Improved adenoviral vectors for gene therapy of Duchenne muscular dystrophy

M.A. Hauser\textsuperscript{a}, A. Amalfitano\textsuperscript{a}, R. Kumar-Singh\textsuperscript{a}, S.D. Hauschka\textsuperscript{b}, J.S. Chamberlain\textsuperscript{a,*}

\textsuperscript{a}The Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618, USA
\textsuperscript{b}The Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

Accepted 17 April 1997

Abstract

We have been exploring the feasibility of gene therapy for Duchenne muscular dystrophy by characterizing parameters important for the design of therapeutic protocols. These studies have used transgenic mice to analyze expression patterns of multiple dystrophin vectors, and have been accompanied by the development of viral vectors for gene transfer to dystrophic \textit{mdx} mouse muscle. Analysis of transgenic \textit{mdx} mice indicates that greater than 50\% of the fibers in a muscle group must express dystrophin to prevent development of a significant dystrophy, and that low-level expression of truncated dystrophins can function very well. These results suggest that gene therapy of DMD will require methods to transduce the majority of fibers in critical muscle groups with vectors that express moderate levels of dystrophin proteins. Strategies for the development of viral vectors able to deliver dystrophin genes to muscle include the use of muscle specific regulatory sequences coupled with deletion of viral gene sequences to limit virus-induced immune rejection of transduced tissues. These strategies should enable production of adenoviral vectors expressing full-length dystrophin proteins in muscle. © 1997 Elsevier Science B.V.

Keywords: \textit{mdx} mice; Adenovirus; Gene therapy; Dystrophin; Encapsidated mini-chromosome; Creatine kinase; Duchenne muscular dystrophy

1. Introduction

Development of gene therapy for Duchenne muscular dystrophy (DMD) and other types of muscular dystrophy will require detailed knowledge of what end-points must be achieved for successful therapy. Identification of these end-points will enable testing delivery systems for the ability to restore normal muscle function following gene transfer. In the case of DMD the obvious goal is to restore sufficient production of the protein dystrophin to restore normal muscle strength. However, the dystrophin gene itself is the largest known, and muscle represents approximately 70\% of the body mass. Is it necessary to transfer dystrophin to every muscle cell in the body for effective gene therapy? How much dystrophin must be produced in different types of muscles? What types of dystrophin mini-genes might suffice to produce functional dystrophin proteins without transfer of enormous gene cassettes? What types of vectors could carry and properly express these gene cassettes in muscle tissue? How can these vectors be engineered to enable long-term gene expression in vivo? Finally, how can these vectors be delivered to the required muscle groups in an efficient and cost effective manner? The answers to few of these questions are currently available, although progress is being made in each of these areas. We describe here studies addressing the levels of dystrophin that must be produced for an effective gene transfer regimen, and we also describe recent progress towards development of an effective gene delivery system for muscle.

Studies to determine the types, amounts, and distributions of dystrophin needed for therapy have been approached by generating transgenic animals that express various types of dystrophin on an otherwise dystrophin deficient background. We have performed these studies using the \textit{mdx} mouse, a mutant with a chain terminator mutation in exon...
23 of the murine dystrophin gene [1]. Five types of mdx mice are available that display differential patterns of expression of the various dystrophin isoforms [2,3]. Each of these mutants share the feature of a failure to express any dystrophin in muscles, and is therefore a good model system with which to study the feasibility of gene therapy for DMD.

In a parallel series of studies we have begun to modify adenovirus vectors to develop a gene delivery system for muscles of patients who have already developed dystrophic systems. These vectors are attractive for gene transfer to muscle as they can be prepared in high titers (large amounts), can efficiently infect muscle tissues, and have a moderately large cloning capacity. However, adenoviruses trigger a potent immune response in vivo, and therefore have been unable to achieve long-term gene expression in muscle or other tissues. Furthermore, while adenoviruses can hold up to 8 kb of exogenous DNA, a larger cloning capacity will be needed to transfer dystrophin cassettes.

