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Exception patients in Duchenne and Becker muscular dystrophy

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The Ohio State University, 1993

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EXCEPTION PATIENTS IN DUCHENNE AND BECKER MUSCULAR DYSTROPHY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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To J.H., M.C., and K.K. who have always been supportive, loving and an inspiration to me.
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LIST OF ABBREVIATIONS

Ab = antibody
bp = base pair
cDNA = complementary DNA
DNA = deoxyribonucleic acid
ELISA = enzyme-linked immunosorbent assay
dNTPs = deoxynucleotide triphosphates
kb = kilobase
kD = kilodalton
mg = milligram
µl = microliter
µM = micromolar
mM = millimolar
ml = milliliter
Mgb = megabase
mRNA = messenger ribonucleic acid
nM = nanomoles
PCR = polymerase chain reaction
RNA = ribonucleic acid
rpm = rotations per minute

x
RT-PCR = reverse transcription polymerase chain reaction

tRNA = transfer ribonucleic acid

v/v = volume per volume

w/v = weight per volume

5' = five prime

3' = three prime
CHAPTER I
INTRODUCTION

THESIS OVERVIEW

Duchenne Muscular Dystrophy (DMD) is an X-linked muscle wasting disorder caused by mutations in the 2Mgb gene encoding the 427 kD protein dystrophin. The entire 14 kb cDNA has been cloned and sequenced (Koenig, et al., 1988). DMD patients generally do not produce dystrophin and have mutations that disrupt the translational reading frame (out-of-frame mutations). Becker Muscular Dystrophy (BMD) patients, the milder allelic form of DMD, produce dystrophin with mutations that do not disrupt translational frame (in-frame mutations). The aim of this project has been to investigate DMD and BMD patients who are exceptions to this frame rule in order to provide a clearer understanding of the genotype/phenotype relationship. In addition, the mechanism of dystrophin production in patients with frameshift mutations has been investigated. Lastly, the results provide insights into possible functional domains of dystrophin.

This research is divided into three parts. The first two parts present patients who are translational frame
rule exception patients. Chapter II describes several types of in-frame and out-of-frame DMD, BMD and intermediate patients. The exon 3-7 frameshift deletion patients are the most common type of exception patient and have been studied in the greatest detail. Therefore, the exon 3-7 frameshift deletion patients are presented separately in Chapter III. A comparison of the significance of the results from all the translational frame exception patients is presented in Chapter V. In the third part, the mechanisms responsible for the occurrence of rare dystrophin-positive fibers in DMD patients are presented in Chapter IV.

Several types of translational frame rule exception patients were studied at the DNA, RNA and protein levels, including in-frame and out-of-frame patients with very large deletions. The most common type of translational frame exception patients are patients with an out-of-frame exon 3-7 deletion. One possible explanation for dystrophin production in exon 3-7 deletion patients is that aberrant splicing could make an in-frame message at the RNA level. In order to determine whether aberrant splicing could account for the restored reading frame in exon 3-7 deletion patients, we amplified mRNA isolated from muscle of patients with genomic exon 3-7 deletions. In all cases, the expected PCR product was amplified
between exons 1-8, 2-8, 2-16, and 8-16. One patient showed an extra PCR product when amplified between exons 2-16 and 8-16, which had a deletion of exon 9 as well as exons 3-7. In no case did the sequence indicate restoration of translational frame by aberrant splicing. Therefore another mechanism, such as reinitiation or ribosomal frameshifting was proposed to account for the dystrophin production. To test this, we produced antibodies to regions preceding and following the proposed reinitiation site in exon 8 and to the amino-terminus. These antibodies were used to demonstrate that a truncated form of dystrophin is being produced in these patients by initiation from ATG in exon 8 and not from the original ATG in exon 1.

