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Gene therapy for muscular dystrophy: evaluation of a muscle-specific promoter for adenovirus-mediated gene transfer

Nancy Larochelle
Department of Neurology and Neurosurgery
McGill University, Montreal
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ABSTRACT

Replication-defective (E1+E3 deleted) human adenovirus vectors are promising means of therapeutic gene delivery to skeletal muscle cells. Since the tropism of adenovirus is non-selective, muscle-specific expression of systemically administered vectors can only be achieved by the use of a tissue-specific promoter/enhancer that is small enough to fit the insert capacity of the vector. We have generated a replication-defective adenovirus recombinant (AV) in which the reporter gene (firefly luciferase) was driven by a truncated (1.35 kb) muscle creatine kinase (MCK) promoter/enhancer. Highly efficient and muscle-specific transgene expression was demonstrated in immunodeficient mice after local injection of AV into muscles at early age. Luciferase levels produced by AVMCKlux compared favourably to those in parallel experiments from injection of AVRSVlux in which *lux* expression is driven by the ubiquitously active LTR sequences of RSV. In nonmuscle tissues (brain, liver, kidney, lung), the transgene expression was extremely low even though in these tissues *in situ* polymerase chain reaction showed as high an infectivity of the cells by the AV as in muscle, and high levels of expression were obtained with AVRSVlux. The relatively small size, the good efficiency and the muscle specificity of the MCK promoter/enhancer would make it ideal to drive the 6.3 kb (truncated) dystrophin cDNA in first generation AV (with a limited (8 kb) insert capacity) designed for gene therapy of Duchenne muscular dystrophy.

RÉSUMÉ

Les vecteurs d'adénovirus humain défectif pour la réplication (délétion dans E1+E3) sont prometteurs pour distribuer un gène thérapeutique dans les cellules de muscle squelettiques. Bien que le tropisme d'adénovirus est non sélectif, l'expression spécifique d'un gène aux muscles par l'administration systémique des vecteurs peut être obtenue par l'utilisation d'un promoteur/enhancer spécifique au tissu qui est assez petit pour s'ajuster avec la capacité d'insertion du vecteur. Nous avons généré un adénovirus défectif pour la réplication dans lequel le gène rapporteur (luciférase) est supporté par un promoteur/enhancer écourté de la créatine kinase musculaire (MCK)(1,35 kb). L'efficacité élevée de l'expression du transgène a été démontrée dans les jeunes souris immunodéficiente après une injection locale d'adénovirus dans les muscles. Les niveaux de luciférase obtenus avec AVMCKlux sont favorablement comparable à ceux des expériences faites en parallèle avec injection d'AVRSVlux, dans lequel l'expression de la luciférase est supporté par les séquences LTR de RSV (Rous sarcoma virus), un promoteur constitutif. Dans les autres tissus (cerveau, foie, reins, poumons), l'expression du transgène était très basse, malgré le fait que le *in situ* PCR (Polymerase chain reaction) montre une infection élevée des cellules avec adénovirus. La taille relativement courte, la bonne efficacité et la spécificité restreinte aux muscles du promoteur/enhancer de MCK, le rendent idéal pour exprimer le cDNA de la dystrophine (6,3 kb) dans la première génération d'adénovirus (avec la limite d'insertion de 8 kb) désignée pour la thérapie génique pour la dystrophie musculaire de Duchenne.

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LIST OF ABBREVIATIONS

ADP= adenosine diphosphate

ATP= adenosine triphosphate

AV= adenovirus

BMD= Becker muscular dystrophy

CMV= cytomegalovirus promoter

DAG= dystrophin-associated glycoprotein

DAP= dystrophin-associated protein

DGC= dystrophin-glycoprotein complex

DMD= Duchenne muscular dystrophy

GA= gastrocnemius

ITR= inverted terminal repeat

lacz= β-galactosidase gene

lux= firefly luciferase

MCK= muscle creatine kinase

PCR= polymerase chain reaction

PFU= plaque forming unit

RSV LTR= Rous sarcoma virus long terminal repeat

TA= tibialis anterior

TREX= transcriptional regulatory element x

PREFACE

The present results involving immunoincompetent mice (*scid*) have been published: Larochelle N., *et al.* Efficient muscle-specific transgene expression after adenovirus-mediated gene transfer in mice using a 1.35 kb muscle-creatine kinase promoter/enhancer, *Gene Therapy* 1997; **4**: 465-472. The present thesis consists of four chapters and an overview of each chapter is summarized below.

Chapter 1. "Introduction", provides an overview of the disease of Duchenne muscular dystrophy, and the protein involved, dystrophin. I discuss the most promising therapy, gene therapy, and the different requirements involved to achieve efficient therapy. This chapter also includes the rationale and the important objectives for the planned experiments.

Chapter 2. "Materials and Methods", describes the methodology used in the experiments presented in chapter 3.

Chapter 3. "Results": this chapter provides the results which were obtained from *in vivo* studies of AVMCKlux recombinant in immunoincompetent and immunocompetent mice.

Chapter 4. "Discussion", provides a brief integrative summary of the major findings derived from the previous chapter.

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INTRODUCTION

In hereditary diseases, identification of the defective gene has provided new insights into the role of the normal protein and into the importance of reactions secondary to the primary defect. One such example is the discovery of the dystrophin protein, the absence of which leads to Duchenne muscular dystrophy, and its milder form, Becker muscular dystrophy.

Pathology

Duchenne muscular dystrophy (DMD) is a progressive skeletal muscle disease which is inherited as an X-linked recessive trait and affects 1/3500 boys. The symptoms begin at a young age, around 3 to 5, as a delay in walking. At 9 to 12 years of age, the affected boys lose ambulation and start using a wheelchair. By about age 20, respiration is compromised and mechanical ventilation is needed. The women and girls are carriers and some of them can manifest limb weakness (summarized in Rowland 1995).

There is a milder form of muscular dystrophy, called Becker muscular dystrophy (BMD). It resembles DMD by the fact that it is X-linked, the level of serum creatine kinase is high and there is muscle weakness. The principal differences are age at onset (usually after 12) and the rate of progression, which is quite slow. These patients are still walking at 20 years of age and often until much later (summarized in Rowland 1995).

Gene and protein involved

The study of DMD/BMD has led to the discovery of a protein that is encoded by a gene on the short arm of the X-chromosome (Koenig et al. 1987)(Figure 1). Genetic linkage analyses were used to isolate a genomic clone that was absent from a DMD patient (Francke et al. 1985 and Kunkel et al. 1985). The pERT87 clone was found to be tightly linked to the DMD gene and was the starting point for a bidirectionnal genomic walk on human recombinant phage libraries (Kunkel et al. 1986, Monaco et al. 1985 and Monaco et al.1986).

The entire gene and its 14 kb mRNA have been cloned (Koenig et al. 1987). Antibodies against fusion proteins or synthetic peptides were soon produced and used for the initial identification of this protein, which has been called dystrophin (Arahata et al. 1988, Bonilla et al. 1988, Hoffman et al. 1987, Watkins et al. 1988 and Zubrzycka-Gaarn et al. 1988) (Figure 1).

Analyses using immunohistochemistry and immunoelectron microscopy revealed the localization of the DMD gene product to be the on cytoplasmic face of normal skeletal and cardiac sarcolemma. Immunoblot analysis detected a protein in normal skeletal and cardiac muscles, which was absent or reduced in DMD specimens (Arahata et al. 1988, Bonilla et al. 1988, Koenig et al. 1988, Watkins et al. 1988 and Zubrzycka-Gaarn et al. 1988).

Dystrophin was predicted to be a rod-shaped protein composed of 4 structural domains. The 240-amino acids in the amino terminal domain show high homology to actin binding proteins such as α-actinin and β-spectrin. The second domain contains a series of 25

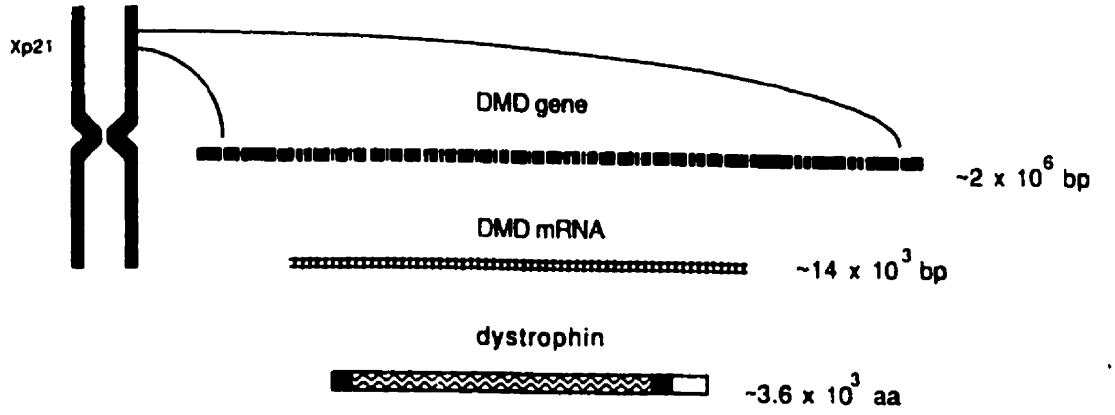


Figure 1. Schematic drawing showing the relative position of the Duchenne muscular dystrophy gene within the Xp21 region of the X-chromosome, an enlargement of this locus indicating the exons that define the approximately 2.5 megabases of Duchenne genomic locus, transcription of the Duchenne gene into the $14X10^3$ bp Duchenne mRNA, and translation of this mRNA into the 427 kDa dystrophin (adapted from Hoffman et al. 1989).

repeats of 109 amino acids in the form of a triple helix. A cysteine-rich domain composed of 150 amino acids has homology to the carboxyl terminal region of Dictyostelium α-actinin, which contains 2 potential Ca^{2+}-binding sites. The last domain, containing 420 amino acids in the carboxy terminal region, has no homology to previously characterized sequences (Koenig et al. 1988 and Koenig et al. 1990)(Figure 2A).

Dystrophin was initially reported to constitute only 0,002% of the total skeletal muscle, but it is now known to constitute 2% of total sarcolemmal protein and 5% of subsarcolemmal cytoskeletal protein, suggesting that it plays a major structural role in the cell membrane of skeletal muscle (Hoffman et al. 1987, Ohlendieck et al. 1991a and Ohlendieck et al. 1991b). Dystrophin mRNA was detected in non-muscle tissues at a lower level, like in brain (10-33% of the amount in striated muscle), in spleen (5-10%) and with even lower levels in lung, testis and kidney, and is absent in liver (Nudel et al. 1988 and Nudel et al. 1989).

