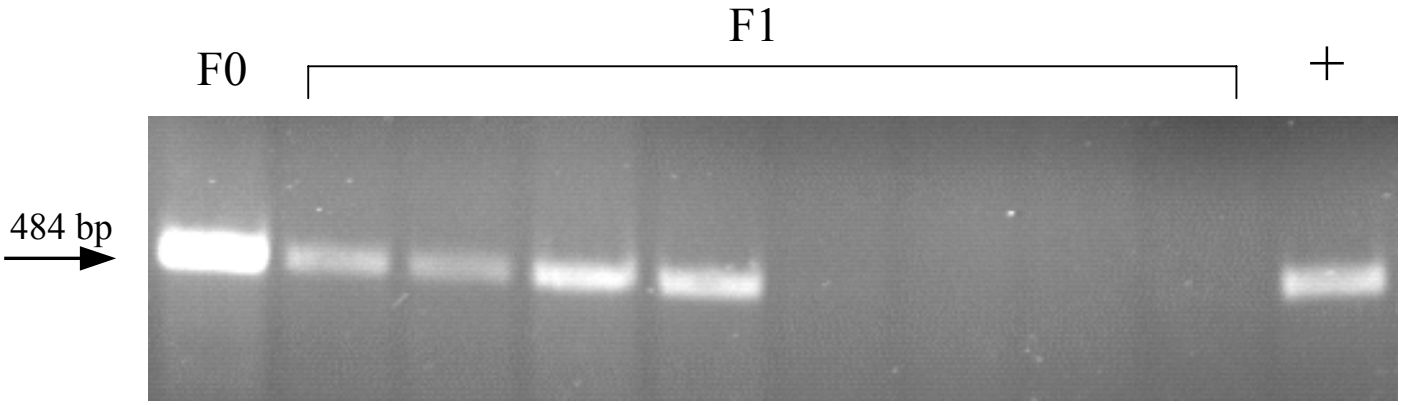


Figure 14. Result of PCR performed on genomic DNA extracted from mouse tails of a founder animal (F0) and its respective offsprings (F1). PCR products were separated electrophoretically on 2% agarose gel containing EtBr. Plasmid DNA containing luciferase transgene was used as positive control (+). The expected amplification product at 484 bp is indicated.