We have begun development of modified adenoviral vectors able to express dystrophin genes in muscle. Parameters being studied include use of MCK regulatory sequences for muscle specific expression, and deletion of viral gene sequences in an attempt to limit viral vector induced immune rejection of transduced tissues. Preliminary results indicate that the MCK enhancer can function efficiently from within adenoviral vectors. Attempts to limit viral immunogenicity have focused on two strategies. The first method involves deletion of essential replication genes from viral vectors accompanied by viral growth on complementing packaging cell lines. The second approach involves development of helper dependent adenoviral mini-chromosomes that can transduce muscle but that do not contain any viral genes. Both strategies will enable production of adenoviral vectors expressing truncated or full length dystrophin proteins in muscle. However, further studies will be required to determine whether these vectors can evade host immune recognition.

2. Materials and methods

Mouse mdx mutants were obtained from the Jackson Labs (Bar Harbor, ME). Dystrophin cDNA clones were isolated and sequenced as described [4,5]. Adenovirus isolates were propagated on human 293 cells using standard protocols [6]. Encapsidated mini-chromosomes were grown and purified as described [7].

2.1. Analysis of transgenic mice

Transgenic mice were generated by microinjection into F1 hybrid zygotes from C57BL/6J X SJL/J parents as described [8]. Possible transgenic mice were identified by the polymerase chain reaction (PCR) using primers specific for dystrophin. Expression of dystrophin in mice was monitored by Western blot analysis of 100 μg of total protein extract as described [9]. Truncated dystrophin was extracted from muscle by resuspending powdered frozen muscle in 4 volumes of RIPA homogenate solution containing 5 mM EDTA, 1 mM Pefabloc SC, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 0.7 μg/ml pepstatin [9]. Histological 4 μm sections were prepared from muscle tissue fixed in 2% formaldehyde and 2% glutaraldehyde, embedded in glycol methacrylate, and stained with haematoxylin and eosin. Sections from 3–4-month-old mice were photographed, and the percentage of centrally nucleated myofibers was determined by dividing the number of myofibers containing one or more centrally located nuclei by the total number of nucleated fibers. Myofibers with no nuclei in the plane of section were not counted. An average of 1400 myofibers were counted for each muscle group. For measurement of mechanical properties, bundles of intact fibers were excised from 3–4-month-old mouse diaphragm muscles. Forces were determined during maximum isometric tetanic contraction in vitro at 25°C, then normalized to total cross-sectional area [10,11].

3. Results

3.1. Expression of dystrophin in transgenic mdx mice

To explore the levels of dystrophin needed to prevent dystrophy in mdx mice, we generated transgenic animals expressing various levels of mouse dystrophin. These animals expressed dystrophin under the control of muscle creatine kinase (MCK) enhancer plus promoter elements [12,13]. This promoter is inactive in myoblasts or non-muscle cell types, but becomes transcriptionally activated after terminal differentiation of myoblasts into post-mitotic myocytes [14].

Selected lines of mice that expressed a range of levels of dystrophin were tested for dystrophic symptoms. The primary assay used was the percentage of muscle fibers with centrally-located nuclei, which is a hallmark of fibers that have undergone at least one cycle of degeneration and regeneration. Muscle mechanical properties were also tested in animals with non-dystrophic morphological appearances. Transgenic mdx mice with high levels of dystrophin, up to 50 times wild-type levels, displayed muscle tissue morphologically and functionally indistinguishable from normal muscle [15]. However, animals with low levels of dystrophin displayed an intermediate morphological picture, with only a partial prevention of the dystrophic process. Fig. 1 displays central nuclei counts and force generating capacities from diaphragm muscles of mdx animals with variable levels of dystrophin. Approximately 20% of the normal levels of dystrophin can prevent the appearance of most dystrophic symptoms, as few fibers were observed to contain central nuclei and specific force generating capacity was not significantly different from normal.

Some of our lines of transgenic animals display a mosaic
pattern of expression of the dystrophin transgene, which results from position effect variegation [9,16,17]. Analysis of the dystrophic symptoms in these mosaic animals revealed that if a large fraction of fibers in a muscle group express dystrophin, then few morphological abnormalities developed. In contrast, muscles with few dystrophin expressing fibers develop obvious dystrophic symptoms [9]. These results suggest that a significant alleviation of dystrophic symptoms could result from delivery of low levels of dystrophin to the majority of fibers in targeted muscles.

