

# Gene therapy of muscular dystrophy

Jeffrey S. Chamberlain\*

Department of Neurology, K243b HSB Box 357720, University of Washington School of Medicine, Seattle, WA, 98195-7720, USA

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**Development of gene therapy for the muscular dystrophies represents a daunting challenge requiring significant advances in our knowledge of the defective genes, muscle promoters, viral vectors, immune system surveillance and methods for systemic delivery of vectors. However, tremendous progress has been made in developing improved viral vectors and avoiding immune reactions against gene transfer. This review summarizes recent progress and highlights problems that must be solved before an effective treatment is available.**

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The muscular dystrophies (MDs) are a heterogeneous group of disorders caused by mutations in any one of a large number of genes (1). Since almost all types of muscular dystrophy arise from single-gene mutations, genetic therapy, involving replacement or modification of a gene, has emerged as a promising approach for treatment. Gene therapy requires delivery of a new gene to the vast majority of muscles in the body—a daunting challenge, since muscle tissues makes up >40% of body mass. Most current research is focused on identifying the correct version of a gene to deliver, and on developing methods for safe and efficient delivery to muscle. Neither task is simple: many of these genes are enormous and display complex expression patterns, and successful delivery must overcome considerable physical and immunological barriers. This review summarizes current approaches to gene therapy of Duchenne muscular dystrophy (DMD), the most common form of MD, which serves as a model for development of therapies for other types of dystrophy.

## THE DYSTROPHIN COMPLEX AND MUSCULAR DYSTROPHY

DMD and the allelic Becker MD arise from defects in the dystrophin gene (2). Dystrophin is the central component of a large complex of proteins important for membrane stability and force transduction from muscle fibers (3–6) (Fig. 1). Defects in several members of the dystrophin–glycoprotein complex (DGC) have been shown to lead to different forms of limb-girdle muscular dystrophy (LGMD) and congenital muscular dystrophy (7,8). Muscular dystrophies arising from DGC defects are recessively inherited, suggesting that gene replacement could be an effective treatment that would not require correction of the mutant allele.

Most studies exploring DMD gene therapy have been conducted animal using models, such as the *mdx* mouse or the *cxmd* dog (9–11). Transgenic animal studies in the *mdx*

mouse revealed that striated muscle-specific expression of either a full-length or a number of truncated dystrophin cDNAs completely prevented the development of dystrophy (12–14). Muscles from these transgenic animals display complete restoration of the DGC, which is destabilized and largely absent from dystrophin-deficient muscles (15). Interestingly, restoration of the DGC by itself has little effect on the dystrophic pathology unless a mechanical link to the cytoskeleton is also formed by dystrophin (16–19). The ability to rescue the dystrophic pathology by gene replacement in muscle has spurred efforts aimed at developing optimized dystrophin expression cassettes and methods for their delivery to muscle.