Dystrophin is associated with a large oligomeric complex of sarcolemmal proteins including a 156 kDa glycoprotein (156 DAG)(α-dystroglycan), a 59 kDa protein (59 DAP)(syntrophin), a 50 kDa glycoprotein (50 DAG)(α-sarcoglycan or adhalin), a 43 kDa glycoprotein (43 DAG)(β-dystroglycan), a 43 kDa glycoprotein (43 DAG)(β-sarcoglycan), a 35 kDa glycoprotein (35 DAG)(γ-sarcoglycan) and a 25 kDa protein (25 DAP)(δ-sarcoglycan) (Ervasti et al. 1990, Ervasti et al. 1991, Sunada et al. 1995 and Yoshida et al. 1990). The first half of the C-terminus and cysteine-rich region of dystrophin binds to β-dystroglycan, and the N-terminus attaches the protein to cytoskeletal actin (Ervasti et al. 1991 and Matsumura et al. 1994). Dystrophin-glycoprotein complex can bind both actin and laminin suggesting that dystrophin serves as a link between the actin cytoskeleton and the extracellular matrix (Ervasti et al. 1993)(Figure 3).

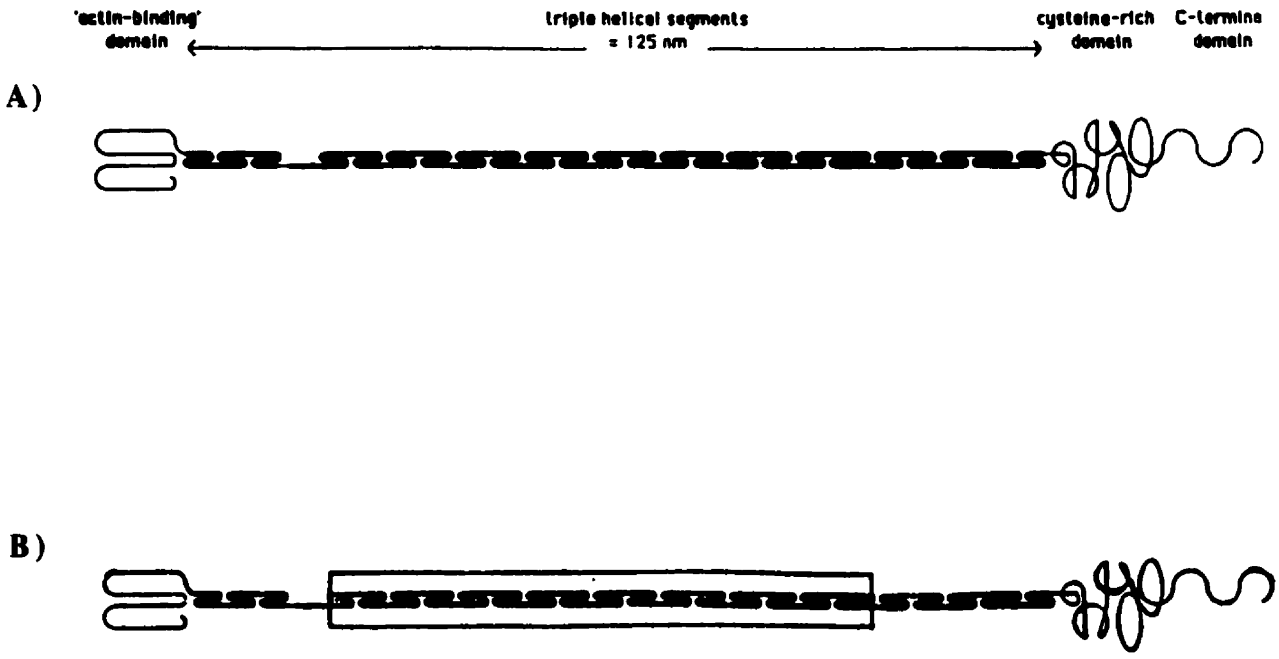


Figure 2. A) Schematic representation of the predicted organization of the 427 kDa dystrophin into four domains. The broad loops shown for the actin-binding, cysteine-rich and carboxyl domains represent potentially more globular structures than the rod-shaped domain. The 25 triple helical segments of the rod domain are predicted to be about 125 nm in length when compared to the same repeat structure of spectrin (adapted from Koenig et al. 1988). B) Representation of the truncated 229 kDa dystrophin found in a patient afflicted with a mild form of Becker muscular dystrophy, where exons 17 to 48 are removed (represented by the square) (adapted from England et al. 1990).

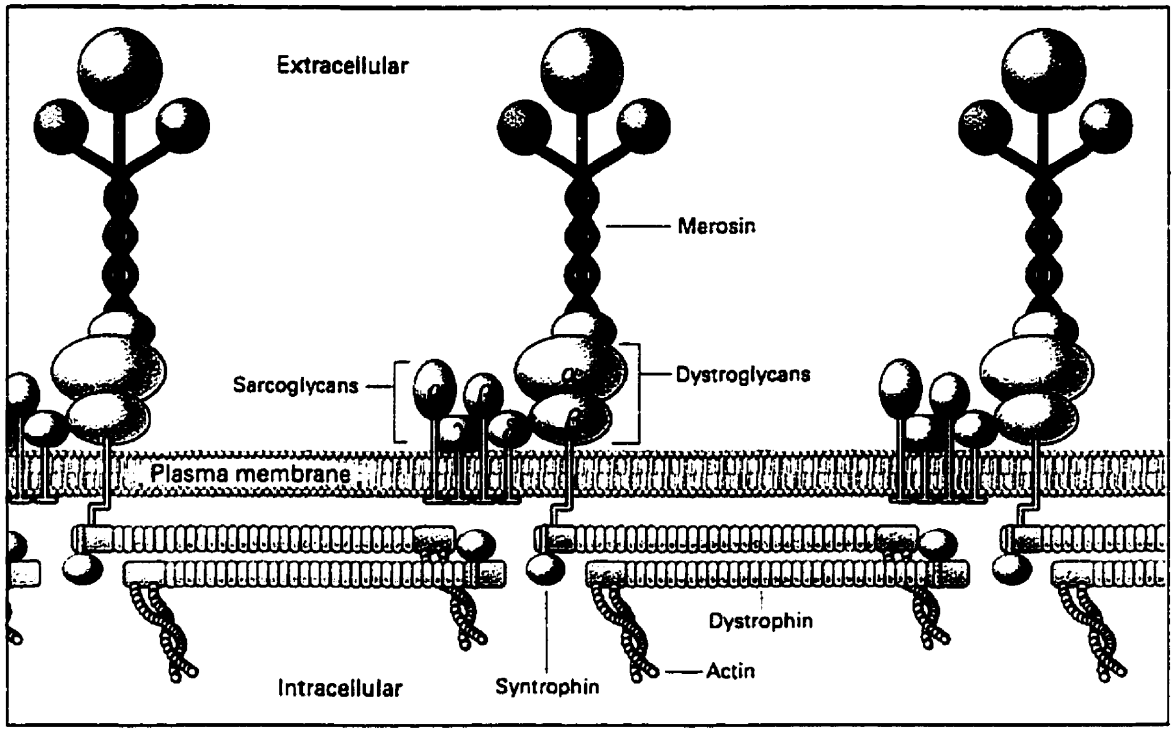


Figure 3. Schematic model of the dystrophin-glycoprotein complex as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix (adapted from Duggan et al. 1997).

Dystrophin plays an essential role in maintaining the integrity of skeletal and cardiac muscle cells (Ahn et al. 1993). Immunohistochemical analysis revealed a drastic reduction in all of dystrophin-associated proteins (DAPs) in DMD patients of various ages. The absence of dystrophin causes the disruption of the linkage of the DAPs to the subsarcolemmal actin cytoskeleton, which leads to an important reduction in all of the DAPs. The resulting disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix may lead to sarcolemmal instability and eventually to muscle cell necrosis and muscle weakness (Matsumura et al. 1993 and Ohlendieck et al. 1993). This may be the case especially during muscle contraction, which may cause physical breaks of the sarcolemma (Ohlendieck et al. 1993). The contribution of dystrophin to membrane structural integrity suggests that myofibers undergoing great physical stress could be the most deleteriously affected by the absence of dystrophin (Watkins et al. 1988). The deficiency of dystrophin causes recurrent necrosis of muscle fiber segments (Carpenter et al. 1979) and while regeneration is vigorous, it cannot fully keep up with necrosis and, as a result, progressive loss of muscle fibers occurs resulting in severe weakness and death usually by 25 years of age . Furthermore, the regenerating fibers, arising from the proliferation of mononuclear myoblast-like cells, do not always attain normal size (Carpenter et al. 1979).

In Becker muscular dystrophy, immunohistochemistry has shown reduced and/or patchy dystrophin staining along the sarcolemma and immunoblot analysis has shown dystrophin of abnormal size, normally shorter for BMD and/or of reduced quantity, in contrast to the lack of dystrophin in DMD (Arahata et al. 1989, Arahata et al. 1991, Matsumura et al. 1994 and Monaco et al. 1988). Immunohistochemistry has shown a correlation between the reduction in dystrophin and DAPs staining in BMD patients having in-frame deletions in the central rod domain of dystrophin. The reduction in the DAPs was milder in BMD patients than in typical DMD patients lacking the C-terminal domain of dystrophin, indicating that this

rod domain is not crucial for the interaction with the DAPs (Matsumura et al. 1994). This suggests that in-frame mutations of the dystrophin gene having minimal effects on the interaction with the DAPs will not result in a significant loss of the DAPs and/or the disruption of the linkage to the extracellular matrix. Dystrophin dimers with defects in the rod domain may not have a normal function or may be unstable, and this may lead to a mild reduction in the density of the dystrophin-glycoprotein complex (DGC). This could explain the mild phenotype of these BMD patients (Matsumura et al. 1994). However, dystrophin dimers with defects in the rod domain are incapable of forming a continuous network beneath the plasma membrane and instead form isolated patches of networked dystrophin next to patches of membrane with little or no dystrophin (Arahata et al. 1989).

Dystrophin lacking the C-terminal domains was reported by immunohistochemistry to be localized properly to the sarcolemmal region in unique patients afflicted with DMD (Hoffman et al. 1991, Matsumura et al. 1993, Matsumura et al. 1994 and Récan et al. 1992). But, despite the proper intracellular localization of truncated dystrophin, the phenotype of these DMD patients was quite severe. This means that the C-terminal domain is important for the correct function of dystrophin and is absolutely required for membrane attachment (Hoffman et al. 1991 and Suzuki et al. 1992). In addition, Northern blot analysis revealed the normal production of the DAP mRNA in skeletal muscle of the dystrophic *mdx* mouse which has a premature termination codon and therefore lacks the C-terminal domain. This suggests that the DAPs are synthetized but may not be properly assembled and/or integrated into the sarcolemma or may be degraded in the absence of dystrophin (Ibraghimov-Beskrovnaya et al. 1992).