3.2. Optimization of MCK expression cassettes

Although high levels of dystrophin production in muscle tissue were not observed to cause toxic side effects [15], it is not clear whether problems might occur if dystrophin were to be expressed in non-muscle tissues. Furthermore, gene transfer and dystrophin expression in non-muscle tissues, such as liver or hematopoietic cells, could contribute to development of an immune response against infected cells. We have therefore been developing adeno viral vectors that would express dystrophin in a muscle specific manner. For these studies we have chosen to use gene regulatory cassettes derived from the muscle creatine kinase (MCK) gene. MCK has numerous advantages for use in an expression cassette. Detailed studies have been performed to determine which parts of the gene are needed for high level activity in muscle [13,18,19]. In addition, only a single muscle isoform of MCK exists, whereas many other highly expressed muscle genes, such as α-actin, are encoded by a separate genes active only in specific muscle types. MCK is expressed in all striated muscles, which are the principal targets for DMD gene therapy. MCK is also the most abundant soluble protein expressed in muscle tissue [20]. Highly active regulatory cassettes have been prepared that are only a few hundred bases in size [13,19,21], and mutagenesis of these cassettes has been shown to increase their activity to levels greater than that of the wild-type gene (Hauschka et al., unpublished).

To develop an optimal MCK cassette for use in adeno viral vectors, we have introduced a variety of gene fragments into an adeno viral plasmid shuttle vector. This shuttle vector contains portions of the adenovirus early region 1 genes, from 0–1 and 9–16 map units, and can be used to transfer genes into adenoviruses by homologous recombination in human 293 cells [6]. Shuttle vectors with the MCK regulatory elements linked to a β-galactosidase reporter gene were tested by transfection into myogenic cells and by direct injection into mouse rectus femoralis muscle. Each of the tested vectors produced moderate to high levels of β-galactosidase in both systems (Fig. 2). One construct, which contains only 358 bps of promoter sequences linked to a mutagenized version of the MCK enhancer element was found to generate the highest levels of reporter gene expression. This latter construct was approximately 6-fold less active than the human cytomega-

![Fig. 1. Dystrophic symptoms in transgenic mdx mice expressing various levels of dystrophin. Numbers at the bottom refer to the amount of dystrophin in transgenic mdx mouse muscle relative to wild-type mice (1X). A) Percentage of muscle fibers containing centrally-located nuclei. Mice were assayed at 3 months of age. Diaphragm muscle from transgenic mdx mice expressing as little as 20% of normal levels of dystrophin display only slightly more fibers with centrally-located nuclei than do wild-type C57Bl/10 mice. B) Maximum specific force generated by diaphragm muscles of 3–4-month-old control and transgenic mdx mice. The diaphragm muscles of mdx mice display a dramatic reduction in measured force generation. Diaphragm muscle from transgenic mdx mice expressing as little as 20% of normal levels of dystrophin can generate a force that is not statistically different from that of normal C57BL/10 mice (t-test with unequal variance, P>0.05) (see also [9]).](image-url)

lovirus enhancer plus promoter (CMV), which is the strongest known promoter. This MCK element appears to provide muscle specific activity, even though the shuttle vector contains the adenovirus E1 region enhancer. These results suggest that the mutant –358 MCK construct could serve as a strong and specific regulatory element to control dystrophin expression in vivo from within adenovirus, a hypothesis that is currently being tested.

3.3. Generation of replication defective adenoviral vectors

Another approach to limiting immunogenicity of adenoviral (Ad) vectors is to develop vectors that express minimal amounts of viral proteins. Currently available adenoviral vectors have been shown to trigger a potent cytotoxic T-cell mediated immune response that is primarily directed against viral late gene encoded proteins [22,23]. These late genes are under transcriptional control of the adenovirus major late promoter, which has been shown to be minimally active prior to the onset of viral replication [24]. Therefore, if viral replication can be eliminated, the major late promoter should not become activated to an appreciable degree.

Our approach to developing new vectors has been to eliminate genes that are critical for adenovirus replication. Three major genes are required for viral replication, and each are encoded in the E2 region of the viral genome. These genes are the single stranded DNA binding protein (DBP, coded by the E2a gene), the DNA polymerase (POL) gene, and the preterminal protein (pTP) gene, the latter two being products of the E2b gene. Removal of these genes from an Ad vector would require development of a complementing cell line that could provide these proteins in trans to enable growth and preparation of the deleted viruses in vitro. Therefore, our early efforts have focused on the
development of Ad packaging cell lines that express the E2b genes (the DBP gene is toxic when constitutively expressed).