## CHALLENGES TO GENE THERAPY FOR DMD

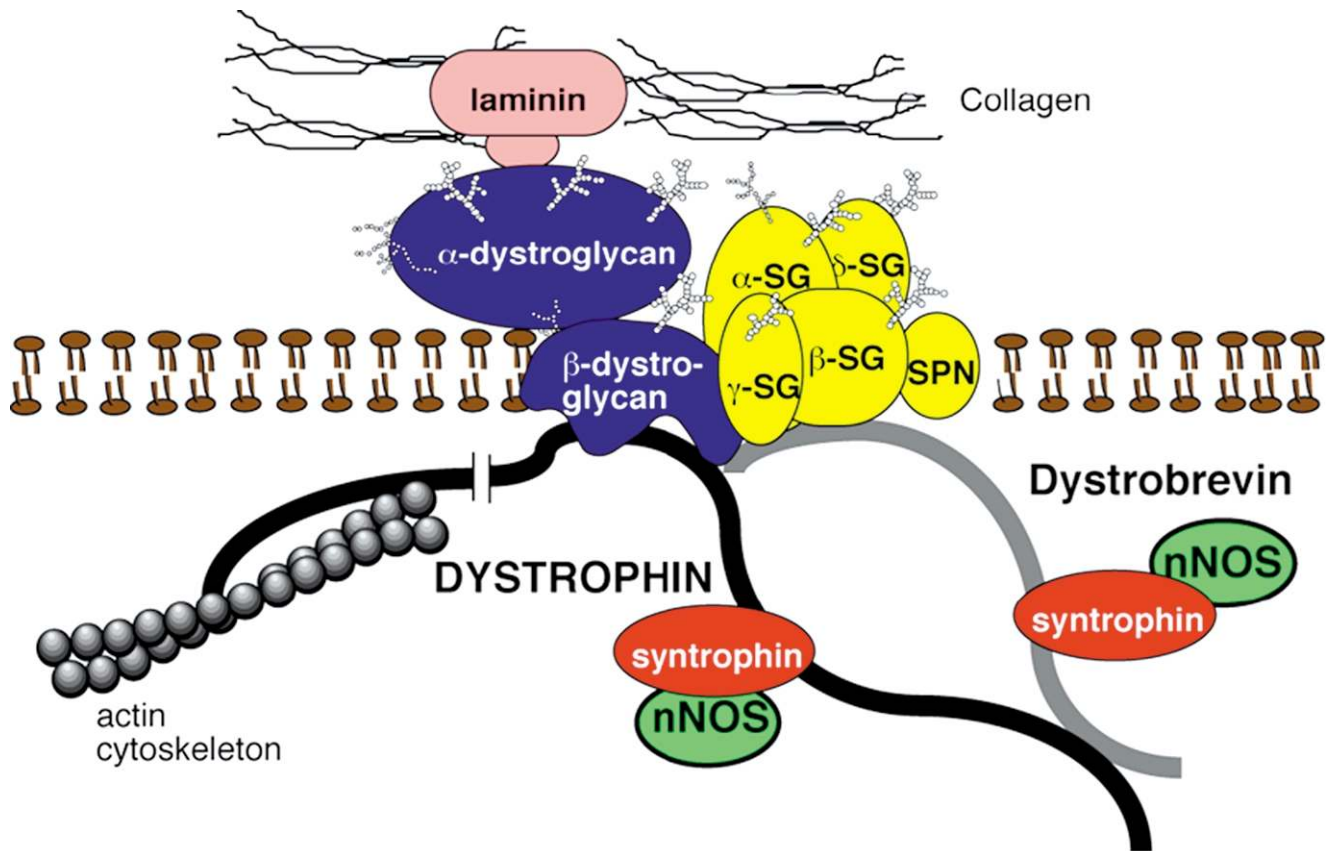
Gene therapy for DMD will require efficient delivery of a dystrophin expression vector to most of the striated muscles of the body. The dystrophin gene is 2.4 Mb in size, necessitating the generation of mini-gene cassettes that can express therapeutic levels of a functional protein. A delivery vector must be identified that can carry these expression cassettes and transduce striated muscle. Finally, muscle transduction must not trigger toxic or immunological reactions that are harmful to the patient or that lead to further muscle damage. Each of these areas is the subject of intensive research, and the remainder of this article will summarize recent progress.

## DYSTROPHIN EXPRESSION CASSETTES

The muscle isoform of dystrophin is encoded on a 14 kb mRNA, and transgenic animal studies have shown that this cDNA can fully prevent dystrophy in *mdx* muscles (12). Generating an expression cassette from a cDNA requires the inclusion of gene regulatory regions active in muscle, such as from the muscle creatine kinase (MCK) gene, a polyadenylation signal and an intron. While considerably smaller than the

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\*To whom correspondence should be addressed. Tel: +1 2066166645; Fax: +1 2066168272; Email: jsc5@u.washington.edu



**Figure 1.** Dystrophin and the dystrophin–glycoprotein complex in muscle. Dystrophin is a cytoskeletal protein that links the  $\gamma$ -actin filaments to the extracellular matrix via the dystroglycan/sarcoglycan complexes. The C-terminal domain of dystrophin binds syntrophin and dystrobrevin, the latter of which also binds the sarcoglycan complex. Loss of dystrophin results in destabilization and loss of the DGC.

