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# Gene Transfer Therapy for Heritable Disease: Cell and Expression Targeting

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# Gene transfer therapy for heritable disease: cell and expression targeting

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## SUMMARY

Gene therapy is defined as the delivery of a functional gene for expression in somatic tissues with the intent to cure a disease. Different gene transfer strategies may be required to target different tissues. Adenosine deaminase (ADA) deficiency is a good gene therapy model for targeting a rare population of pluripotent hematopoietic stem cells capable of self-renewal. We present evidence for the highly efficient gene transfer and sustained expression of human ADA in human primitive hematopoietic progenitors using retroviral supernatant with a supportive stromal layer. A stem cell-enriched ($CD34^+$) fraction was also successfully transduced.

Duchenne muscular dystrophy (DMD) is also a good model for somatic gene therapy. Two of the challenges presented by this model are the large size of the gene and the large number of target cells. Germline gene transfer and correction of the phenotype has been demonstrated in transgenic *mdx* mice using both a full-length and a truncated form of the dystrophin cDNA. We present here a deletion mutagenesis strategy to truncate the dystrophin cDNA such that it can be accommodated by retroviral and adenoviral vectors useful for somatic gene therapy.

## 1. INTRODUCTION

To date, clinical therapy for most genetic diseases has been limited to supportive care and, when an enzyme defect has been identified, protein replacement. Recent developments in the understanding of the molecular basis of numerous genetic diseases has facilitated novel experimental approaches to therapy. We present advances directed toward clinically effective gene transfer therapy for two single-gene disorders, adenosine deaminase (ADA) deficiency and Duchenne muscular dystrophy (DMD). Each provides quite different challenges.

Hematopoietic stem cells can be purified, grown *in vitro*, and will repopulate and reconstitute the human hematopoietic system when administered intravenously. ADA deficiency, which results in a severe combined immune deficiency, therefore serves as a model for gene therapy with a high likelihood of success. In contrast, DMD results from a structural gene defect in muscle cells which comprise 75% of the human body. Myoblasts (muscle precursor cells) lack migration and targeting properties, and gene therapy of DMD is therefore likely to be achieved by organ gene delivery *in vivo*, rather than cellular delivery or gene delivery *in vitro*.

## 2. BACKGROUND

### (*a*) *Adenosine deaminase deficiency*

ADA deficiency, an autosomal recessive disorder, accounts for 25% of severe combined immunodeficiency (SCID). This metabolic enzyme catalyses the irreversible deamination of adenosine and deoxyadenosine (dAdo) to inosine and deoxyinosine, respectively. In the absence of ADA, dAdo accumulates in many tissues and the accumulation of dAdo and its metabolites, particularly deoxyadenosine triphosphate, inhibits DNA synthesis, resulting in profound T-cell and subsequent B-cell dysfunction. Approximately 100 families with ADA-deficient SCID have been identified. Untreated, ADA-deficient children usually die before age 2 from overwhelming infection (Kredich *et al.* 1989).

Complementary DNA (cDNA) sequences of normal human ADA have been cloned and characterized (Daddona *et al.* 1984) allowing analysis of seven mutant ADA alleles from five ADA-deficient individuals (Kredich *et al.* 1989). Five of these alleles have a single base mutation resulting in amino acid substitutions that presumably decrease ADA activity. The absence of enzyme activity in a hemolysate of the

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patientsa erythrocytes is diagnostic for this disease. Assay of ADA in cultured amniotic cells, fetal blood, and chorionic villi allows prenatal diagnosis and carrier detection (Kredich *et al.* 1989).

### (*b*) *Duchenne muscular dystrophy*

DMD, an X-linked recessive disorder affecting skeletal and cardiac muscle and brain, is the most common form of muscular dystrophy in children affecting 1 in 3500 male births. DMD and its milder allelic form, Becker muscular dystrophy (BMD), are caused by mutations in the dystrophin gene, which codes for a cytoskeletal structural protein. DMD is a degenerative disorder that begins with skeletal muscle dysfunction early in life, progressing to loss of ambulation, respiratory compromise, cardiac dysfunction and death by the second or third decade of life. A proportion of DMD patients also exhibit mental deficiency that cannot be entirely explained as secondary effects of social environment and functional disability. The phenotype of BMD presents later than DMD and has a more gradual and variable degenerative course (Clemens & Caskey 1992).