DNA analysis demonstrates a deletion or a duplication at Xp21 in 60-70% of cases of either Duchenne or Becker diseases. Point mutations account for the remainder, but these

have been difficult to identify (Davies et al. 1983, Koenig et al. 1987 and Rowland 1995). A relationship has been found between the site of the deletion and the clinical syndrome, as seen in analysis of BMD. More severe types tend to be related to deletions in either the C-terminal and cysteine rich regions of the gene containing 79 exons (Koenig et al. 1989). Milder Becker syndromes are associated with deletions in the central part of the rod domain and the N-terminal regions (Koenig et al. 1989). In most typical Becker cases, deletions involve exon 45, and a hot spot for Duchenne mutations is located in the first 20 exons (Koenig et al. 1989). Because of the huge size of dystrophin gene, 2,5 megabases, the frequency of mutation is very high and the deletion breakpoints are heterogenous (Davies et al. 1983, Kunkel et al. 1986).

Mouse models for muscular dystrophy

The mouse model for DMD is the *mdx* mouse, which is dystrophin deficient (Matsumura et al. 1994 and Sicinski et al. 1989). DNA sequence analysis of normal and *mdx* dystrophin cDNAs revealed a single base substitution in *mdx* mice when compared to normal animals. A cystosine is replaced by a thymine resulting in a termination codon in place of a glutamine codon. The mutation in an exon to a stop codon results in premature termination of translation at 27% of the length of the dystrophin polypeptide (Sicinski et al. 1989). *mdx* mice have similar biochemical and histological defects compared with DMD patients, although they don't display severe progressive myopathy (Bulfield et al. 1984 and Torres et al. 1987). *mdx* mice are viable but have elevated plasma creatine kinase level (Bulfield et al. 1984). In these mice, there is excessive atrophy with loss of normal muscle fibers. Furthermore, there is a variation in fiber size, degeneration of some fibers, and a marked concentration of densely-stained sarcolemmal nuclei with phagocytic cells in place of lost fibers (Bulfield et al.

1984 and Karpati et al. 1988). Regeneration of muscle after injury takes a similar form, whatever the cause of fiber necrosis. Debris is removed by macrophages and proliferation of satellite cells with the formation and fusion of myoblast proceeds, leaving the presence of central nuclei as an indicator of previous damage. The capacity for regeneration in *mdx* does not seem to be impaired and may explain the lack of muscle weakness and the normal life span (Torres et al. 1987). The necrosis-regeneration process of *mdx* skeletal muscle fibers starts at 15 days of age and is still present in 360-day-old animals, with a high prevalence between the ages of 45 and 60 days (Karpati et al. 1988 and Torres et al. 1987).

Therapy for Duchenne muscular dystrophy

We can now appreciate the critical role and function of dystrophin in muscle cells and consider an available therapy. There has been no effective treatment for these diseases until now (Rowland 1995). By introducing dystrophin in DMD muscle fibers, prevention of the repetitive cycle of necrosis and eventual fiber loss can be obtained. A recombinant adenovirus type 5 containing cDNA for dystrophin minigene under the control of CMV or RSV promoter directly injected into a limb muscle (single injection) of young *mdx* mice showed more than 50% positive immunostained fibers, with the correct localization of the protein to the sarcolemma (Acsadi et al. 1996, Ragot et al. 1993 and Vincent et al. 1993). In addition, the presence of the protein could considerably decrease the damage produced by overall mechanical stress in many neighbouring muscle fibers, even if gene transfer did not occur in all fibers of the muscle (Deconinck et al. 1996). The transgene expression protected the myofibers from necrosis (Acsadi et al. 1996, Chi Lee et al. 1993, Deconinck et al. 1996, Phelps et al. 1995, Vincent et al. 1993 and Wells et al. 1995). Furthermore, overexpression of dystrophin cDNA in transgenic *mdx* mice, under the control of full length muscle creatine

kinase (MCK) promoter/enhancer (6,6 kb), showed that a great excess of dystrophin in muscle is not toxic and that the transgene product, dystrophin, restores DAPs at the membrane, and can correct dystrophic symptoms (Cox et al. 1993). The dystrophin glycoproteins complex is probably properly assembled at the membrane (Chi Lee et al. 1993).

DMD results from the absence of dystrophin and because the gene is cloned, a probable function of the gene product is known, and a mutation in only this gene results in morbidity or mortality, gene therapy is the most promising therapy at this point to provide the normal gene product for DMD.

Gene therapy consists of introducing a normal gene into target cells to provide its normal protein product and correct the deleterious consequences of the genetic mutation (Karpati et al. 1994). Although in this muscle disease, cardiac muscle cells and the central nervous system are also affected (Ahn et al. 1993 and Sanyal et al. 1982), the most severely affected tissue is still skeletal muscle (Karpati et al. 1994). For this reason, we must develop a gene transfer technique based on the characteristics of skeletal muscle fibers. A muscle fiber is a large, elongated cell containing up to thousands of myonuclei and each myonucleus contains a full genome whose normal expression is essential for the structural and functional integrity of the entire cell (Grounds 1991). Also, one important point is that myonuclei of mature muscle fibers do not divide, these are post-mitotic cells (Karpati et al. 1994). The dystrophin gene in a given myonucleus gives rise to dystrophin that is situated only in the immediate vicinity of that nucleus. In this case, the majority of nuclei must acquire a normal allele if most of the muscle fiber is to contain dystrophin (Karpati et al. 1993a). Instead of the whole cell, only a segment of a muscle fiber could become necrotic, while the the remaining portions would survive (Carpenter et al. 1979). The final point to consider is that muscle

fibers are surrounded by a well developped extracellular matrix (basal lamina) that could create a barrier to gene transfer (Karpati et al. 1994).

The 3 major elements to consider for the construction of an expression vector to be used in gene therapy are: which vector to use, the gene to introduce (DNA template) and the promoter and/or enhancer to drive gene expression.

Vector for gene therapy in DMD

Many gene delivery systems have been tested and have been found inefficient for gene transfer to muscle fibers. The utilization of a plasmid expression vector (Acsadi et al. 1991, Davies et al. 1993 and Wolff et al. 1990), microprojectiles (Yang et al. 1990), cationic liposomes containing the therapeutic gene or receptor-mediated endocytosis (Curiel et al. 1991 and Wagner et al. 1991) are some examples of methods which have failed. The intramuscular injection of plasmid, where the expression vector contained constitutive promoters (Rous sarcoma virus or Cytomegalovirus) in 5' of dystrophin cDNA or Becker-like cDNA (Acsadi et al. 1991, Davies et al. 1993 and Wolff et al. 1990) is one example of inefficient gene delivery system.

Ex vivo technique for gene therapy entails the *in vitro* transfection of cultured myoblast with a dystrophin cDNA. The transduced (or transfected) myoblasts are then reinjected into the host muscle where they may fuse with the host fibers, carrying myonuclei with functioning dystrophin genes into the host fibers. Myoblast transfer has been found to be also ineffective for gene transfer (Karpati et al. 1993b).

The next candidate as a vector for gene therapy is the viral one. Retroviruses require the target cell to be capable of replication for efficient transduction and the maximum length of insert is relatively small (7 kb) (Temin 1989). The Herpes virus has a large insert capacity (152 kb), but its tropism for muscle cells is poor. Furthermore, the virus tends to shut-down the insert gene (Suhr et al. 1993). The low insert capacity (4,7 kb) of adeno-associated viruses also makes them unattractive (Suhr et al. 1993).

Presently, the most promising vectors for therapeutic gene therapy to skeletal muscle fibers are human adenoviruses (Kozarsky et al. 1993, Quantin et al. 1992 and Ragot et al. 1993). The major advantages for using the virus are: first, the availability of a replication defective version (Berkner 1988 and Quantin et al. 1992); homologous recombination can accomodate the therapeutic gene insert (Berkner 1988 and Quantin et al. 1992); there is a low risk of toxicity to muscle cells (Quantin et al. 1992); it is feasible to produce high titers up to 10^{12} particles/ml (Berkner 1988 and Quantin et al. 1992); adenovirus can infect myoblast, myotubes and regenerating fibers (Acsadi et al. 1994a and Acsadi et al. 1994b); the internalization receptor and ligand sequences are identified (Pierschbacher et al. 1984 and Wickham et al. 1993); no major integration occurs into host genome ($<10^{-4}$) (Van Doren et al. 1984) and under certain circumstances, an excellent longevity of insert gene expression can be obtained (Vincent et al. 1993). On the other hand, the major disadvantages of using first generation human adenoviral vectors are: the insert size is limited to 8 kb (Acsadi et al. 1995 and Berkner 1988); they are not highly infective for mature muscle fibers (Acsadi et al. 1994a); possible direct toxicity at high dose and risk of destruction of transduced cells by the host through immunologic mechanisms (Dai et al. 1995).

Adenovirus contains one copy of a double-stranded, linear DNA molecule of 36 kb which contains in each termini, the 100 bp inverted terminal regions (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 (Berkner 1988). The regulation of late gene expression and the transport of mRNA to the cytoplasm is driven by E1B region gene products. This region is also required for replication of the viral DNA (Berkner 1988). 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 absolutely necessary for viral replication but it is involved directly in modulating the immune response of the host *in vivo* (Berkner 1988). Finally, the protein products of the E4 region are required for efficient viral DNA replication as well as late gene expression (Acsadi et al. 1995 and Berkner 1988). Adenovirus is able to package a maximum of 105% of its total genome length, which corresponds to an additional 2 kb sequence (Acsadi et al. 1995 and Berkner 1988).

The primary receptors for the viral penton base coat protein by which adenovirus is internalized into cells has been identified as integrin dimers containing α_V-β_3/β_5 monomers (Wickham et al. 1993 and Wickham et al. 1994). These integrins are much more abundant in myoblasts than in myotubes or mature muscles fibers (MacCalman et al. 1992). Furthermore, in mature muscle fibers, whatever small amout of α_V-β_3/β_5 integrin is present is likely to be occupied by its natural ligands, vibronectin or fibronectin of the extracellular matrix. The active ligand in the penton for the integrins is the tripeptide RGP (arginine, glycine and proline)(Pierschbacher et al. 1984). Adenoviruses enter the cell by receptor-mediated endocytosis and end up in endosomes, where the fiber is separated from the virion. The nucleocapsid enters the cytoplasm and reaches the nuclear membrane where the capsid is removed. The viral genome then enters the nucleus, by passing through the nuclear pores (Acsadi et al. 1995).

In the first generation of adenoviral vectors, deletions of the E1 and E3 genes render the adenovirus (AV) replication-defective but do not affect its packaging capacity and infectivity (Acsadi et al. 1995 and Berkner 1988). This deletion of E1+E3 creates "room" of 8 kb for therapeutic gene inserts which can be introduced by homologous recombination (Ghosh-Choudhury et al. 1986, Lamarche et al. 1990 and Van Doren et al. 1984). A special cell line, 293 cell line, has been established by stable transfection of human embryonic kidney cells with adenovirus type 5 DNA containing the full length E1 region. Replication-defective adenovirus can then replicate in these cells (Ghosh-Choudhury et al. 1986).