By transformation of human 293 cells with two expression vectors encoding the Ad-POL gene and the Ad-pTP gene, we were able to identify a cell line that expressed high levels of RNA for both Ad-POL and pTP (Fig. 3). These cell lines are able to support growth of Ad vectors with temperature sensitive mutations in either the POL gene or the pTP gene, indicating that functional levels of these proteins are being co-expressed in the 293 cells [25,26]. These cell lines therefore should be able to support growth of vectors with deletions in either of the genes, or in both (Fig. 4). Construction of such vectors is currently being carried out with the packaging cell lines to enable testing of the immunogenic properties in vivo. In addition to displaying reduced immunogenicity, these vectors will have an increased cloning capacity, facilitating insertion of large genes such as dystrophin.

### 3.4. Isolation of encapsidated adenovirus minichromosomes

Another approach to developing vectors that can not express viral proteins is to eliminate all the viral genes from the vector. Adenovirus contains more than forty genes, many of which are toxic when constitutively expressed. Therefore, it is unlikely that a packaging cell line could be developed that supplies sufficiently high levels

---

**Fig. 2.** MCK expression vectors. Five versions of MCK promoter elements were cloned into plasmid shuttle vectors upstream of a β-galactosidase reporter gene to compare their relative strength. Also tested was a vector containing the CMV promoter. The scale along the top most construct gives the relative size in bps of each construct, with the numbering relative to the MCK gene transcription start site (+1)[12]. The MCK basal promoter is located between bases +1 and –80[13]. Minx refers to a synthetic intron derived from adenovirus. The lower most MCK construct provides the greatest amount of activity in myogenic cells both in vitro and in vivo, and is approximately 6-fold less active than the CMV promoter.

<table>
<thead>
<tr>
<th>Tissue Culture (β-gal)</th>
<th>Direct IM injection (β-gal)</th>
<th>Adenovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>3300</td>
<td>-1256</td>
<td>-1050</td>
</tr>
<tr>
<td>E</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>++</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>E'</td>
<td>lacZ</td>
<td>+</td>
</tr>
<tr>
<td>CMV</td>
<td>lacZ</td>
<td>+++</td>
</tr>
<tr>
<td>CMV</td>
<td>lacZ</td>
<td>+++</td>
</tr>
<tr>
<td>CMV</td>
<td>lacZ</td>
<td>+++</td>
</tr>
<tr>
<td>CMV</td>
<td>lacZ</td>
<td>+++</td>
</tr>
<tr>
<td>CMV</td>
<td>lacZ</td>
<td>+++</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** Northern blot analysis of total RNA isolated from human 293 cell lines before and after transfection with various expression vectors. Left: RNA from the cell lines indicated at the top was separated on agarose/formaldehyde gels, transferred to nitrocellulose membranes, and hybridized with a fragment from the adenovirus DNA polymerase (Ad-POL) gene. No Ad-POL mRNA was detected in parental 293 cells, but transcripts at 5 and 7 kb were observed at various levels in three transfected cell lines designated B6, B9, and C7. These two sized mRNA arise from the use of alternate poly A addition signals within the expression vector transfected into the cell lines [25]. The B9 also displayed an aberrantly large Ad-POL of unknown origin. Right: hybridization of similar northern blots with a probe from the adenovirus pre-terminal protein (pTP) gene. The C7 cell line also expresses the pTP mRNA, which is not found in 293 cells or B6 cells. These results indicate it is possible to develop Ad packaging cell lines that express not only the Ad E1a and E1b genes (as do the parental 293 cells), but also the Ad-POL and Ad-pTP genes [26]. These cell lines enable growth of viral isolates lacking Ad-POL and Ad-pTP activity.
of all Ad proteins to propagate a vector with no viral genes. However, such a vector could be produced in large quantities if it was grown in the presence of a second virus that supplied the missing viral genes. This strategy could yield a purified vector lacking all viral genes if it methods were available to separate the helper virus from the gene-depleted virus.