The cloning of the cDNA for the dystrophin gene (Koenig *et al.* 1987) revolutionized DNA diagnostic strategies for DMD. Use of dystrophin cDNA probes for Southern hybridization demonstrates large deletions or duplications in approximately 70% of DMD patients (Darras *et al.* 1988; Baumbach *et al.* 1989; Gilgenkrantz *et al.* 1989; Hu *et al.* 1989; Koenig *et al.* 1989; Angelini *et al.* 1990; Hu *et al.* 1990). Approximately 98% of the deletions can also be detected by the simpler technique of polymerase chain reaction (PCR) exon amplification (Chamberlain *et al.* 1988, 1989; Beggs *et al.* 1990; Multi Center Study Group 1992). The remaining 30% of DMD cases are probably due to mutations below the detection threshold of Southern and PCR studies. There are two reports of DMD patients with point mutations causing premature chain termination within the dystrophin gene. These mutations were detected by prediction of the mutation site based on a truncated protein fragment observed by Western analysis, followed by DNA sequencing (Bulman *et al.* 1991) and by identification of a new *Hin*dIII site within exon 48 followed by DNA sequencing (Clemens *et al.* 1992). Strategies to screen for point mutations within the dystrophin gene are also being developed (Kilimann *et al.* 1992; Roberts *et al.* 1992). Despite the remarkable advances in DNA diagnostics for DMD and the application of these strategies to prenatal diagnosis (Ward *et al.* 1989; Clemens *et al.* 1991), a high spontaneous mutation rate within the gene will perpetuate this devastating disorder.

## 3. CURRENT OPTIONS FOR THERAPY

### (*a*) *Adenosine deaminase deficiency*

Bone marrow transplantation (BMT) from a human lymphocyte antigen (HLA)-identical donor is the preferred treatment for ADA deficiency, and while associated with morbidity, can result in complete cure. Unfortunately, fewer than 30% of the patients have an HLA matched sibling. Enzyme replacement is an attractive mode of therapy because dAdo is freely diffusible, and therefore the ADA enzyme does not need to enter the target cells to be partially effective. Weekly injections of bovine ADA protein conjugated to polyethylene glycol (PEG-ADA) have been shown to reverse the biochemical abnormalities, and restore some T-lymphocyte function. Neverthless, total immune reconstitution has not been demonstrated and anti-PEG-ADA antibodies have been demonstrated in some patients(Kredich *et al.* 1989).

### (*b*) *Duchenne muscular dystrophy*

To date, the only treatment available to DMD patients is symptomatic, comprising physical therapy, tendon release to relieve joint contracture, and spine surgery for scoliosis (Brooke *et al.* 1989). These procedures may improve the quality of life, but do not prevent the inevitable degeneration and death. Recent studies exploring immunosuppression with prednisone show promise for slowing the course of muscle degeneration, but not for eradication of the disease (Mendell *et al.* 1989; Fenichel *et al.* 1991; Griggs *et al.* 1991). There is a clear need for more effective treatment for patients with DMD. Two strategies currently under study as potential somatic therapies for the disease are myoblast transfer and gene transfer (discussed below).

## 4. ADVANCES TOWARD GENE THERAPY

Gene therapy is defined as the transfer of a functional gene into the cells of an organism for the treatment of disease (Miller 1990; Levine *et al.* 1991; Mulligan 1991; Verma *et al.* 1991). In general, a disease that might be amenable to gene therapy should be a single-gene disorder of recessive inheritance and be well understood at the molecular level. The biology of the target tissue should be known and gene transfer into the target tissue should result in levels of expression adequate to cure the disease. ADA deficiency is an ideal model for gene therapy targeting hematopoietic stem cells (HSCs) based on the results from BMT, suggesting that transfer of the normal ADA gene into HSCs would be sufficient to correct the enzymatic defect. Only 20% of the normal ADA level in the blood would be needed for treatment and it is plausible that *in vivo* selection of even a few corrected stem cells would take place. In DMD, the defects are well understood at the molecular level. However, further understanding of the function of dystrophin in tissues is necessary for successful gene transfer strategies. *Ex vivo* gene transfer into candidate target cells, myoblasts, has been attempted. The *mdx* mouse provides an animal model for DMD gene therapy.