When an adenoviral recombinant was injected into adult mice, the expression of transgene declined rapidly, compared to a more sustained expression after injection into young mice (Acsadi et al. 1996, Ragot et al. 1993 and Vincent et al. 1993). This can be due to a partial tolerance of adenovirus-derived molecules by the immune system (Schwartz 1993). The recombinant adenovirus containing reporter genes inserts (*lacz* or luciferase) are efficient in transducing the muscles cells of very young mice (Acsadi et al. 1994a and Quantin et al. 1992), that contain myoblasts and myotubes only. However, the gene uptake efficiency is 2 magnitudes less in muscle of adult mice which contain only mature fibers (Acsadi et al. 1994a). By inducing the regeneration of adult muscle fibers into myotubes and myoblasts, the transduction efficiency is increased (Acsadi et al. 1994a).

A first major disadvantage of using adenovirus is that humoral and cellular immune reactions may interfere with sustained transduction efficiency of adenoviral vectors (Acsadi et al. 1996, Dai et al. 1995, Yang et al. 1994a and Yang et al. 1995b). It has been shown that cytotoxic CD8+ lymphocytes play a critical role in this process (Dai et al. 1995, Quantin et al. 1992, Yang et al. 1994a, Yang et al. 1994b and Yang et al. 1995b). In addition, a humoral response to adenovirus prevented efficient transduction of the target cells after readministration

of adenovirus (Dai et al. 1995, Quantin et al. 1992, Van Ginkel et al. 1995, Yang et al. 1994a, Yang et al. 1994b and Yang et al. 1995b). Antibodies are produced against both the viral proteins and transgene. Injection of naked DNA into *mdx* muscle (dystrophin) did not evoke an immune reaction while after injection of the same transgene inserted into an adenoviral vector, immune reactions are observed (Acsadi et al. 1991 and Acsadi et al. 1996). It is probable that gene transfer by injection of plasmid DNA did not lead to high level expression and it was not sufficient to evoke an immune reaction (Acsadi et al. 1991). If the promoter is not specific to muscle cells (Gorman et al. 1982), the expression of the transgene can occur in many cells, including antigen-presenting cells like macrophages or dendritic cells (Acsadi et al. 1996). The use of a muscle specific promoter/enhancer may prevent immune reaction, since mature muscle fibers, to which the transgene expression is limited, are poor antigen presenting cells (Dalakas 1995).

It has been shown recently that the immune response of the host (mice) against adenovirus-transduced cells and the vector can be mitigated or avoided by various measures of immunosuppression such as administration of IL-12, CTLA4Ig, cyclosporin and FK506 (Fang et al. 1995, Kay et al. 1995, Lochmüller et al. 1995, Vilquin et al. 1995 and Yang et al. 1995a). IL-12 administration blocked humoral responses selectively and did not lead to a prolongation of transgene expression (Yang et al. 1995a). Short-term CTLA4Ig administration did not prevent the formation of low-level neutralizing antibodies, which presumably prevented efficient secondary transduction 8 weeks after the initial gene transfer (Kay et al. 1995). Long-term CTL4AIg administration (21 days) did not block humoral and cellular responses against adenoviral and β-gal proteins (Guérette et al. 1996). Although cyclosporin A is an inhibitor of activated T-lymphocyte proliferation (Kronke et al. 1984), a daily treatment was ineffective in augmenting long-term transgene expression after adenovirus injection into mouse diaphragm (Petrof et al. 1995). FK506 interferes with the signal

transduction between the T cell receptor and nucleus, thus preventing T cell proliferation, differentiation and cytokine production (Sawada et al. 1987 and Schreiber et al. 1992). A short duration of treatment with FK506 (30 days) after adenovirus-mediated dystrophin transfer into mature *mdx* muscle significantly prolonged dystrophin expression for up to 2 months (Lochmüller et al. 1996). In addition, FK506 significantly suppressed the humoral immune response of the animals against the vector after the initial adenovirus administration (Lochmüller et al. 1996). Thus, with some immune suppression regimens, sustained expression of transgenes inserted into the adenoviral vector can be achieved.

Adenoviral vectors, with their characteristics, are the most promising delivery system for gene therapy for skeletal muscle fibers. In the first generation of adenovirus the insert size capacity is limited to 8 kb, but the newly-developed adenoviral vectors can accomodate up to 10 kb, that have a realistic promise for gene therapy in DMD (Acsadi et al. 1995 and Wang et al. 1995).

DNA template for gene therapy in DMD

In the case of development of gene therapy for muscular dystrophy, the dystrophin gene is the only possible gene to use. A second major drawback of using first generation adenovirus (produced by deletion of E1 and E3 regions) as a vector, is its limited insert capacity of 8 kb, precluding the use of the full length dystrophin cDNA (14 kb) (Acsadi et al. 1995 and Berkner 1988). A patient has been found to show a mild phenotype of Becker dystrophy (still walking at 61 years old) in whom the dystrophin polypeptide, lacking exon 17 to 48 (5106 bp, 229 kDa), was correctly localized in the muscle cells (England et al. 1990)(Figure 2B). But, by using this 6,3 kb minigene, only a conversion of the DMD to

BMD phenotype can be expected at best (Wells et al. 1992).

It has been shown in *mdx* transgenic mice that the dystrophin minigene is enough to protect muscle fibers from necrosis, despite the fact that its expression level was low (Wells et al. 1992). Based upon findings in heterozygote carriers of the disease and transgenic mice, it is estimated that approximately 30 to 40% of the normal amount of dystrophin must be expressed in an even distribution in order to prevent major myopathic manifestations (Hoffman et al. 1992 and Watkins et al. 1989), and well-distributed dystrophin seems to be more important than the level of expression (Phelps et al. 1995. The heterozygote fibers seem to be rescued from segmental necrosis by redistribution of dystrophin (Watkins et al. 1989).

Since the dystrophin minigene of 6.3 kb of length is small enough to fit in adenoviral vectors and the truncated protein is able to protect muscle fibers from subsequent necrosis, this is DNA template of choice.

Promoter and/or enhancer for gene therapy in DMD

The ideal promoter and/or enhancer for driving expression of therapeutic genes for skeletal muscle fibers should be highly active in target cells, and show specific activity in muscle cells to minimize possible toxicity and immunogenicity in non-target cells (Karpati et al. 1994). In addition, for use in first adenoviral vectors, the regulatory sequences should be confined to a DNA fragment of small size.

The natural muscle promoter of dystrophin (Ahn et al. 1993 and Klamut et al. 1990) should theoretically be an ideal promoter but it was found that this promoter is very weak or even inactive (Klamut et al. 1990). The constitutive viral promoters (Rous sarcoma virus late

promoter, RSV-LTR, or the cytomegalovirus promoter, CMV) are very active in muscle cells (Wolff et al. 1991) and are relatively small (<1 kb), but their activity is not restricted to muscle cells (Gorman et al. 1982).

Many muscle-specific genes have been cloned and their regulatory sequences are being mapped and characterized. These include α-skeletal actin (Bergsma et al. 1986, Muscat et al. 1987 and Walsh et al. 1988), α-cardiac actin (Minty et al. 1986, Miwa et al. 1987 and Mohun et al. 1986), troponin I (Konieczny et al. 1987), myosin light chain 2 (Arnold et al. 1988), myosin heavy chain (Bouvaget et al. 1987) and muscle creatine kinase (MCK) (Cox et al. 1993, Jaynes et al. 1986, Jaynes et al. 1988 and Johnson et al. 1989)· α-skeletal actin promoter is weak (Manthorpe et al. 1993), while myosin light chain 2, myosin heavy chain, and troponin I promoters are fiber-type specific (Arnold et al. 1988, Bouvaget et al. 1987 and Pette et al. 1990), which is a drawback for gene therapy in DMD (Karpati et al. 1994). Muscle creatine kinase has high expression levels in muscle and its promoter/enhancer region is small enough to make it suitable for inclusion in adenoviral vectors. Creatine kinase plays a central role in cellular energy metabolism by catalyzing the reversible transfer of high energy phosphate bonds from creatine phosphate to ADP to generate ATP (Bessman et al. 1985). Muscle creatine kinase 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 (Cox et al. 1993).

High level expression of MCK is restricted to differentiated skeletal and cardiac muscle (Johnson et al. 1989 and Urdal et al. 1983). The full length MCK promoter/enhancer (6.5 kb) consists of a muscle specific enhancer (E1) (-1050 to -1256 bp), the basal promoter in the 5' untranslated region upstream of the first exon, and a second enhancer (E2)(+738 to +1599) in the first intron (Jaynes et al. 1986, Jaynes et al. 1988 and Johnson et al. 1989). Translation

begins in exon 2, 3.2 kb downstream of the transcription initiation site (Jaynes et al. 1986). While sequences between -4800 and -1800 bp do not significantly affect MCK gene expression (Sternberg et al. 1988), the E1 enhancer influences overall MCK expression and confers muscle specificity (Johnson et al. 1989). Although the E2 enhancer is not essential for MCK gene activity, it does increase expression levels of the basal promoter (Buskin et al. 1989). Transgenic mice carrying wild-type or mutated 5'-flanking regions of the mouse MCK gene were generated (Donoviel et al. 1996). Adult transgenic mice containing -3300 MCKCAT construct, showed high CAT activity in only skeletal and cardiac muscles. Wild-type -1256MCKCAT transgene exibited high-level CAT activity in skeletal and cardiac muscle but extremely low activity in liver (Donoviel et al. 1996). 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 (Johnson et al. 1989). 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 (Chamberlain et al. 1985, Kwiatkowski et al. 1985 and Ritchie et al. 1991).

Several *cis*-elements in the first enhancer (E1) have been identified that regulate expression of MCK: CArG, AP2, A/T rich, left and right E boxes, MEF-2 sites, and TREX (Transcriptional regulatory element x) (Amacher et al. 1993, Buskin et al. 1989, Fabre-Suver et al. 1996 and Mueller et al. 1989). This 206 bp enhancer region has been delineated into several motifs which, when mutated, lead to diminished promoter activity in skeletal and cardiac muscle (Amacher et al. 1993). Mutation of the MCK right E box (MEF-1) site decreases enhancer activity more dramatically in skeletal (40 fold) than in cardiac muscle cells, while mutations of the CArG site or the A/T-rich site seem more deleterious in cardiac (10-20 fold) than in skeletal muscle cells (2-10 fold) (Amacher et al. 1993). In contrast to these

differential effects, mutations of the MEF-2 site or the left E box site decrease enhancer activity to about the same extent in both cell types (2-5 fold) (Amacher et al. 1993). Unlike the other MCK enhancer control elements, the AP2 site seems to repress transcription in cultured skeletal and cardiac muscle cells as its mutation leads to increased expression in both cell types (50 %) (Amacher et al. 1993). 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 (Fabre-Suver et al. 1996).