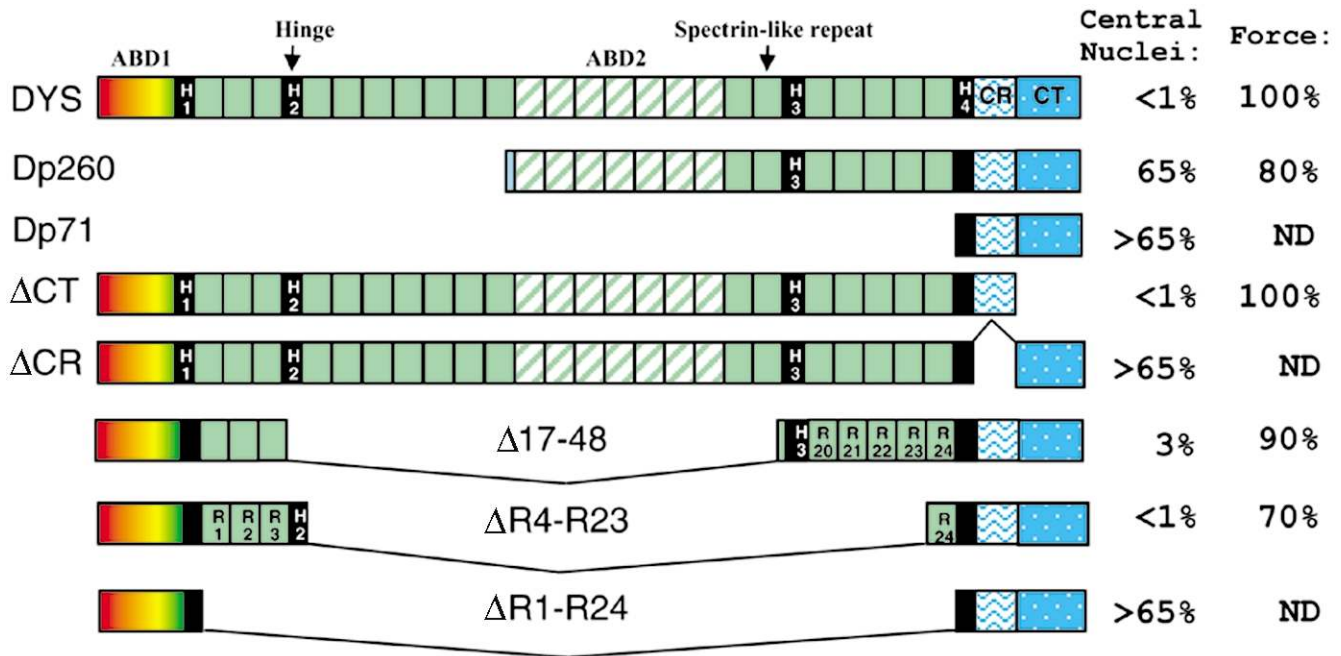
natural gene, these full-length dystrophin expression cassettes cannot fit into most viral vectors. Consequently, numerous studies have focused on identifying truncated, yet functional, versions of dystrophin. The idea that truncated dystrophins could be functional came from observations that some mildly affected BMD patients have deletion mutations that remove large portions of the gene (20,21).

Two large regions of dystrophin can be truncated with minimal impact on function: the central rod (Rod) domain and the C-terminal (CT) domain (Fig. 2). The CT domain is composed of 277 amino acids that are minimally required for dystrophin function (22–24). The Rod domain makes up nearly 80% of the protein and contains 24 spectrin-like repeats. Micro-dystrophin clones with less than three repeats display little function (25,26), whereas proteins with four or more repeats display surprisingly high activity (26–28). One of the smallest of these constructs ( $\Delta$ R4-R23), which contains the first three and the last of the 24 spectrin-like repeats, is able to reverse many of the morphological abnormalities of dystrophic muscle when delivered to young adult mice (28). This remarkable construct can be carried on a 3.6 kb cDNA. Of course, these micro-dystrophins have only been tested in the *mdx* mouse, whose limb muscles develop weakness and lose mass at a much slower relative rate than do the corresponding human muscles. Nonetheless, some micro-dystrophins are

remarkably functional in the mouse diaphragm, which is a very good model for the human disease (28).