Highly efficient gene transfer is required for gene therapy. The retroviral vector is the best characterized viral vector for human gene transfer (Levine *et al.* 1991; Mulligan 1991). Retroviral vectors transduce dividing cells in culture with almost 100% efficiency




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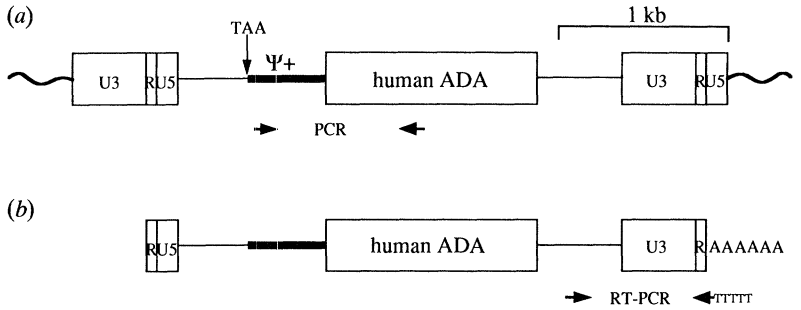


Figure 1. Structure of integrated retroviral vector (*a*) and transcript from the vector (*b*). In this vector, ΔN2stADA, the start codon of gag$^+$ was mutated to a stop codon (TAA). Human ADA cDNA is transcribed from the 5′ LTR. The primers for the provirus PCR (*a*) and vector mRNA RT-PCR (*b*) are indicated as arrows. Wavy lines indicate flanking chromosomal DNA.

and integrate into the host genome stably without causing gross rearrangements.

### (*a*) *In adenosine deaminase deficiency*

A gene replacement therapy trial in two patients with ADA deficiency began recently at the National Institutes of Health (Culver *et al.* 1991). The ADA gene transfer strategy targets peripheral T-cells that have been stimulated *in vitro*. Both patients have shown improvement clinically, as well as in *in vitro* and *in vivo* immune function. However, because only mature T-cells are transduced, not all of the immune function indices have normalized (Anderson 1992). Also, the limited life span of T-cells necessitates repetitive infusions of transduced T-cells, raising serious concerns about clonality of the immune cells and the possibility of insertional mutagenesis of cancer-related genes by repeated retroviral transfections.

The HSC, which constitutes 0.01–0.1% of human bone marrow (BM) cells, has been regarded as an ideal target tissue for human gene therapy because it is pluripotent and undergoes self-renewal. In theory, all hematopoietic cell lineages would demonstrate the transduced gene after the transfection of even a few HSCs. Cells bearing the CD34 antigen represent a population of bone marrow cells thought to contain the most primitive progenitors. Retroviral vectors efficiently transfer genes into mouse HSCs; we have also used them for gene targeting to human HSCs.

A retroviral vector, ΔN2stADA (figure 1) (Scarpa *et al.* 1991), which utilizes the retroviral LTR to drive human ADA, was used for transduction studies. Low density mononuclear cells from normal BM samples were transduced either by co-cultivation with the virus producing cell line or by virus-containing supernatant in the presence of a supportive autologous stromal layer. Transduced BM cells were kept in myeloid long term culture (LTC) and a colony-forming assay on methylcellulose-containing medium was performed as described previously (Cournoyer *et al.* 1991). Polybrene, recombinant human interleukin 3 (rhIL-3) and rhIL-6 (gifts from Dr S. C. Clark, Genetics Institute) were added during transduction in both protocols. Additionally, CD34$^+$ cells were selected on anti-CD34 selection devices (Applied Immune Sciences), plated onto previously grown autologous stroma and infected with retroviral supernatant. The cells were maintained in LTC before analysis.