These six enhancer regulatory elements are the target sites for an array of DNA binding factors. The CArG motifs have been shown to bind serum response factors (Shore et al. 1995). The left and right E boxes contain the consensus core sequence CANNTG, which is the target for skeletal muscle-specific determination factors of the MyoD family of transcription factors (Apone et al. 1995). The MEF-2 site and the A/T-rich site are both rich in adenine and thymidine (Cserjesi et al. 1992, and Gossett et al. 1989). The MEF-2 site is the target of MEF2 proteins and BBF-1, a serum-inducible factor (Shore et al. 1995, Yu et al. 1992 and Zhou et al. 1993). MEF2 proteins have been shown to cooperate in transactivation with members of the MyoD family of transcription factors (Funk et al. 1992, Kaushal et al. 1994, Molkentin et al. 1995 and Olson et al. 1995) and bind with lower affinity to the MCK A/T-rich site (Cserjesi et al. 1994). The mouse MCK A/T-rich element is also a DNA-binding site for both the homeoprotein MHox and Oct-1 factor (Cserjesi et al. 1992, and Cserjesi et al. 1994).

Muscle creatine kinase promoter/enhancer expression level is high and restricted to differentiated muscle cells. Furthermore, the E1 enhancer of MCK is sufficient to confer muscle specificity (Johnson et al. 1989) and this fragment is small enough to be included in

adenoviral vectors.

Rationale for the study and objectives

A gene therapy approach for Duchenne muscular dystrophy consists of the introduction of dystrophin into skeletal muscle fibers to protect cells from necrosis, and their eventual loss that leads to muscle weakness. Presently, the most promising vector for gene delivery are human adenoviruses. Since the tropism of the virus is not restricted to muscle cells only, we must develop an adenoviral vector that contains a promoter/enhancer sequence to regulate gene expression in order to avoid immune reactions, which may interfere with sustained transduction efficiency and to limit expression of dystrophin in non-muscle organs, where its expression is much lower. The muscle creatine kinase promoter/enhancer confers high level and restricted expression in differentiated muscle cells. The E1 enhancer of MCK is sufficient to confer muscle specificity and this fragment is small enough to be included in adenoviral vectors.

The main objectives of this study consist in:

1) evaluation of tissue specificity of the MCK promoter/enhancer in adenoviral vectors by comparison with a strong and constitutive promoter (LTR RSV) *in vivo* in immunoincompetent mice

2) evaluation of long term reporter gene expression of both promoters in adenoviral vectors *in vivo* in immunocompetent mice

The sequence -1354 to +1 from the transcription initiation site of the MCK

promoter/enhancer, containing the promoter and E1 enhancer was previously cloned into adenoviral transfer plasmid in our laboratory. A recombinant adenoviral vector containing this promoter/enhancer driving the luciferase reporter gene had been obtained (AVMCKlux) in our laboratory. Luciferase transgene has been used because the expression of this reporter gene is easy to detect by a sensitive chemiluminescent assay, and the length of the gene is relatively short (<2kb) to be included in adenovirus vector (de Wet et al. 1987).

For the evaluation of muscle-specific expression of luciferase driven by the MCK promoter/enhancer, young mice were used because muscle transducibility is higher than in older mice (Acsadi et al. 1994a and Huard et al. 1995b). I used immunodeficient mice (*scid*) to minimize immune problems. The severe combined immunodeficient (*scid*) mice have impaired differentiation of both T and B lymphocytes, so they are unable to produce specific antibody (Bosma et al. 1983).

As a control, I used an adenoviral recombinant that contained RSV (Rous sarcoma virus) promoter (AVRSVlux) instead of the MCK promoter/enhancer. The RSV promoter is a constitutive and non-muscle-specific promoter (Gorman et al. 1982). This can eliminate the potential problem of differential stability of luciferase protein in muscle cells compared to other organs. In addition, this control permits the evaluation of the efficiency of the MCK promoter compared to the strong RSV promoter.

To determine whether the utilization of a muscle-specific promoter in an adenoviral vector can lead to long term expression by avoiding immune reaction, via limiting expression of the transgene to muscle fibers, which are poor antigen presenting cells, the AVMCKlux recombinant construct has been used in immunocompetent (adult) mice. Utilization of adult *mdx* mice is a good choice because 1) muscle transducibility is higher than in adult normal

mice (Acsadi et 1994a), due to the presence of regenerating muscle fibers, and 2) adult animals are immune competent.

The ultimate goal is to use dystrophin cDNA driven by a muscle-specific promoter/enhancer in an adenoviral vector, with an increased insert capacity to fit the expression cassette (adenovirus full E1+E3 deleted) to avoid immune reaction, and to limit the transgene expression to muscle fibers.

MATERIALS AND METHODS

Construction of recombinant adenovirus

The muscle creatine kinase promoter/enhancer driving the expression of the luciferase gene was cloned as an expression cassette into a plasmid containing sequences from human adenovirus type 5. The MCK fragment contains the region from -1354 to +1 bp from the transcription initiation site. The fragment was amplified by PCR, using the full length MCK promoter/enhancer unit (6.5 Kb) as a template (a gift by Jeffrey S. Chamberlain, University of Michigan Medical School) (Cox et al. 1993), and Bgl II and Hind III sites were added for cloning purposes.

Homologous recombination of replication defective (E1+E3 deleted) human type 5 adenovirus

The adenoviral plasmid cassette was linearized with ClaI digestion, then purified and co-transfected with ClaI-cut, E1+E3 deleted adenovirus by standard $CaPO_4$ transfection into 293 cells. The plates were overlaid by 1% Seaplaque agarose in 10% bovine calf serum supplemented DMEM, and plaques were obtained 7-14 days after transfection. The recombinant was already generated when I began the project. The plaques were purified two consecutive times according to the results of the analysis of reporter gene expression and Southern and slot blotting to verify the content of the recombined adenoviruses (MCK promoter/enhancer, luciferase gene, presence of E2 and absence of E1 regions in adenovirus

(Lochmüller et al. 1994)).

Large scale production, purification and titration of recombinant adenoviruses

The plaque-purified adenoviral recombinant was used to infect 293 cells grown in one liter spinner cultures at a density of 10^6 cells/ml. 30-40 hours later, when the cytopathic effect was complete, the cells were collected and pelleted. The cell-sludge was freeze-thawed 3 times, then the debris was separated by low speed centrifugaton and rinsed with 10 mM Tris-Cl (pH 7.9). The virus-containing supernatant was layered onto 2 discontinuous CsCl gradients and centrifuged at 50 000Xg for 2 hours in a Beckman XL-90 ultracentrifuge. The viral band was then collected and diluted in 2.5 ml phosphate buffered saline (PBS) and desalted on a Sephadex G25 column. The optical density of the harvested fractions (500 ul) was measured by spectrophotometry (at 260 nm) and titrated by standard plaque assay and expression assay. The latter was carried out by infecting 293 cells with a serial dilution of virus ranging from 10^{-2}-10^{-12}, then analyzed for luciferase assay expression as described below. The virus stock was used immediately or stored in a 1% BSA and 10% glycerol buffer at -70°C.

Animals and injection techniques

Experiments were performed on groups of *scid* mice (Jackson Laboratories, Bar Harbour, Me). The study was conducted in accordance with the guidelines of the Canadian Council of Animal care. 4-7 day old *scid* 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. Quadriceps, tibialis anterior and gastrocnemius muscles (4 animals) were injected percutaneously with 5 µl of AVMCKlux suspension ($7X10^{11}$ particles/ml). In addition, liver (6 animals), kidney (6 animals), brain (6 animals) and heart (5 animals) were directly injected with 20 µl, 20 µl, 20 µl and 30 µl of AVMCKlux suspension, respectively. All mice were euthanized at 10 and 30 days post-injection. The level of luciferase activity for the lung was determined after systemic injection of 4-7 day old *scid* mice injected directly into left cardiac ventricle as described (Huard et al. 1995a).

To compare the efficacy of the MCK promoter/enhancer with RSV (Rous Sarcoma virus) promoter, tibialis anterior, quadriceps and gastrocnemius of 4-7 day old *scid* mice were injected with 5 µl of each viral suspension, at the same titer; the right side of 5 mice were injected with AVMCKlux and left side with AVRSVlux. In addition, the liver, kidney and brain of 6 *scid* mice were also directly injected with 10 µl of AVRSVlux suspension, while the left cardiac ventricle of 4 newborn *scid* mice was injected with 25 µl of AVRSVlux suspension. All mice were euthanized after 10 days.

To evaluate long-term expression of reporter gene into immunocompetent animals, 21 adult *mdx* mice (30-50 days of age) were injected in both tibialis anterior and gastrocnemius with 15 µl of AVMCKlux suspension ($7X10^{11}$ particles/ml) and 16 animals were injected with AVRSVlux suspension ($7X10^{11}$ particles/ml). Mice were sacrificed at 10, 30 and 60 days.

Analysis of reporter gene expression

For luciferase quantitation, the dissected organs were lysed in 1.5 ml Eppendorf tube in 200 µl of lysis buffer (100 mM potassium phosphate (pH 7.8), 1mM dithiothreitol, and 0.1% Triton X-100) by homogenizing the tissue with a plastic pestle, then centrifuged in a microcentrifuge, at 12 000 RPM and at 4°C. An 87.5 µl portion of the 200 µl supernatant was analysed for luciferase activity in a buffer containing 10 µl of 1M K_2HPO_4 at pH 7.4, 1µl of 1M $MgCl_2$, 2.5 µl of 0.1 M ATP and 35 µl of 3 mmol luciferin [Analytical Luminescence Laboratories (ALL) with a BioOrbit luminometer (LKB-Fisher)]. 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. Each reading corresponds to the total amount of luciferase (pg) for the injected tissue. The data were not expressed in term of luciferase activity per microgram of total protein, since the weight of the tissue increases with aging. In this case, the luciferase quantification would had been diluted out. The data were then statistically analysed by ANOVA (Analysis of variance) tests.

RESULTS

The part of muscle creatine kinase promoter/enhancer (MCK) that was used to generate the adenoviral recombinant AVMCKlux is described in Figure 4. The 1.35 kb fragment containing the MCK E1 enhancer and promoter, but not the E2 enhancer found in the first intron, was cloned into an expression vector to drive the firefly luciferase reporter gene (*lux*). Luciferase transgene has been used because the expression of this reporter gene is easy to detect by a sensitive chemiluminescent assay, and the length of the fragment is relatively short (< 2 kb) for inclusion in adenovirus vector (de Wet et al. 1987). After *in vivo* recombination in 293 cells (E1-complementing cell line) with human adenovirus type 5 carrying deletions of the E1 and E3 regions, high titer adenoviral recombinants were produced ($7X10^{11}$ particles/ml).