## VECTORS FOR MUSCLE GENE THERAPY

Efforts to deliver dystrophin to muscle have focused on four vectors: adenoviruses (Ad), retroviruses, adeno-associated viruses (AAV) and plasmids (29). Ad vectors have a relatively large cloning capacity, can be grown to the highest titers and display relatively efficient infection of muscle (30). Conventional Ad vectors have been used to deliver dystrophin mini-genes to *mdx* and *cxmd* muscles (31–37). Unfortunately, these vectors can elicit a robust cellular immune response against viral and some transgene proteins, so their use has been limited to studies in immune-compromised animals. Such studies have shown that muscles can be transduced with high levels of mini-dystrophin, at least near the site of injection, and that transduction can prevent the development of pathology in *mdx* mouse limbs. To overcome immunological problems, ‘guttled’ Ad vectors have been developed that use a minimal vector backbone lacking all viral genes (38–40). Deletion of the viral genes also creates a larger cloning capacity, allowing full-length dystrophin cassettes to be carried (41). Full-length dystrophin is efficiently expressed



**Figure 2.** Domain structure of full-length and mini-dystrophins. The figure shows the N-terminal and central actin-binding domains (ABD), the 24 spectrin-like repeats that are interrupted by 4 'hinge' regions (hinge 4 contains a 'WW' domain), the cysteine-rich domain that binds  $\beta$ -dystroglycan, and the C-terminal domain that binds syntrophins and the dystrobrevins. Also shown are the Dp260 isoform (expressed in retina), the Dp71 isoform (expressed in many cell types), and various mini- and micro-dystrophins that have been tested in animal models for DMD. The size of each protein is shown, together with a summary of the effectiveness each displays when expressed in transgenic *mdx* mice.

from gutted vectors in muscle, and they elicit few of the immunological problems seen with conventional Ad (Fig. 3) (42–44). However, Ad vectors do not integrate into the host genome, so their ability to persist for long periods of time is unclear. Muscle gene therapy using Ad vectors will therefore require either repeat delivery of vector to patients or development of methods that enable vector integration into the host genome. Two studies have recently described hybrid vectors between Ad and retroviruses or AAV, approaches that could enable integration of a dystrophin cassette (45,46).