PCR and reverse transcriptase-PCR (RT-PCR) were used to examine the integration and expression, respectively, of ADA-retrovirus vector in human hematopoietic colonies derived from LTC (figure 1). Human clonogenic progenitors, which are present at week 0, disappear by death or differentiation after five weeks in myeloid LTC. Therefore, colonies formed after that time are derived from primitive progenitors, which are presumed to be closely related or identical to HSCs present at the time of transduction (Sutherland *et al.* 1989; Sutherland *et al.*, 1990). Granulocyte–macrophage (GFU-GMS) colonies demonstrated approximately 90% transduction at week 0, 50% transduction at week 3 and 50% later than week 5, indicating that approximately 50% of primitive hematopoietic progenitors were transduced by the co-cultivation method (figure 2*a*). The percentage of transcript-positive colonies was 33% at week 0, 22% at week 3 and 14% at week 6 (figure 2*a*). At week 6, the transcript was also detected in 25% of colony-forming unit-erythroids (GFU-ES) (data not shown). The percentage of provirus-positive and transcript-positive colonies transduced with viral supernatant was equivalent to that of co-cultivation (figure 2*b*). While transduction with viral supernatant is preferred to co-cultivation, previous results with viral supernatant have demonstrated poor transfer efficiency (Eglitis *et al.* 1988; Bodine *et al.* 1991; Cournoyer *et al.* 1991). The stromal support appears to play a major role in efficient gene transfer by viral supernatant. The CD34$^+$ fractions from normal individuals, as well as from an ADA-deficient patient, were also transduced successfully, suggesting that the viral supernatant protocol is applicable to a clinical trial in ADA-deficient patients (data not shown).

To detect the increased ADA activity after retroviral gene transfer, an ADA conversion assay was performed on collected non-adherent cells in LTC (Aitken *et al.* 1980). The activity of purine nucleoside phosphorylase (PNP) was used to standardize the amount of protein in each samples. We observed a 3.6-fold increase of ADA to PNP activity at week 5 post-transduction that was maintained at week 9 (figure 3). In the case of ADA(−) BM, the ratio of




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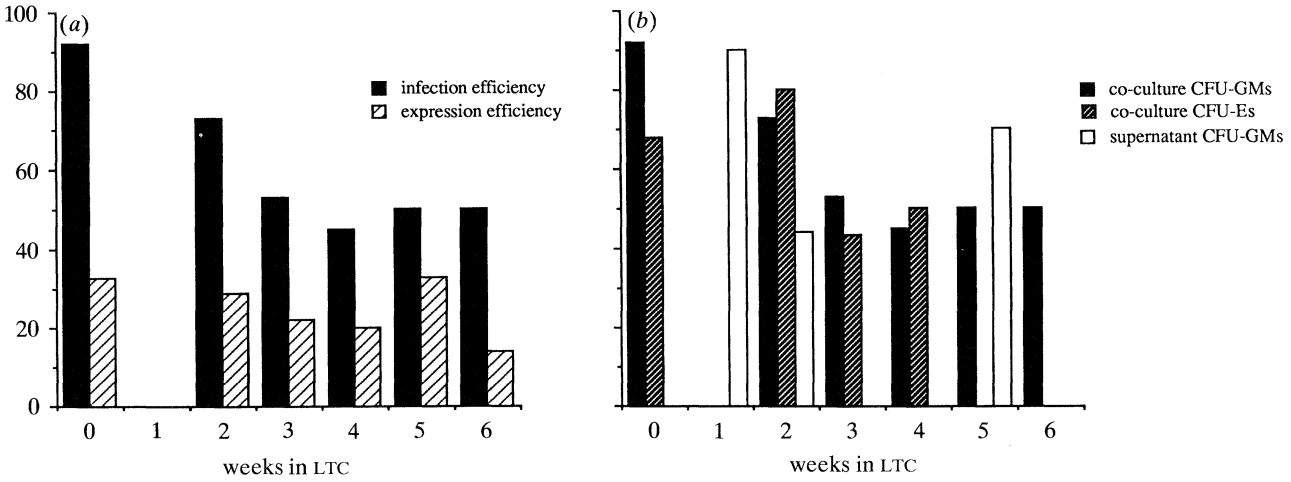
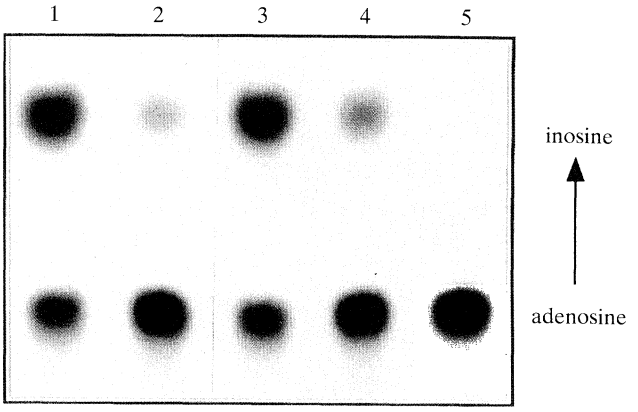



Figure 2. (*a*) Gene transfer and expression efficiency of the retroviral vector in GFU-GMS transduced by co-cultivation. (*b*) Comparison of gene transfer efficiency by co-cultivation protocol and viral supernatant protocol.