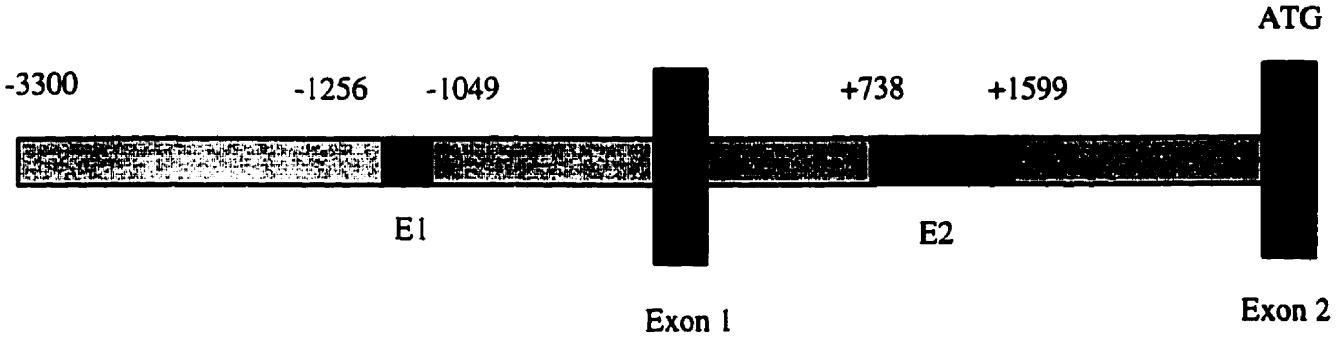
To test the tissue specificity of the regulatory sequences which were present in the adenoviral recombinant, a series of *in vivo* experiments were carried out in young *scid* mice. AVMCKlux was injected directly into skeletal muscle (Gastrocnemius, tibialis anterior and quadriceps), heart, brain, liver and kidney, which were sampled at 10 and 30 days post-injection. Luciferase activity was measured by chemiluminescent assay. Young mice were chosen to enhance transduction efficiency of muscle by adenovirus (Acsadi et al. 1994a), and the use of immunodeficient animals (*scid* mice) minimized immune reaction that compromises longer-term expression, as demonstrated previously (Acsadi et al. 1996). The severe combined immunodeficient (*scid*) mice have impaired differentiation of both T and B lymphocytes, so they are unable to produce specific antibody (Bosma et al. 1983). As shown in Figure 5A, 10 days after direct injection with AVMCKlux, skeletal muscle had the highest

Figure 4. **A)** Depiction of the construct to generate AVMCKlux, the full length muscle-creatine kinase promoter/enhancer and the construct analyzed in transgenic mice by Johnson et al. 1989. **B)** Description of AVMCKlux recombinant.

A

MCK lux transfer cassette

MCK 5' region (Johnson et al. 1989)



Transgene -3300 MCK-CAT (Johnson et al. 1989)

B

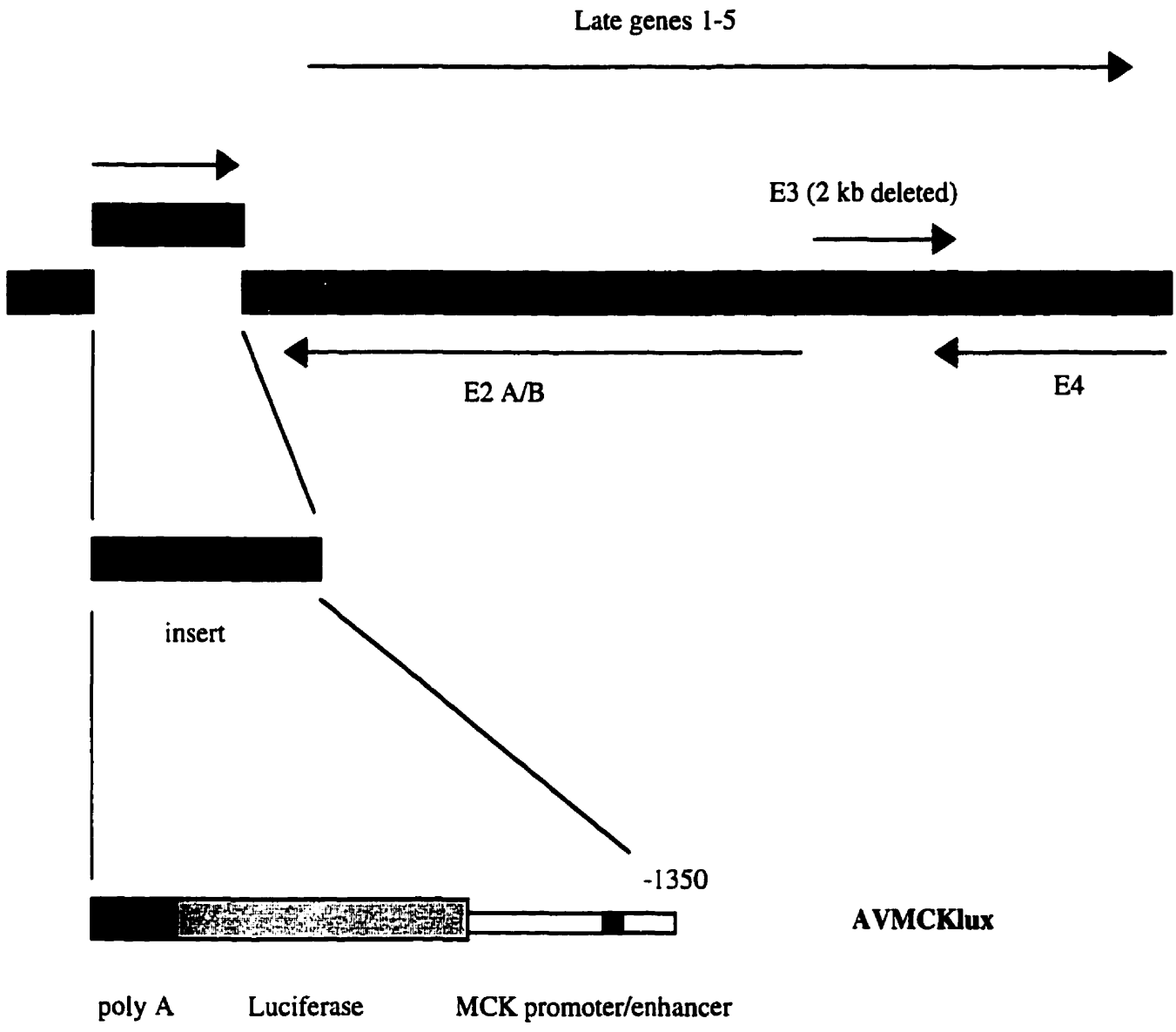

Late genes 1-5
E3 (2 kb deleted)
E2 A/B
E4
insert
-1350
AVMCKlux
poly A
Luciferase
MCK promoter/enhancer

Figure 5. Expression of luciferase in various tissues of young *scid* mice analysed at **A)** 10 days and **B)** 30 days after injection of AVMCKlux as described in Materials and Methods. Total luciferase activity (mean +/- standard error) was calculated as described in Materials and Methods and has been plotted on a logarithmic scale.

activity, ~ 1000 pg of luciferase, approximately 10^4-fold higher than was detected in liver. Only heart and lung (after systemic injection through the heart) had any significant luciferase activity, although the levels were more than 50 to 500-fold lower in comparison to skeletal muscle.

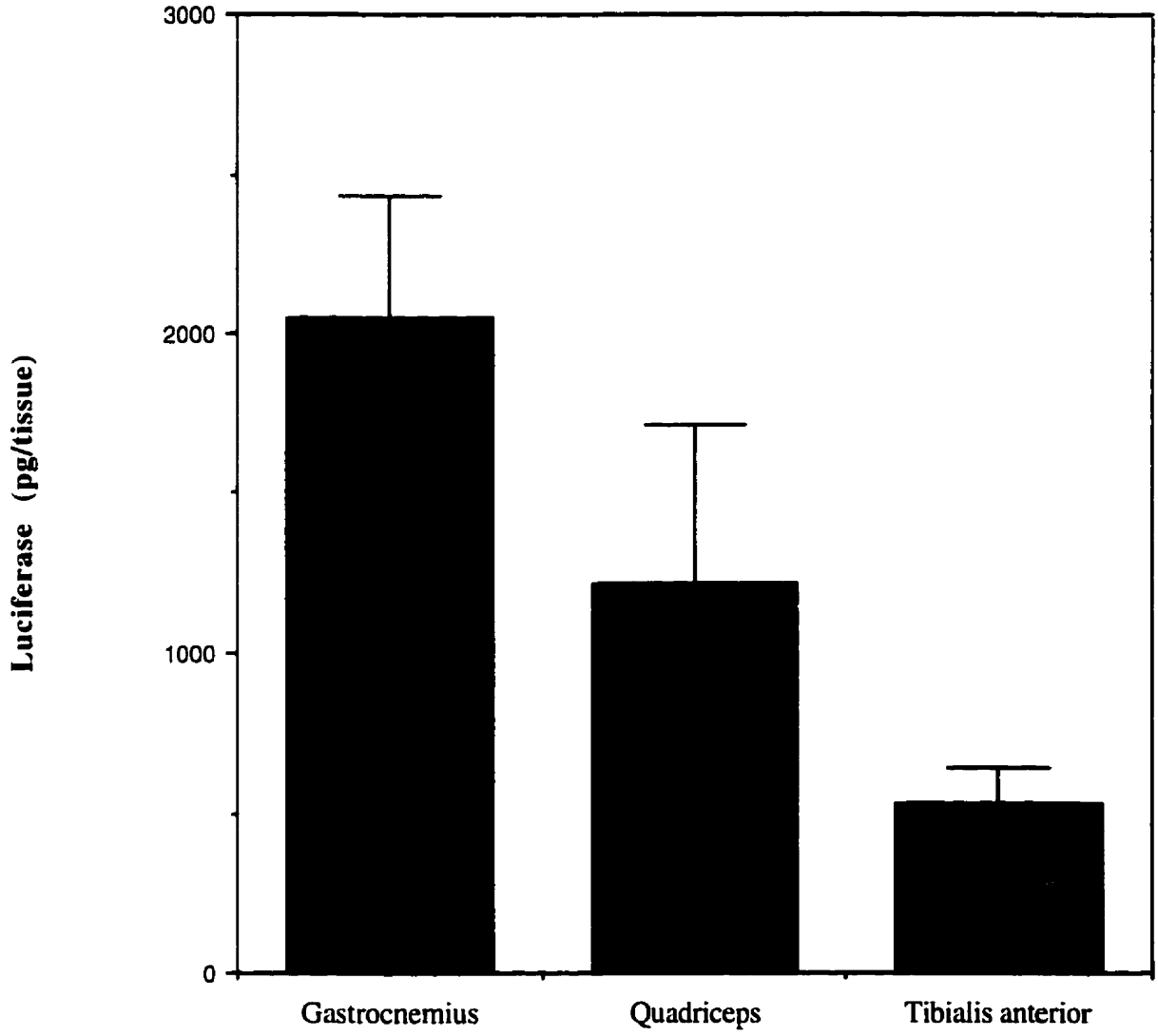
The use of immunoincompetent mice was to prevent immune reaction and, as expected, the level of luciferase activity at 30 days post-injection into skeletal muscle was stable, ~1000 pg, while in other organs it was still barely detectable, as shown in Figure 5B.