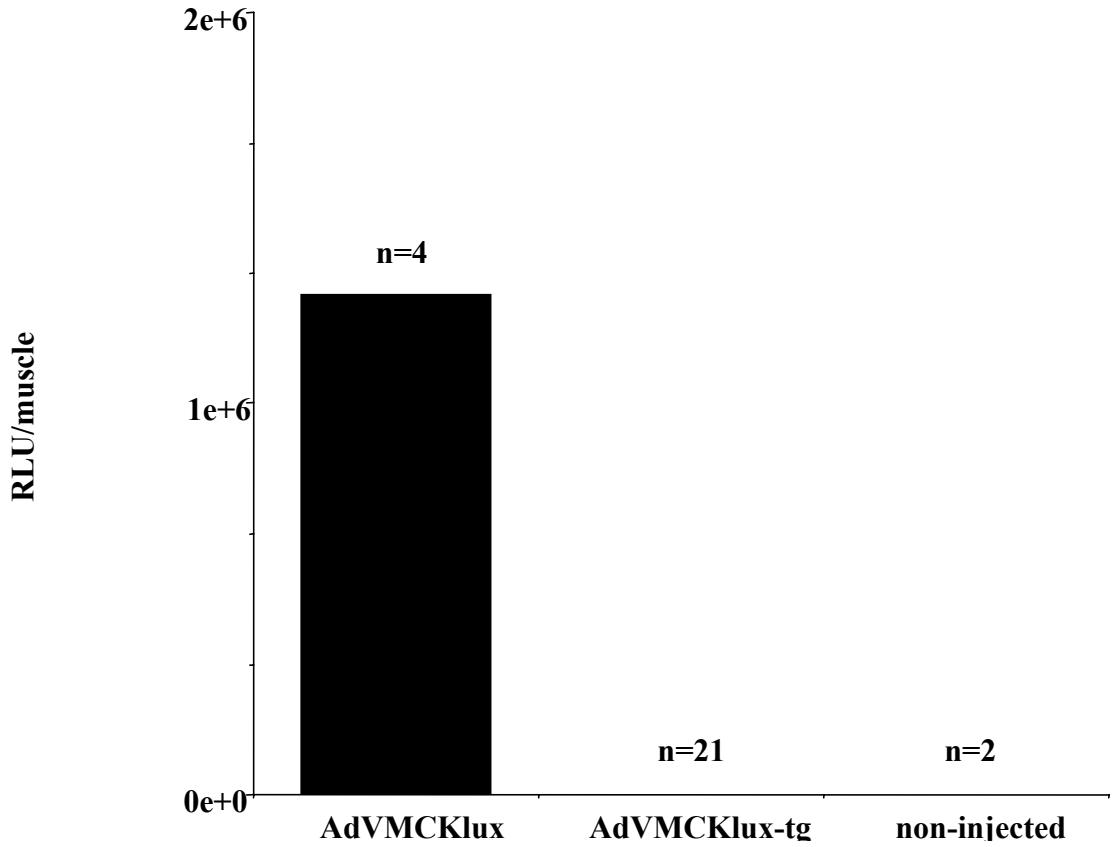


Figure 15. Luciferase expression measured in relative light units (RLU)/muscle in newborn non transgenic mice injected in both TAs with AdVMCKlux. Also, luciferase expression in muscle tissues of AdVMCKlux-tg animals and in negative non-injected controls.