ADA to PNP activity was 21.0 in the transduced BM and 0.22 in untransduced BM ten weeks post-transduction (data not shown). Although there could be a small amount of contaminating long-lived mature cells in this fraction, the large increase of ADA activity at this late stage of LTC suggests strong expression of the transduced ADA gene in cells differentiated from transduced progenitors. Development of a mouse model of human ADA deficiency, in progress in our laboratory, is also important to evaluate the efficacy of our vector. The expression from the LTR promoter of Moloney murine leukemia virus (Mo-MuLV) is sustained in both clonogenic progenitors and mature cells derived from transduced primitive progenitors *in vitro*. Transplantation of transduced BM into *Bg/nu/xid* mice (Dick *et al.* 1991) or transduced BM depleted of mature T- and B-lymphocytes and BM from mice with severe combined immunodeficiency into lethally irradiated Balb/c mice (Lubin *et al.* 1991) would be ideal for evaluation of ADA gene replacement into lymphocytes. *Bg/nu/xid* mice were used to evaluate the efficacy of ADA gene transfer into peripheral T-cells of an ADA deficient patient (Ferrari *et al.* 1991). Long-term survival of vector-transduced human lymphocytes as well as restored immune functions, as indicated by the presence of human immunoglobulin and antigen-specific T-cells, was demonstrated in recipient animals (Ferrari *et al.* 1991).

### (*b*) *Duchenne muscular dystrophy*

The cloning of the full-length dystrophin cDNA




| | | | | | |
|---|---|---|---|---|---|
| ADA activity | 1.27 | 0.12 | 1.30 | 0.26 | --- |
| PNP activity | 0.67 | 0.27 | 0.19 | 0.30 | --- |
| ADA/PNP | 1.82 | 0.51 | 2.90 | 0.79 | --- |
| increase | | 3.6 | | 3.7 | |

Figure 3. Proviral induced ADA activity in normal BM. A ratio of ADA to PNP activity (ADA/PNP) is an average of duplicated assays. ADA and PNP activities are described as (nanomole conversion) per hour per $10^3$ cells. Lanes : 1 and 2, 5 weeks post-transduction; 3 and 4, 9 weeks post-transduction; 5, no extract; 1 and 3, transduced BM; 2 and 4, untransduced BM.

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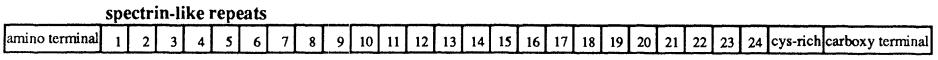


Figure 4. Schematic representation of the structure of dystrophin predicted from the cDNA sequence.

that was shown to express dystrophin in cell culture (Acsadi *et al.* 1991; Dickson *et al.* 1991; Lee *et al.* 1991a) has facilitated two general experimental approaches toward gene replacement therapy for DMD: (i) demonstration of germline correction in transgenic mice; and (ii) studies toward somatic cell therapy, both by myoblast transfer and gene transfer.

Germline correction of dystrophin deficiency in animal models provides an experimental demonstration of the potential for gene replacement therapy for DMD patients. A transgenic mouse with a single copy integration of the full-length dystrophin cDNA driven by the muscle creatine kinase promoter with the E1 and E2 enhancers was bred onto the dystrophin-deficient *mdx* genetic background. Transgene expression of both messenger RNA and protein was found in the expected pattern of tissues (Lee *et al.* 1991*b*). Another transgenic mouse was generated using a deleted dystrophin cDNA construct derived from a BMD patient and driven by the constitutive promoter elements of the Mo-MuLV (Wells *et al.* 1992).