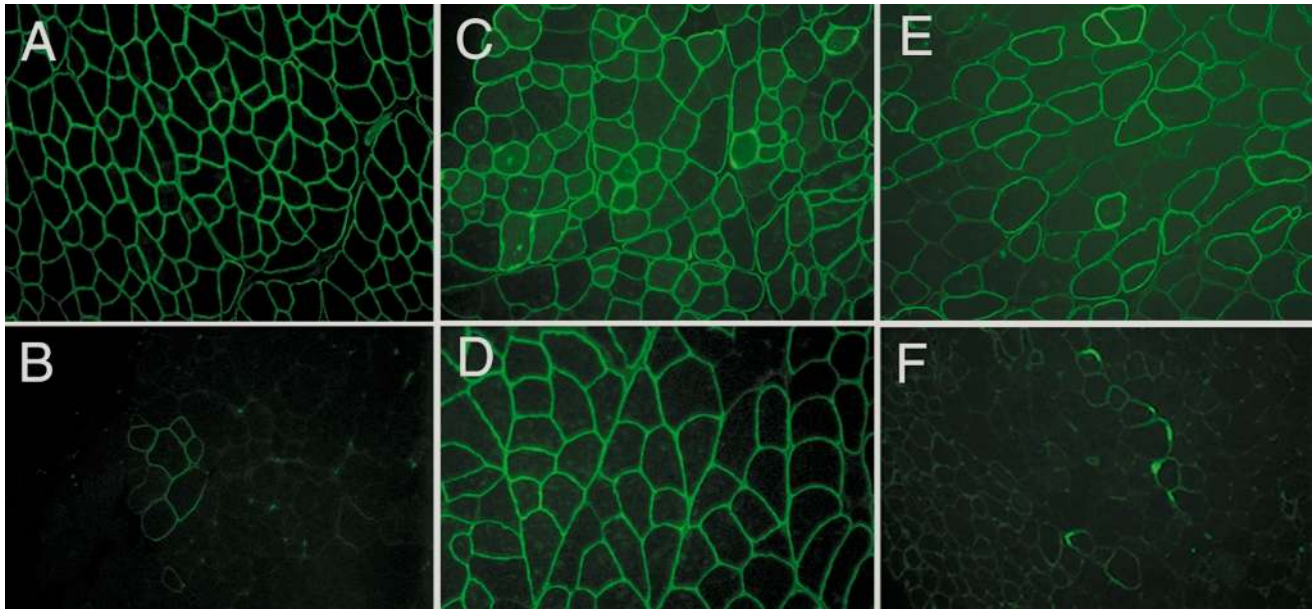
Different classes of retroviruses have cloning capacities between 7 and 11 kb, so these vectors are limited to delivering only the mini- and micro-dystrophins (47). Retroviruses are difficult to grow in large quantities, preventing robust transduction of muscle by direct injection of vector. Nonetheless, all types of retroviruses efficiently integrate into the host genome, potentially allowing persistent gene transfer. Lentiviral vectors display robust infection of a variety of stem cells, and may be of interest for stem cell-based gene delivery (48).

AAV is of great interest for muscle gene therapy as it efficiently infects skeletal muscle and can persist for years, at least in healthy mice (49,50). Persistence is likely a result of nuclear retention signals in the vector genome, since recombinant AAV does not appear to integrate to an appreciable extent (51). Gene delivery with AAV might therefore also require periodic 'boosting' to maintain high-level expression. Nonetheless, these vectors can be grown to titers approaching those of Ad vectors, and serotypes 1, 5 and 6 are particularly efficient at transducing skeletal muscle (52–54).

Naked plasmid DNA displays a remarkable ability to transfer genes to muscle (55,56). Plasmids display minimal immunogenicity and toxicity, and have an extremely large cloning capacity. The primary disadvantage of plasmids is their relatively poor transduction efficiency under typical delivery protocols. However, several laboratories have been working to improve this efficiency by using high-pressure injection and/or electroporation (57–59). Retention of plasmids will be an important consideration, and, as with Ad and AAV, it may be necessary to either repeat administration or modify the plasmids to enable integration. A human clinical trial assessing the safety of plasmid-mediated dystrophin delivery is currently underway in France, although no results have been released (60).

## IMMUNOLOGICAL HURDLES TO GENE THERAPY OF DMD

Innate and acquired immune defense mechanisms can block or limit the extent of gene transfer, lead to destruction of 'rescued' cells, and in severe cases cause systemic reactions that can result in death (61–66). Serious problems from innate immune responses are generally observed only with high doses of virus administered intravascularly, and may not be of great concern for many vectors and delivery routes (67,68). Nonetheless, this concern is one reason that clinical gene therapy trials are conducted only after extensive animal testing and are initiated using very low vector doses. Acquired immunity involves humoral and cellular responses that are



**Figure 3.** Transduction of adult, immunocompetent *mdx* mouse muscles by dystrophin and utrophin expression vectors. The figure shows cross-sections of C57Bl/10 (A) or *mdx* mouse TA muscles that had been (B and F) sham-injected (no vector), or (C, D and E) injected with viruses expressing different dystrophin or utrophin cDNAs. At intervals following injection, the mice were sacrificed and muscle sections were stained with antibodies against dystrophin (A–D) or utrophin (E and F). Gutted Ad vectors express full-length mouse dystrophin (C) or mouse utrophin (E) for at least 3 months after injection; (D) AAV vectors express micro-dystrophin for at least 5 months after injection.