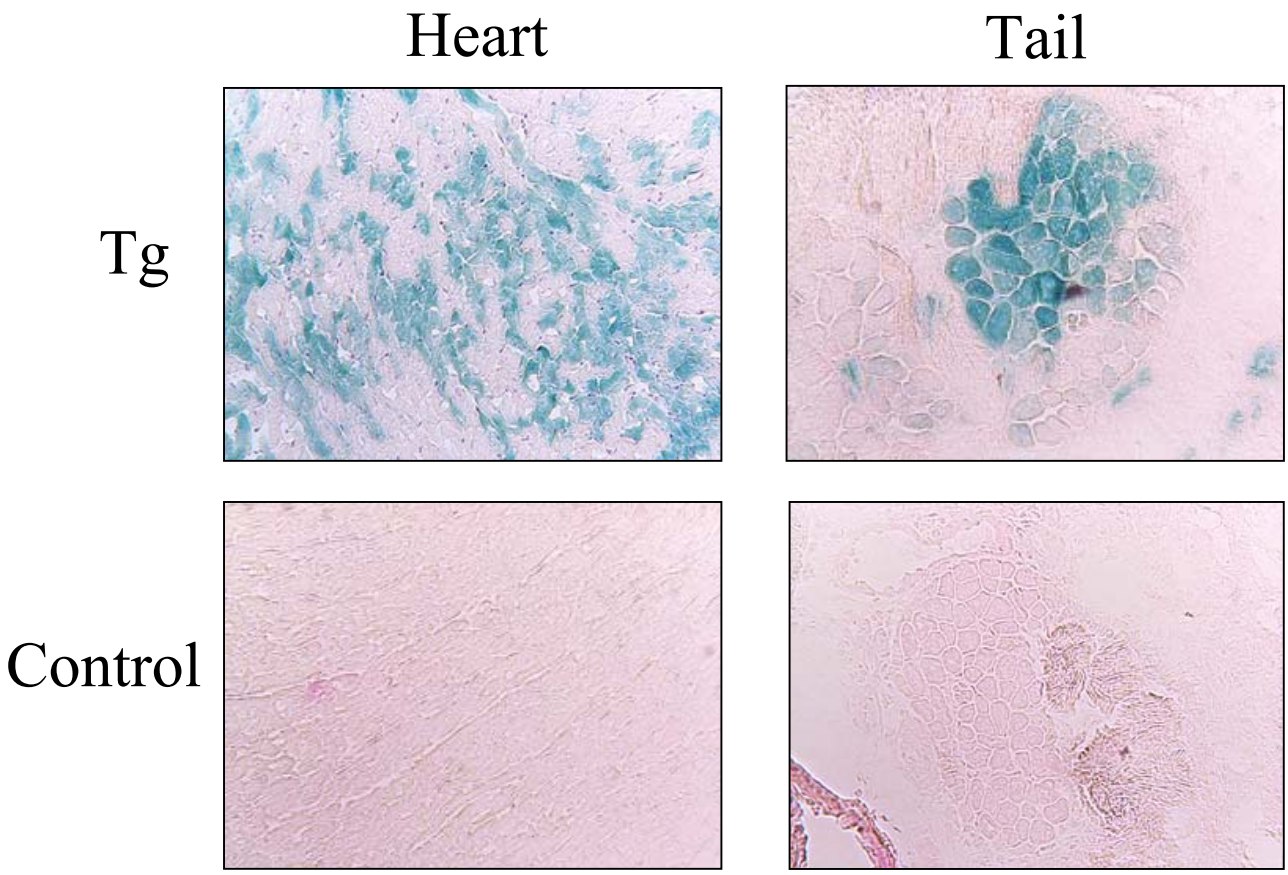


Figure 16. Histochemical β-galactosidase staining on tissue sections from the transgenic founder for AdVCMVlacz (Tg) and negative control. Photomicrographs showing transgene expression in about 50% of heart cells and in muscle cells from tail section (Magnification 200X).

Table 2. Results of AdV-transgenesis following microinjection of different AdV vectors into the perivitelline of early embryos.

	AdVCMVGFP	AdVMCKlux	AdVCMVlacz
Titer (infectious units/100 pl)	5-10	10-100	20-1000
Transgenic founders/viable animals	3/21 (14%)	19/24 (79%)	1/34 (3%)
Transmission to F1/transgenic founders	2/3 (66%)	13/13 (100%)	0/1
Number of offsprings positive/total	11/20 (55%)	71/113 (63%)	n/a
AdV integration confirmed by	Southern blot	nd	nd
Transgene expression	no	no	yes

nd= not determined

n/a= not applicable

Discussion

Recombinant viruses such as retrovirus, adenovirus and adeno-associated (AAV) virus are frequently used to transfer genes into mammalian cells, they also serve as gene therapy vectors for human disease. The best vector for a given application is determined by many factors, but mainly through the biological interactions of the vector with the host. Chromosomal integration of vector DNA has been described for retroviral and AAV-based vectors, and allows for long-term persistence of a transgene even in dividing cells, but brings about the additional risks of cancerogenesis and germ-line transmission. Therefore, the rate and pattern of integration (random vs. site-specific integration) of a given vector is an important safety characteristic. Adenoviral vectors (AdV) are generally regarded as non-integrating vectors. The lack of genomic integration together with a host immune response leads to transient gene expression in numerous gene transfer applications. However, there has been recent demonstration of low-level AdV integration *in vitro* [173]. Furthermore, there have been conflicting reports of AdV-mediated transgenesis and germ-line transmission following infection of mammalian oocytes [175;176]. Therefore, we investigated the biology of AdV-mediated gene transfer to fertilized mouse embryos and discuss the consequences for transgenic animal biology and gene therapy.

Efficient AdV-infection of fertilized mouse oocytes occurs only if the barrier of the zona pellucida is surmounted, an intact zona pellucida prevents infection [176;209]. We demonstrate that infection of fertilized mouse oocytes is dependent on AdV titer, efficient infection is obtained at MOI's (multiplicity of infection) of 10-100 that is similar to other primary, mammalian cells susceptible for AdV such as fibroblasts or myoblasts [168;218]. Infectability of cells by AdV depends largely on the expression of primary (CAR, i.e. Coxsackie and Adenovirus Receptor) and secondary ($\alpha v\beta 3$ integrins) receptors on the cell surface [89;92;97]. Therefore, we speculate that these receptors may be expressed on fertilized mouse embryos. Very high AdV titers led to strong transgene expression, but also to developmental arrest and degeneration of early embryos. This may be caused by toxic effects of either transgene or AdV gene expression, or by the high load of toxic AdV capsid proteins. However, we were able to determine an intermediate range of AdV titers that led to moderate gene expression, high survival rate of embryos, and ultimately transgenic offspring.