Studies aimed at somatic therapy for DMD patients began with myoblast transfer. This is a form of cellular somatic therapy designed to exploit the normal biology of skeletal muscle regeneration to correct a genetic defect. Precursor mononuclear muscle cells (commonly called myoblasts or satellite cells) fuse with other myoblasts and existing muscle fibres to produce the multinucleate syncytia of differentiated skeletal muscle (Grounds 1991). The concept underlying myoblast transfer therapy for DMD is that normal donor myoblasts injected into degenerating, dystrophin-deficient muscle will fuse with the degenerating muscle fibres, provide dystrophin which is presumed to be necessary to maintain cytoskeletal integrity, and aid in the regeneration of skeletal muscle. Promising results were observed in animal studies in which normal mouse donor myoblasts, injected into *mdx* mouse muscle, fused with host dystrophin-deficient fibres forming mosaic myofibres (Law *et al.* 1988; Karpati *et al.* 1989*a*,*b*; Partridge *et al.* 1989; Morgan *et al.* 1990). Pilot human myoblast transfer studies, in which myoblasts derived from a muscle biopsy taken from a related donor are injected into selected, small muscles of patients with DMD, are now in progress (Law *et al.* 1990, 1991; Huard *et al.* 1991; Miller *et al.* 1992). To date, myoblast transfer studies in humans have not yielded promising results, perhaps due in part to immune rejection of the donor myoblasts by HLA class I and class II specific alloreactive T cells and natural killer cells (Karpati *et al.* 1988; Bao *et al.* 1990; Hohlfeld & Engel 1990*a*,*b*; Mantegazza *et al.* 1991). Furthermore, a major limitation of myoblast transfer as a potential therapy for DMD is that it can only address the skeletal muscle manifestations of the disease. In addition, DMD patients have widespread skeletal muscle degeneration in many muscle groups and transplanted myoblasts may not migrate far from the injection site.

Other researchers have attempted to introduce the deficient genetic material more directly. One experimental attempt of somatic gene therapy for DMD *in vivo* was the intramuscular injection of human dystrophin DNA constructs into *mdx* mice resulting in low-level human dystrophin expression (Acsadi *et al.* 1991). These results are encouraging for the prospect of human gene therapy in DMD patients because they demonstrate that myoblasts or muscle fibres will incorporate and express exogenous DNA *in vivo*. Another means of direct gene transfer under investigation utilizes particle bombardment technology to introduce marker genetic material into cultured muscle cells and muscle tissue *in vivo* (Yang *et al.* 1990). To date, neither of these direct gene transfer strategies into muscle have achieved the efficiency of gene transfer that would be required for clinical efficacy.

The use of retroviral and adenoviral vectors holds promise as an efficient method of somatic gene transfer for DMD patients. The successful application of retroviral-mediated gene transfer into myoblasts has been demonstrated by transfer of the human growth hormone gene into mouse myoblasts (Barr & Leiden 1991; Dhawan *et al.* 1991) and by transfer of the human multidrug transporter gene into rat myoblasts (Salminen *et al.* 1991). Furthermore, retrovirus-infected myoblasts have been injected into recipient animal muscle resulting in expression of the gene insert that was sustained for one to three months (Barr & Leiden 1991; Dhawan *et al.* 1991; Salminen *et al.* 1991). An adenoviral vector containing the β-galactosidase reporter gene under the control of muscle-specific regulatory sequences has been used to demonstrate efficient transfection of myotubes in culture and of muscle after direct injection *in vivo* (Quantin *et al.* 1992).

Several unique challenges are presented by the application of retroviral and adenoviral vectors to gene transfer for DMD patients. First, the dystrophin cDNA is approximately twice the maximum insert size of both vectors. Second, the protein product of the transferred gene must be targeted to tissues that normally express dystrophin, and within muscle cells must localize to the cell membrane to perform its presumed cytostructural function.

Four domains of protein structure have been predicted from the DMD cDNA sequence: (i) an amino terminal domain; (ii) a repeat domain predicted to have a rod-like structure; (iii) a cysteine-rich domain; and (iv) a carboxy terminal domain (figure 4). The first three domains have homology to two known cytoskeletal proteins, α-actinin (domains one and three) and spectrin (domain two) (Koenig *et al.* 1988).