AVMCKlux efficiently transduced different groups of muscles, such as the gastrocnemius, quadriceps and tibialis anterior which all expressed high levels of luciferase activity 10 days after direct injection (Figure 6). There was no significant difference in luciferase activity between the different muscle types.

In conclusion, the results obtained demonstrated high level of luciferase expression in muscle driven by MCK promoter/enhancer and very low level in other tissues. This strongly suggests that this MCK promoter/enhancer construct in adenovirus recombinant is muscle-specific. However, two other possibilities can explain low luciferase level in non-muscle tissues: first, non-muscle cells are not infected by adenoviral recombinant, or second, *lux* transcript and/or protein are not as stable as in muscle fibers.

To verify that the different tissues could be infected with adenoviral recombinant and that transcript and/or protein stability do not play a role in the luciferase level obtained, AVRSVlux recombinant was directly injected in skeletal muscles (gastrocnemius, quadriceps and tibialis anterior) on one side of young *scid* mice, with AVMCKlux recombinant being injected on the other side. Groups of animals were also injected in liver, kidney, brain and

Figure 6. Expression of luciferase in different muscle groups of young *scid* mice analysed 10 days after direct injection of AVMCKlux. Total luciferase activity (mean +/- standard error) was calculated as described in Materials and Methods.


Luciferase (pg/tissue)
3000
2000
1000
0
Gastrocnemius
Quadriceps
Tibialis anterior

heart with one or the other adenovirus recombinant. The RSV promoter is constitutively expressed everywhere and serves as a positive control (Gorman et al. 1982). All tissues infected with AVRSVlux recombinant showed high levels of luciferase activity compared to tissues injected with AVMCKlux, as reflected in the low MCK/RSV ratios obtained with non-muscles tissues (Figure 7). In support of the results in Figure 5, all the examined tissues, except for skeletal muscle, had extremely low luciferase activity after injection with AVMCKlux, with less than 5% of the expression detected with AVRSVlux (Figure 7). In skeletal muscle, 40% of the luciferase levels obtained with AVRSVlux could be attained with AVMCKlux, attesting to the effectiveness of MCK promoter/enhancer sequences incorporated into AVMCKlux.

Humoral and cellular immune reactions may interfere with sustained transduction efficiency of adenoviral vectors (Acsadi et al. 1996, Dai et al. 1995, Yang et al. 1994a, Yang et al. 1995b). The use of a muscle specific promoter/enhancer may prevent immune reaction, since mature muscle fibers, to which the transgene expression is limited, are poor antigen-presenting cells (Dalakas 1995). To evaluate the longer-term expression of luciferase reporter gene, two groups of adult *mdx* mice were injected in skeletal muscle (tibialis anterior and gastrocnemius) with AVMCKlux and AVRSVlux recombinants. *mdx* mice were used because adult animals are immune competent and muscle transducibility is higher than in adult normal mice (Acsadi et al. 1994a), due to the presence of regenerating muscle fibers.

As mentioned earlier, when an adenoviral recombinant is injected into adult mice, the expression of the transgene declines rapidly, compared to a more sustained expression after injection into young mice (Acsadi et al. 1996, Dai et al. 1995, Quantin et al. 1992, Vincent et al. 1993 and Yang et al. 1994a). In Figure 8A, where *mdx* adult mice had been injected with AVRSVlux recombinant, luciferase activity is high at 10 days (~2400 pg in tibialis anterior)

Figure 7. Comparison of luciferase activities (ratio MCK lux/RSV lux in %) after direct injection of various tissues of *scid* mice with either AVMCKlux or AVRSVlux.

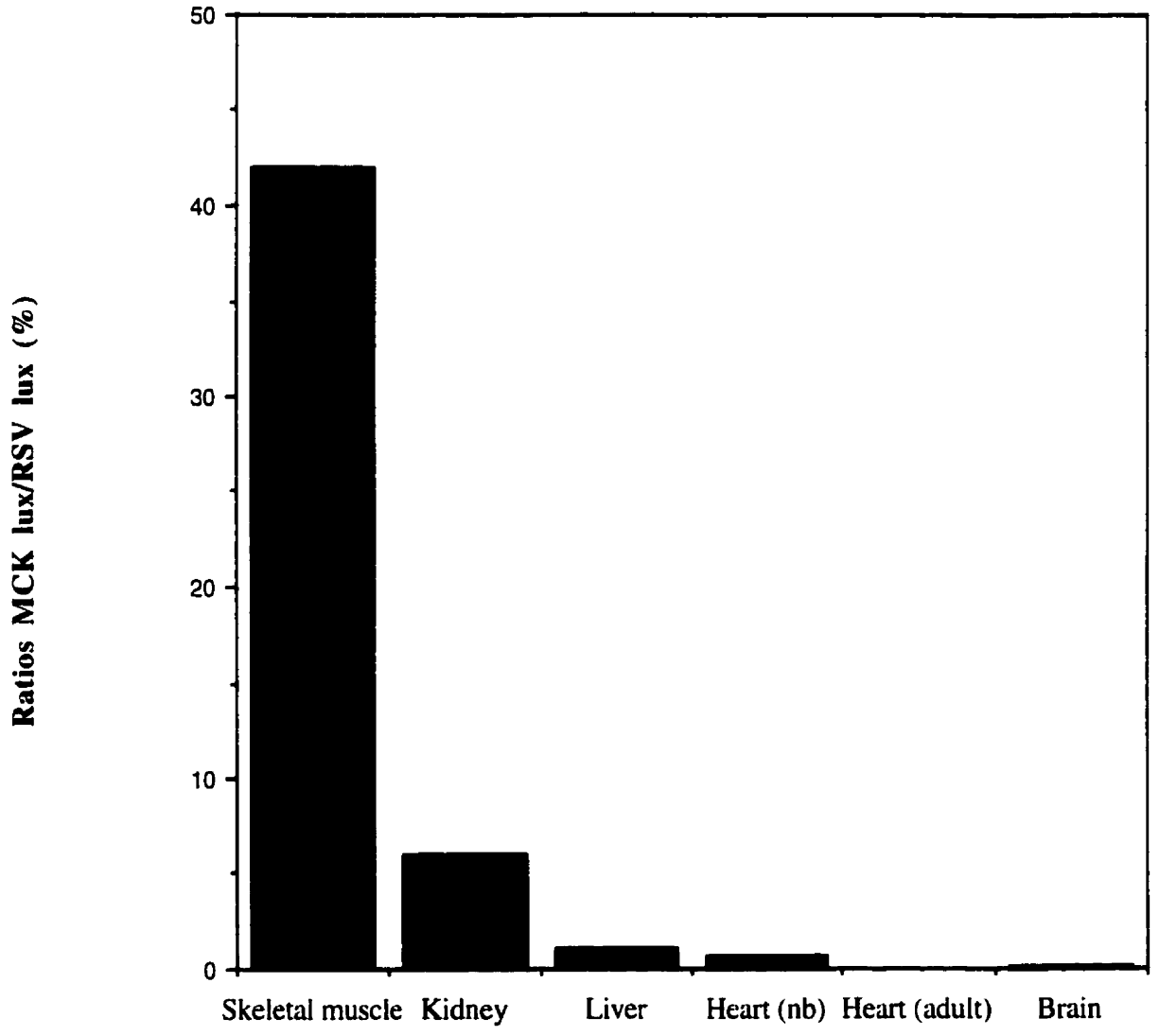
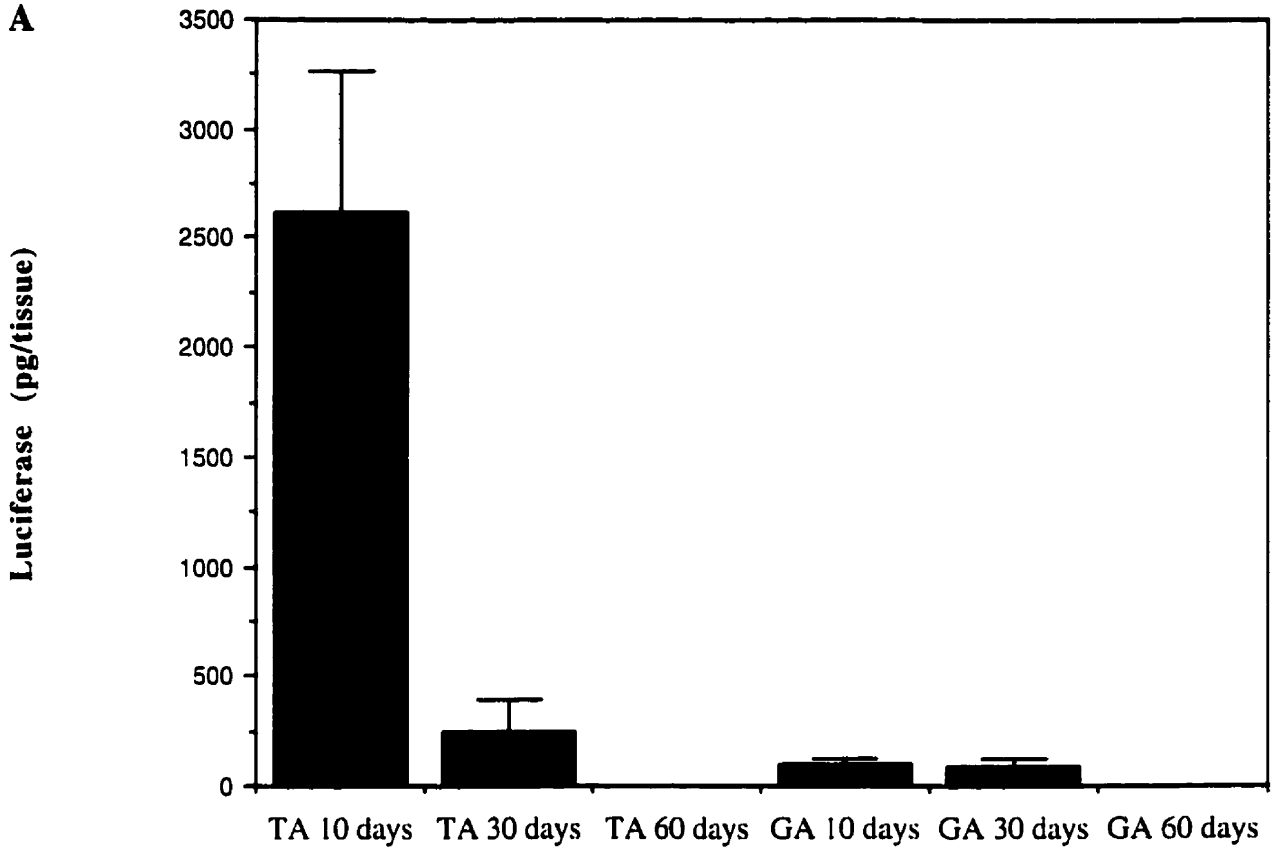
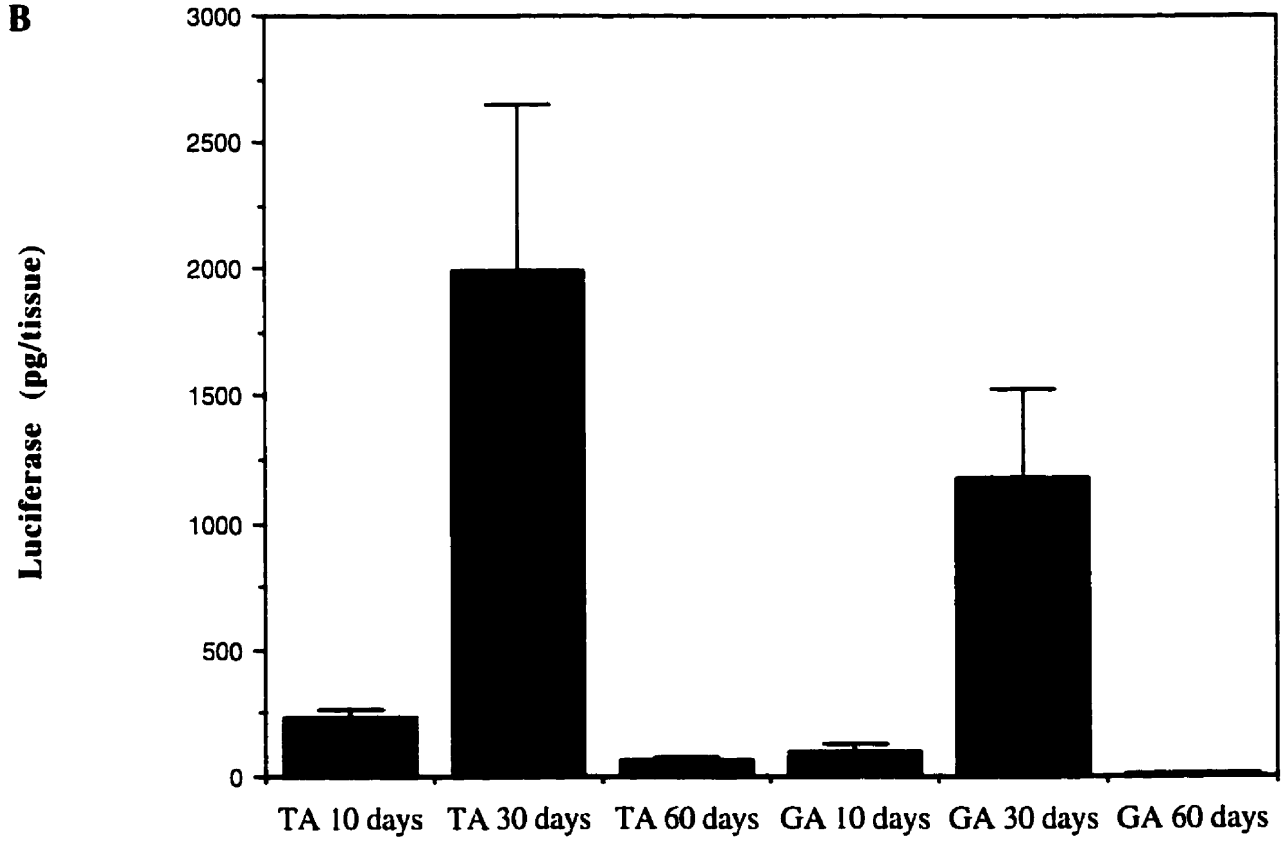