activated through B- and T-cell-mediated pathways. A humoral immune response typically generates circulating antibodies against vector or transgene-encoded proteins. These antibodies can block further vector delivery to muscle, but in general are not a great concern for long-term expression of an intramuscular protein. In contrast, cellular immune responses can lead to cytotoxic T-lymphocyte (CTL)-mediated destruction of otherwise ‘rescued’ tissues, and it is critical to avoid them in a clinical setting.

The propensity of Ad vectors to elicit a cellular immune response has greatly tempered enthusiasm for their use. Fortunately, several methods have been developed that largely overcome these problems. The first is the use of the gutted Ad system, which greatly limits the CTL response against Ad proteins and enables relatively long-term vector persistence in both liver and muscle (43,44,54,69–71). The second is the use of muscle-specific promoters, which block the CTL response against many transgenes (72–74). Gutted Ad vectors that express full-length dystrophin from the MCK promoter do not display a loss of gene expression for at least 3 months following intramuscular injection into adult mice (Fig. 3) (54). It remains to be shown whether gutted Ad vectors can avoid triggering an innate immune response.

A number of examples have also been found where a cellular immune response can be generated against a protein delivered by AAV. The intracellular localization of the protein is critical for this effect: cytoplasmic proteins display the fewest problems, while transmembrane proteins are the most likely to be immunogenic (75). The news is even worse for dystrophic muscle. While AAV-mediated delivery of many proteins is without obvious immunological consequences, delivery of the same vectors to dystrophic muscles can sometimes lead to

a cellular immune response against the protein product. For example, delivery of either a sarcoglycan or a  $\beta$ -galactosidase gene to a mouse model for LGMD led to rapid destruction of transduced fibers unless a muscle-specific promoter (MCK) was used (74,76,77). Similarly, muscle promoters largely block the immune response against human dystrophin when delivered to *mdx* mouse muscles with AAV (27,28). However, in contrast to results with Ad vectors, muscle promoters do not appear to block fully an immune response against  $\beta$ -galactosidase when delivered to dystrophic *mdx* muscles using AAV (78). Dystrophic muscles have two features that exacerbate the induction of an immune response: ongoing myofiber necrosis that deposits cellular contents into the environment, coupled with greatly elevated levels of immune effector cells (73,79,80). Muscle-specific promoters are highly effective in Ad vectors because they shut down gene expression in macrophages and dendritic cells, which are easily infected by Ad (72,81). In contrast, AAV does not have a high tropism for mature dendritic cells, and the immune response to these vectors in dystrophic muscle primarily results from antigen cross-presentation from necrotic fibers to macrophages and dendritic cells (82,83). While this effect is not fully blocked by muscle promoters, it can be halted or ameliorated by rescue of the dystrophic phenotype (31,76,78).

Other vector systems, such as retroviruses and plasmids, do not typically elicit a robust cellular immune response. Nonetheless, a mild CTL response has been observed against human dystrophin when delivered to *mdx* muscles using intramuscular injection of plasmids—an effect that was not blocked by use of a muscle promoter (84,85). The cellular immune response against vector-encoded proteins is often transient, and might be blocked by immune suppression at the

time of gene delivery (37,86). For example, recombinant AAV and gutted Ad vectors do not carry viral genes. Consequently, viral protein synthesis does not occur *de novo*, and the host immune system is only transiently exposed to the viral proteins that coat the delivered viral particles. Developing a better understanding of the mechanisms that induce immune reactions in dystrophic tissue and of ways to control them is obviously important to the success of gene therapy for MD.

## SYSTEMIC DELIVERY OF GENES TO MUSCLE TISSUE

Recent progress suggests that safe vectors will soon be available for dystrophin delivery. However, there is not yet a method to deliver these vectors efficiently to all the muscles of the body. Crude delivery methods, such as performing hundreds of intramuscular injections, could be used on selected limb muscles to improve mobility and quality of life for patients. However, such methods are not easily applied to the heart or diaphragm, which are critical for long-term survival.