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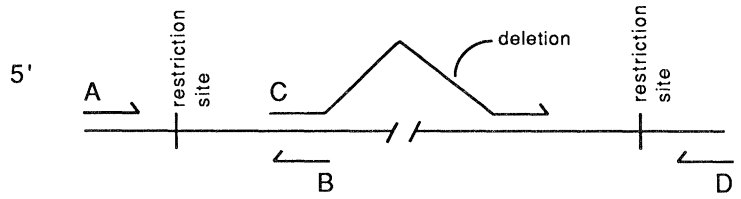


Figure 5. Diagram of the PCR-based deletion mutagenesis strategy. Primer C is a central primer that encodes the deletion such that the 5′ half of the primer is complementary to the dystrophin cDNA sequence just 5′ to the deletion and the 3· half of the primer is complementary to the dystrophin cDNA sequence just 3′ to the deletion. Primer B is complementary to the 5′ half of primer C. Primers A and D are flanking primers that are 5′ and 3′, respectively, of restriction sites used for cloning.

The fourth domain has homology to the autosomal transcript, DMDL (Love *et al.* 1989), which codes for dystrophin-related protein.

To truncate the dystrophin cDNA such that it can be accommodated by retroviral and adenoviral vectors, we are generating recombinant dystrophin cDNA constructs with deletions of various sizes in the spectrin-like repeat region of dystrophin. This region is targeted for deletion mutagenesis because the patients with milder phenotypes generally have in-frame deletions in this region (Gospe *et al.* 1989; England *et al.* 1990). The use of a PCR-based deletion mutagenesis strategy provides control over the exact position of the deletion and therefore, maintenance of the reading frame. Four oligonucleotide primers are designed for each engineered deletion, with the central primer (primer C) encoding the desired deletion (figure 5). PCR is performed with primers A and B and with primers C and D in two separate reactions. Products of the two reactions are mixed and a second PCR is performed with primers A and D. Under the conditions of the PCR, the products of the first PCR reactions denature allowing for the annealing of the strand complementary to primer A with the strand complementary to primer D followed by 3′ extension of each strand prior to amplification by the second PCR. After sequencing the PCR product to ascertain that no errors are introduced by the PCR, the fragment encoding the deletion is cloned into the original 5′ clone of the dystrophin cDNA. Following this, the mutagenized 5′ clone is spliced to the 3′ clone of the dystrophin cDNA to reconstitute a full-length recombinant dystrophin cDNA with a central deletion of part of the spectrin-like repeat region.

Using this approach, we have generated two truncated dystrophin constructs deleting 3–5 kb of the spectrin-like repeat region of dystrophin. By also truncating the 3′ untranslated region, the size of the DNA molecule can be reduced further. The truncated cDNA molecules will be tested for expression and targeting *in vitro* and *in vivo*.

## 5. CONCLUSIONS

Human gene therapy has progressed from theory to reality in a short time. The first gene therapy protocol, for correction of ADA deficiency in peripheral blood lymphocytes, began in September 1990. Now there are more than 20 active or approved clinical protocols and many additional protocols are in various stages of development. Several years ago, targeting of hematopoietic stem cells by retroviral vectors was thought to be the only realistic protocol for human gene therapy. Now, work is focused on a variety of somatic tissues, including muscle cells, and several gene transfer strategies.

The continued characterization of genes that are mutated in single gene disorders will widen the field of inherited diseases that can be approached by human gene therapy. Emphasis must be placed on the development of vectors or other transfer strategies that can deliver and express foreign genes more efficiently. Finally, it is likely that *in vivo* gene transfer will be required in some cases and preferable to *ex vivo* gene transfer, where cells are removed from a patient, the gene is transferred *in vitro* and the corrected cells are transplanted to the patient.