We also demonstrate that AdV sequences frequently integrate into the host genome following infection of fertilized mouse oocytes. The AdV-transgene is transmitted to the progeny according to Mendelian rules. Since all AdV used were replication-deficient, the passage of AdV and transgene sequences to the progeny can only be explained by early integration into the mouse genome. This is confirmed by Tsukui and colleagues who infected murine zona-free eggs with first-generation AdV and demonstrated germ-line transmission of the AdV-transgene [175]. In contrast, Kubisch et al. did not find AdV-mediated transgenesis following infection of fertilized oocytes of various mammalian species. Therefore, different mammalian species appear to differ substantially in their susceptibility to AdV-mediated transgenesis. In addition, we show that approximately one AdV copy is integrated into the mouse genome using Southern blot analysis. Similar findings have been obtained by Tsukui and colleagues [175]. This reinforces our hypothesis that transgenesis is mediated by a "bona fide" infection where only one copy of the AdV genome is delivered by the capsid. Therefore, AdV-mediated transgenesis may be useful where gene dosage is of concern. Conventional transgenesis using naked DNA injection into the pronucleus often results in multiple integrated copies [219].

Tsukui and colleagues observed transgene expression (β-galactosidase) in 2 out of 3 AdV-transgenic lines [175]. In contrast, we did not observe transgene expression in a total of 17 AdV-transgenic lines using different validated promoter and reporter gene constructs (AdVCMVGFP or AdVMCKlux). Since we were able to detect transgene expression in infected embryos but not in adult animals, we hypothesize that transgene expression has been lost during embryonic/fetal development. However, we were not able to demonstrate methylation [220] or histone acetylation [216] of the transgene in AdV-transgenic animals that could have explained the silencing of transgene expression. In addition, mouse genetic background may also affect transgene expression. In general, transgenes inserted into inbred C57BL/6 or BALB/c mice tend to be highly methylated correlating with frequent silencing of transgenes [221]. While our experiments were carried out in B6D2 hybrids that were backcrossed to C57BL/6, Tsukui's experiments were done in a C57BL/6-C3H background. Furthermore, leaky expression from AdV genes may interfere with embryonic/fetal development. Therefore, only embryos that have insertions of AdV and transgene sequences

in genomic regions that do neither allow for leaky AdV gene expression nor for transgene expression may develop fully and give rise to live-born animals. The first-generation AdV used in this study (AdVCMVGFP or AdVMCKlux) contain various early and late AdV genes. Leaky expression of AdV genes from first-generation AdV has been demonstrated in various cells and tissues [101;105]. Indeed, using a third-generation AdV (AdVCMVlacZ) that lacks most viral genes we were able to demonstrate transgene expression in various tissues of an AdV-transgenic animal.

It will remain a matter of further investigations whether AdV-mediated transgenesis may be a useful alternative to generate transgenic animals compared to standard methods. Pronuclear injection does not limit the size of the transgene, the proportion of born transgenic is usually around 10-20%, and many copies of the transgene tend to integrate at one site [219]. In this study, AdV-infection of fertilized mouse oocytes resulted in high levels of transgenesis (up to 79%) and germ line transmission (66%-100%). In addition, low copy numbers of the transgene (approximately 1) are integrated into the genome. Unfortunately, first-generation AdV did not allow for transgene expression in transgenic animals. This problem may be overcome by the use of third-generation AdV.