The simplest method for systemic delivery would be to use intravascular routes, since capillaries intimately surround all muscle fibers. However, vectors do not easily pass through vessel walls. One solution is to disrupt the integrity of the capillary wall to allow vectors to pass through and contact the muscle fiber surface. Vasodilators, such as histamine and papaverine, can achieve this effect, and have enabled impressive gene delivery efficiencies in rodent limbs (87). These drugs are dangerous when used systemically, and this approach must be refined considerably to allow safe use in the clinic. A related method is to inject vectors under high pressure (88). A third approach is to modify the vector capsid proteins to alter their natural tropism so as to bind muscle fibers selectively rather than other organs such as the liver (89–91).

A intriguing method for gene transfer is to use stem cells. Early interest in this concept led to clinical trials of myoblast transplantation, but the cells being used were not well characterized and the studies were not successful (92,93). More recently, stem cells isolated from bone marrow and muscle have been reported to display a low efficiency of muscle formation when delivered by bone marrow transplantation (94–96). While it is not clear if these observations reflect transdifferentiation or non-specific incorporation of cells into myofibers, the results warrant further development. In this scenario, hematopoietic or muscle-derived stem cells could be harvested from a patient, transduced *ex vivo* with an integrating, dystrophin-expressing virus (such as a lentivirus), and then used for bone marrow transplantation back into the patient.

## GENE REPAIR

Several groups are exploring direct methods to repair or modify a mutant dystrophin gene. The most successful approach to date is with chimeroplasts, single-stranded RNA/DNA oligonucleotides that can base-pair with a small target sequence and trigger repair of a mutation. When chimeroplasts were delivered to cultures of the *mdx* mouse myoblasts, up to 5% of the mutant dystrophin genes were repaired (97). Other

approaches for gene repair rely instead on viral vectors for gene targeting (98), or the use of short segments of homology in DNA oligonucleotides (99). Antisense oligonucleotides have also been used to influence exon/intron splicing in the dystrophin gene to either skip a mutant exon or restore an open reading frame (100,101). While these methods are not yet efficient, they offer the potential advantages of simplicity, safety, oral delivery and cost-effectiveness.

## OTHER GENES FOR DMD THERAPY

An alternate approach for therapy of DMD involves delivery or upregulation of utrophin, a dystrophin homologue that can compensate for dystrophin deficiency (102–105). Since utrophin is expressed normally in DMD patients, it would not be expected to elicit an immune response (54,106,107). Similarly, methods that could upregulate its synthesis or lead to uniform expression along the sarcolemma might effect a therapy without gene transfer (108,109). Several groups are conducting small-molecule screens to find an inducer of utrophin synthesis. Upregulation could presumably occur at either a transcriptional or a post-transcriptional level. In this regard, a recent report showed that modification of the glycosylation pattern of  $\alpha$ -dystroglycan can lead to a large increase in utrophin accumulation along the sarcolemma in *mdx* muscles (110). These data imply that the rate-limiting step for utrophin upregulation may not be gene transcription, but creating high-affinity docking sites on the sarcolemma. In this regard, previous studies have shown that utrophin does not effectively compete with dystrophin for sarcolemmal binding (111).

## CONCLUSIONS

Over the past 10 years, the concept of gene therapy for muscular dystrophy has gone from a distant dream to an idea moving rapidly towards clinical trials of safety. During this time, it has become possible to shrink the dystrophin gene from 2.4 Mb to 3.5 kb without a significant loss of functionality. Numerous vectors are now available that can hold these expression cassettes and transduce muscle tissue with minimal immunological or toxic side-effects. A major challenge to an effective treatment remains the need for an efficient, systemic delivery system. Coupled with intriguing advances in alternate areas of study, the possibility of a treatment for DMD and other forms of MD is no longer such a distant challenge.

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