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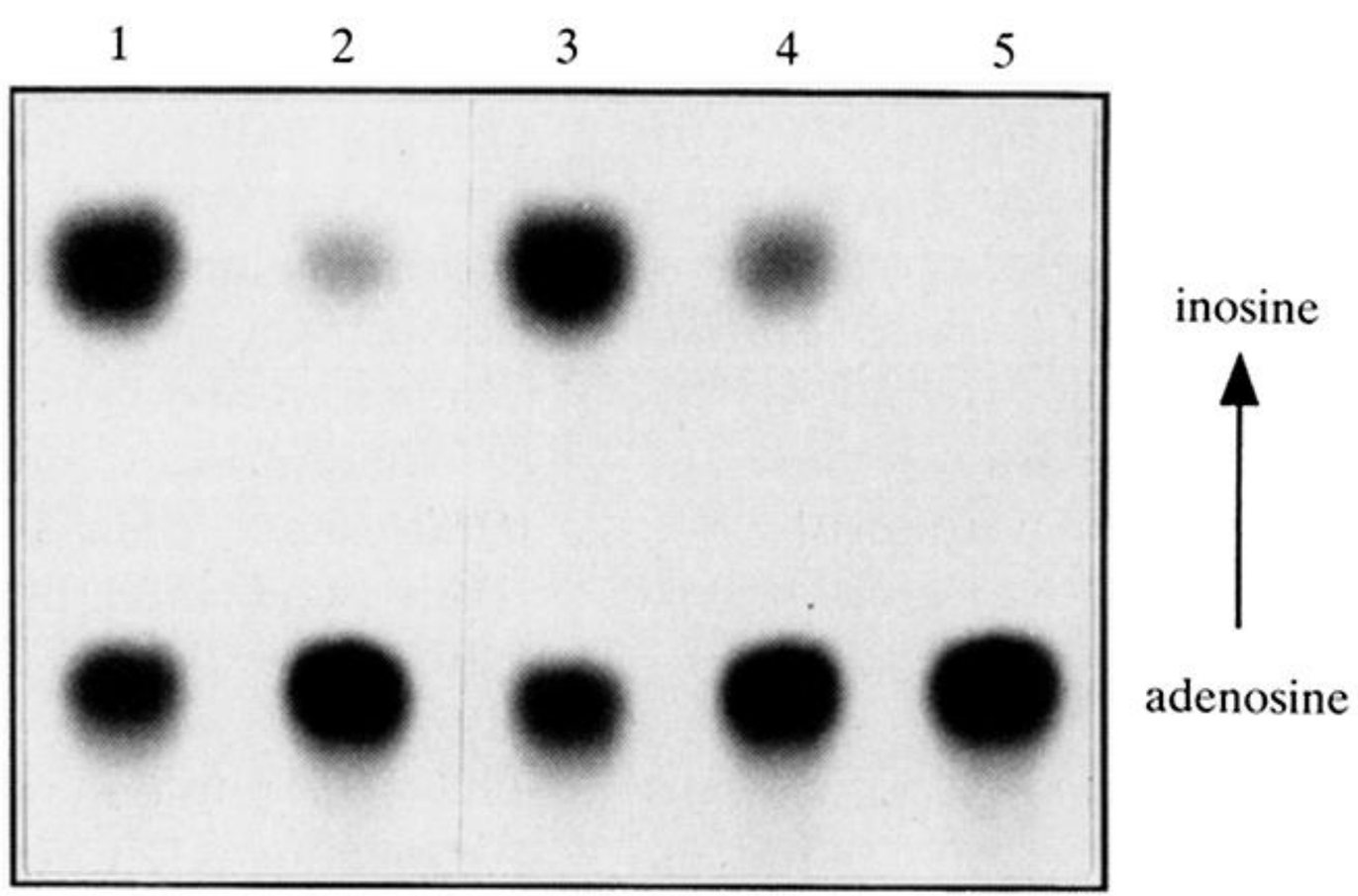


| | | | | | |
|---|---|---|---|---|---|
| ADA activity | 1.27 | 0.12 | 1.30 | 0.26 | --- |
| PNP activity | 0.67 | 0.27 | 0.19 | 0.30 | --- |
| ADA/PNP | 1.82 | 0.51 | 2.90 | 0.79 | --- |
| increase | | 3.6 | | 3.7 | |

gure 3. Proviral induced ADA activity in normal BM. A ratio of ADA to PNP activity (ADA/PNP) is an average of plicated assays. ADA and PNP activities are described as (nanomole conversion) per hour per $10^3$ cells. Lanes : 1 d 2, 5 weeks post-transduction; 3 and 4, 9 weeks post-transduction; 5, no extract; 1 and 3, transduced BM; 2 and 4, ntransduced BM.