The dogma of AdV as a non-integrating vector requires revision. This is an important safety aspect for gene therapy protocols that use AdV in humans. Harui and colleagues had demonstrated low-level genomic integration of AdV *in vitro* [173]. We and others show genomic integration of AdV following infection of fertilized mouse oocytes and transmission to the progeny [175]. However, this requires the removal or bypass of the zona pellucida and reasonably high local AdV titers. In contrast, systemic or local infection of adult animals with AdV did not result in germ-line transmission. Despite direct exposure of mouse spermatogenic cells to high doses of replication-defective AdV prior to *in vivo* fertilization, introduction of the vector into embryos via the fertilizing sperm was not documented [208]. Paielli and colleagues directly injected high titers of replication-competent human AdV into mouse testis. This resulted in transgene expression and possibly replication in the testis, but germ line transmission was not detected in offspring [222]. Furthermore, AdV were directly injected into the mouse ovary or used for infection of zona-free oocytes prior to *in vitro* fertilization [223]. In this study, less than 2% of infected zona-free eggs used for *in vitro*

fertilization showed transgene expression, AdV-mediated transgenesis and germ-line transmission was not observed. After systemic administration high titer AdV is distributed to both ovaries and testis, but does not lead to transmission to offspring [174]. These findings provide strong evidence that the risk of AdV transduction into adult male and female germ cells is an unlikely event in a gene therapy regimen. However, AdV-mediated germ-line transduction may depend upon additional factors such as species differences. Therefore, adequate precautions should be taken in gene therapy protocols of reproductive patients since infection of oocytes or early embryos and subsequent chromosomal integration cannot be ruled out entirely.

CHAPTER V

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Curriculum vitae

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1997-2001 **Ph.D. student at the department of chemistry of the Ludwig-Maximilians-University**; Genzentrum, Munich, Germany (supervisor: PD Dr. med. Hanns Lochmüller) Gene transfer to skeletal muscle using adenoviral recombinants

1995-1997 **Master's degree in Neurological sciences (M.Sc.);** Montreal Neurological Institute, McGill University, Canada (supervisors: Prof. J. Nalbantoglu, Prof. G. Karpati) Gene therapy for neuromuscular diseases: evaluation of a muscle-specific promoter for adenovirus-mediated gene transfer.

1991-1995 **Bachelor degree in Microbiology (B.Sc.);** Laval University, Quebec, Canada (supervisor: Prof. R. C. Levesque) Sequencing and analysis of ribonucleotide reductase in *Toxoplasma gondii*.

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Scholarships

1995	Summer scholarship from the Muscular Dystrophy Association of Canada, Canada
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Publications

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Lochmüller H., Petrof B.J., Pari G., **Larochelle N**., Dodelet V., Wang Q., Allen C., Prescott S., Massie B., Nalbantoglu J., Karpati G. Transient immunosuppression by FK506 permits a sustained high level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic [mdx] mice. *Gene Therapy* 1996; **3**: 706-716.

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List of figure and tables

Abbreviations

AAV	adeno-associated-virus
AdV	adenovirus
Anova	analysis of variance
BMD	Becker muscular dystrophy
bp	base pair
CAR	coxsackie adenovirus receptor
cDNA	complementary (to mRNA) DNA
CMV	cytomegalovirus promoter
DAPs	dystrophin-associated proteins
DGC	dystrophin-glycoprotein complex
dko	double knock-out, dystrophin/utrophin-deficient mouse
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
EF-hand	motif that contains approximately 40 residues and is involved in binding intracellular calcium
E. coli	*Escherichia coli*
EDL	extensor digitorum longus
GFP	green fluorescent protein
GRMD	golden retriever muscular dystrophy
HPRT	hypoxanthine phosphoribosyltransferase
ITR	inverted terminal repeat
kb	kilobase
kDa	kilodalton
lacZ	β-galactosidase gene
lux	firefly luciferase
MCK	muscle creatine kinase
mRNA	messenger RNA
nt	nucleotide

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming unit
RNA	ribonucleic acid
RSV-LTR	rous sarcoma virus long terminal repeat
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
TA	tibialis anterior
WW domain	domain that contains 2 conserved tryptophans (W) spaced by 20-22 amino acids