Ratios MCK lux/RSV lux (%)
50
40
30
20
10
0
Skeletal muscle
Kidney
Liver
Heart (nb)
Heart (adult)
Brain

Figure 8. Expression of luciferase in two different muscle groups of adult *mdx* mice analysed 10, 30 and 60 days after direct injection of AVRSVlux (**A**) or AVMCKlux (**B**). Total luciferase activity (mean +/- standard error) was calculated as described in Materials and Methods.

A


Luciferase (pg/tissue)
3500
3000
2500
2000
1500
1000
500
0
TA 10 days
TA 30 days
TA 60 days
GA 10 days
GA 30 days
GA 60 days

B


Luciferase (pg/tissue)
3000
2500
2000
1500
1000
500
0
TA 10 days
TA 30 days
TA 60 days
GA 10 days
GA 30 days
GA 60 days

and decreases by 10-fold 30 days post-injection, in both muscle types. In contrast, as shown in Figure 8B, using MCK promoter/enhancer in the adenovirus recombinant in parallel experiments, the same luciferase level (~2000 pg) can be achieved; this level of luciferase expression is obtained at 20 days and still present at 30 days post-injection. However, the luciferase expression was found to be decreased 60 days post-injection for both AVRSVlux and AVMCKlux recombinants.

DISCUSSION

In adenovirus-mediated gene transfer, limiting gene expression to specific cell types can theoretically be achieved either by customizing the tropism of the virus, or by restricting expression of the transgene to the target tissue by a tissue-specific promoter. To date, no single cell surface molecule has been identified which is absolutely specific to skeletal muscle, hindering the development of adenoviral vectors in which capsid proteins have been modified for attachment to, and infection of, only skeletal muscle. On the other hand, the regulatory sequences which govern expression of many different muscle-specific genes have been mapped, allowing control of gene expression through the use of these specific *cis*-acting elements. However, in the first generation adenoviral vectors, there are limitations on the size of promoter/cDNA expression cassette that can be inserted and packaged (8 kb). This is even more important for correction of dystrophin deficiency since the smallest cDNA fragment which can be used to functionally replace the full-length dystrophin, the Becker "mini-gene", is 6.3 kb in length. Hence, there is a need for identifying the minimal sequences required for efficient and muscle-specific expression to be included in adenoviral vectors.

In this thesis I have shown that the muscle creatine kinase sequences drive a high level and tissue-specific expression of the luciferase reporter gene in muscle. As expected, with MCK regulatory sequences, high level of reporter gene expression was achieved and maintained for at least 30 days in immunodeficient hosts. To verify that low luciferase level in non-muscle tissues was not due to lack of infection by adenoviral recombinants, an *in situ* PCR was performed by Dr. Ji-En Zhao, of our laboratory. The presence of adenoviral nucleic acid in the injected tissues was confirmed by an *in situ* PCR protocol in which DNA was

amplified with primers specific for the E4 region of adenovirus and subsequently hybridized to E4 probe. The results showed that all tissues were well infected but expression of the luciferase reporter gene was several magnitudes lower (40X to 6000X fold) as determined with this sensitive luciferase assay.

Luciferase levels produced by AVMCKlux compared favourably to those obtained in parallel experiments from injection of AVRSVlux in which *lux* expression is driven by the ubiquitously active LTR sequences of RSV. This is highly significant considering that the LTR of RSV is most active in skeletal muscle (Acsadi et al. 1994a). Taken together, the data indicate that engineering the adenoviral vector to be muscle-specific did not diminish efficiency of expression.

When the pattern of expression observed in Figure 5 is compared with what has been reported with transgenic mice containing different copies of integrated MCK promoter/enhancer constructs, it is evident that AVMCK is regulated in various tissues in a manner similar to those constructs containing the longer fragment of MCK upstream sequences (-3300MCKCAT) (Johnson et al. 1989). One exception seems to be in cardiac muscle where expression levels with AVMCK were low, indicating that additional regulatory sequences may need to be included or excluded eventually to correct the defect in DMD cardiac muscle. In experiments in which animals were injected by the intracardiac route, expression was detected in lung and in the heart. In the -3300MCKCAT transgenic mice, lung and brain have the highest reporter gene levels after skeletal and cardiac muscle (Johnson et al. 1989). This is consistent with the presence of pulmonary myocardiocytes (Jones et al. 1994) and/or alveolar macrophages (Johnson et al. 1989) in lung.

Several groups have used the 206 bp region of the E1 enhancer of MCK in

conjunction with heterologous promoters to obtain muscle-specific expression of transgenes (Dai et al. 1992 and Ferrari et al. 1995). Although these experiments have been successful *in vitro*, regulation *in vivo* is clearly different since recent analysis in transgenic mice have shown that the 1 kb region between the basal promoter and the enhancer is required, producing a 100-fold increase in expression levels of reporter genes (Donoviel et al. 1996). Thus, it is unlikely that the MCK regulatory sequences included in AVMCKlux can be further trimmed. However, the muscle creatine kinase promoter/enhancer used in this study is small enough to drive the Becker-type mini-dystrophin gene in the newly-developed adenoviral vectors with larger (10 kb) insert capacity that have a realistic promise for gene therapy of Duchenne muscular dystrophy (Acsadi et al. 1995 and Wang et al. 1995).

Longer term transgene expression (30 days) is maintained by using the muscle-specific MCK promoter/enhancer when recombinant adenovirus is injected into muscle of adult immunocompetent *mdx* mice (Figure 8B). By using a non muscle-specific promoter in a parallel experiment (AVRSVlux recombinant), transgene expression decreased dramatically at 30 days post-injection. The production of antibodies was detected early on by Elisa technique, done by Carole Allen in our laboratory. Antibodies were produced both against luciferase and the viral proteins in the animals injected with AVRSVlux recombinant. Since RSV LTR promoter is not muscle-specific (Gorman et al. 1982), transgene expression had occured in many cells, including antigen-presenting cells. These results correlate well with the expression level of the transgene. In the serum of animals injected with AVMCKlux, some anti-luciferase antibodies were detected at late time points although viral antibodies were present earlier. Despite the fact that adenovirus is replication-defective, there is still some "leaky" activity of the late adenovirus genes encoding for capsid proteins (Yang et al. 1994b) that can lead to the production of viral antibodies. Anti-luciferase antibody production could be due to the release of this foreign protein by the necrotic activity in the fibers, especially

since prevalence of necrosis is high at this age (45-60 days) (Karpati et al. 1988 and Torres et al. 1987).

Although we cannot rule out the involvement of an immune response in the loss of transduced fibers, low *lux* expression at 60 days post-injection in immunocompetent mice with MCK promoter/enhancer may also be explained by the progressive loss of transduced fibers due to the continuing necrosis-regeneration process. This loss of transduced or transfected fibers has been observed even in young injected immunocompetent mice (Danko et al. 1993 and Vincent et al. 1993). On the other hand, transgene expression was maintained when AVRSVmDys and AVRSVlacz were injected together (or plasmid containing both luciferase and dystrophin, or Becker-like dystrophin gene) where dystrophin minigene expression protected fibers from subsequent necrosis (Acsadi et al. 1996, Danko et al. 1993 and Wells et al. 1992). It would be expected that transduced fibers undergoing necrosis lose the luciferase protein, without previous transduction of satellite cells.

In light of this study, it will be interesting to test the long-term expression and the protection of muscle fibers from necrosis by using the minidystrophin gene driven by this muscle-specific MCK promoter/enhancer (1.35 kb) in the new adenoviral vectors with enlarged insert capacity. I hope sincerely that this work has contributed to the advancement of the formulation of a therapy for Duchenne muscular dystrophy.

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