We have prepared such a virus by incorporating two regions of the adenoviral genome into a plasmid vector. These two regions are the sequences needed for viral replication and for packaging viral DNA into an infectious particle (Fig. 5). The required Ad sequences are the inverted terminal repeats (ITRs), which are located at either end of the Ad genome, and the packaging signals (ψ), which are located immediately adjacent to the left ITR. The Ad E2 region proteins recognize the viral ITRs and replicate any double stranded molecule containing these sequences. The packaging signals interact with additional viral proteins and lead to packaging of the replicated DNA into capsids that can infect most human tissues. We termed these infectious plasmids ‘encapsidated adenovirus mini-chromosomes’, or EAM, since they are infectious encapsidated particles that can replicate in the presence of helper virus. To generate a version of this EAM that could be easily tested, we added two types of non-viral genes. The first was a β-galactosidase reporter gene driven by the CMV promoter. This reporter gene allows titration of the vectors by simply measuring β-galactosidase activity. The second gene we added was the 14 kb dystrophin cDNA driven by the full-length 3300 bp MCK regulatory element. This latter gene allowed us to ask whether an EAM could synthesize full-length dystrophin in muscle tissues.

Transfection of the dystrophin + β-galactosidase plasmid (named pAd5βdys) into human 293 cells together with DNA from a helper virus resulted in the appearance of a cytopathic effect (CPE) on the transfected plates within 10–14 days [7]. For these studies the helper virus was a conventional E1-deleted adenovirus that contained an alkaline phosphatase (AP) reporter gene cloned into the E1 region.

Fig. 4. Schematic illustration of the E2 region of adenovirus. The major late promoter is transcribed from the top DNA strand, while the E2a and E2b genes are transcribed from the bottom DNA strand. By preparing deletions in the E2b region, it is possible to prepare viral isolates lacking either the Ad-POL gene, the Ad-PTP gene, or both. Growth of such isolates requires a complementing cell line such as C7 (Fig. 3).

Fig. 5. Structure of pAd5βdys. This plasmid is derived from pBluescript 11 KS + (Stratagene), and contains the following elements: a β-galactosidase reporter gene under transcriptional control of the human cytomegalovirus immediate early promoter (LaCZ); a 14 kb mouse dystrophin cDNA under transcriptional control of the muscle creatine kinase promoter (dystrophin); and sequences from adenovirus type 5 needed for replication (right and left inverted terminal repeats, RITR and LITR) and encapsidation into infectious particles (ψ). Co-transfection of this plasmid into 293 cells with a helper adenoviral genomic DNA clone leads to encapsidation of the plasmid and generation of infectious particles expressing both β-galactosidase and dystrophin [7].

The appearance of CPE in the transfected cells indicated that infectious particles had been generated and were spreading throughout the plate. These viruses could be either the helper virus alone, the EAM particles, or both. To determine the relative yield of helper versus EAM we infected HeLa cells and assayed for β-galactosidase activity (made by the EAM) or for AP (made by the helper). Both reporter genes were expressed, indicating the production of an EAM in the presence of helper virus [7]. Subsequent serial growth of the viruses followed by CsCl gradient separation of EAM from helper virus resulted in a viral preparation that was >95% pure EAM [7]. Infection of this preparation onto dystrophin minus mdx myogenic cultures resulted in high levels of dystrophin production only in infected cells (Fig. 6). These data indicate that an infectious EAM can be prepared that lacks all viral genes, and which can transduce full-length dystrophin into dystrophic muscle cells.

4. Discussion

Delivery of new genes to dystrophic muscle is a promising technique to treat muscular dystrophy. However, the methods needed to achieve this goal are far from being perfected. Adenovirus vectors are a promising approach for delivery of genes, but methods must be developed to prevent the potent immune response that has so far prevented successful clinical use of these vectors. By combining the approach of using muscle specific regulatory elements with viral vectors depleted of all viral genes, it
may be possible to circumvent the immune response to virally infected cells. Nonetheless, even with these changes significant problems remain.

It remains unclear how any viral vector can be delivered efficiently to all muscles of the human body. Vascular delivery methods could be used to deliver high viral doses to defined muscle groups, and initial therapy trials might be limited to treating only the most critical muscles, such as the diaphragm. However, if methods could be developed that allow viruses to bind only to muscle cells, the efficiency of delivery to muscle would be improved dramatically. In addition, methods must be developed that will allow enormously greater quantities of viral vectors to be grown for use in the clinic. While each of these obstacles remains
significant, progress has been occurring rapidly, and gene therapy for DMD remains one of the best hopes for a treatment.

Acknowledgements

We thank Ann Saulino, Stephanie Phelps, and Catherine Begy for excellent technical assistance. Supported by a grant from the Muscular Dystrophy Association (USA).

References