We and others (Shimizu, T., et al., 1988; Nicholson, L., et al., 1989; Hoffman, E., et al., 1990; Burrow, K., et al., 1991; Klein, C., et al., 1992) have observed rare dystrophin-positive fibers, termed revertants, in approximately 50% of our DMD patients who are typically absent for dystrophin. One possible mechanism for the occurrence of revertant fibers is that a secondary mutation deletes the original mutation. Initial data showed that in some non-deletion patients, the revertants stained positive for amino- and carboxyl-terminal dystrophin antibodies, but did not stain with the antibody
corresponding to amino acids 2305-2554 (Klein, C., et al., 1992, table 1, page 23). We analyzed the region corresponding to the deleted epitope in one of these patients using RT-PCR and sequencing and found a 2bp GG->AT nonsense mutation in exon 51 which creates a new cryptic splice site 2 bp 3' of the mutation, that splices the end of exon 50 into the middle of exon 51 and deletes the original mutation. However, this cryptically spliced mRNA is out-of-frame. Splicing from the end of exon 49 into this cryptic splice site can produce an in-frame mRNA. Primers specific to the exon 49/exon 51 junction were able to detect this mRNA specifically in the patient sample and not in the control sample. We produced an antibody to the end of exon 51 in order to determine if the revertant fibers produce dystrophin used this new cryptic splice site.

In conclusion, the translational frame rule exception patients presented in this study give further insight into the proposed functional domains in the amino-terminus of dystrophin. In particular, patients who delete both actin-binding domains or maintain both actin-binding domains yet delete parts of the rod domain can have mild phenotypes. Whereas patients who delete one of the actin binding domains and part of the rod domain are more severely affected. The study of these patients also indicates that
other factors besides dystrophin production influence phenotype. In addition, our results from the analysis of revertant fibers identified a 2 bp point mutation in a DMD patient and support the theory that these fibers can be produced by a secondary mutation that removes the original mutation. With this understanding of revertant fibers, we were able to develop important antibody controls for the detection of revertant fibers that will be useful during the initial stages of the myoblast transfer studies.
DUCHENNE MUSCULAR DYSTROPHY

Duchenne Muscular Dystrophy (DMD) is one of the most common human genetic diseases, affecting approximately 1/3500 live male births (Emery, 1987). DMD was first described by Meryon in 1852 and subsequently by Little in 1853 and Duchenne de Boulone in 1861 (Worton and Burghes, 1988). DMD is an X-linked muscle wasting disorder and approximately 1/3 of all cases arise via new mutation (Moser, 1984). The disease initially presents as a muscle weakness in the pelvic girdle which then spreads symmetrically towards the extremities. Progressive muscle weakness usually becomes apparent by ages of 3-5 years and the patients are generally wheelchair bound by the age of 12 years. Death usually occurs in the third decade of life due to respiratory failure, but occasionally from cardiac failure or gastrointestinal blockage (Chamberlain and Caskey, 1990). Becker Muscular Dystrophy (BMD) is a milder allelic form of DMD (Worton and Thompson, 1988; Rowland, 1988; Monaco and Kunkel, 1988). Symptoms of BMD are similar to those of DMD, but generally display a later onset and are much less severe. Classification is based on the age of loss of ambulation. DMD patients are wheelchair bound by the age of 12 while BMD patients are still ambulatory at age 16. Patients who demonstrate severity between DMD and BMD patients are referred to as
intermediates or outliers (Brooke, et al., 1983). It should be noted that this disease shows a continuous spectrum of severity and that the definitions are somewhat arbitrary (Malhotra, et al., 1988; Brooke, 1986; Emery, 1987; Rowland, 1988).

THE GENE AND PROTEIN PRODUCT

The DMD gene is the largest gene so far isolated, spanning 2.4 million base pairs of DNA (Burmeister and Lehrach, 1986; van Ommen, et al., 1986, 1987; Kenwrick, et al., 1987; Burmeister, et al., 1988). The gene is now referred to as the dystrophin gene (Hoffman, et al., 1987). Two groups were involved in the cloning of the gene, Monaco, et al, (1986) and Burghes, et al., (1987). The entire cDNA of the gene has been cloned and sequenced (Koenig, M., et al., 1987, 1988) and was shown to encode for a 14kb mRNA with a 11kb open reading frame (van Ommen, et al., 1987; Koenig, et al., 1987). Dystrophin mRNA transcripts have been detected at the highest levels in skeletal and cardiac muscle. It is also detected in smooth muscle at about 2-10% of the level found in skeletal muscle and detected in brain and kidneys at only 1-2% of the level found in skeletal muscle. It is possible to detect the mRNA at extremely low levels (0.004-0.05% of muscle level) in fibroblasts and lymphoblasts due to illegitimate transcription, however no protein is produced.

The protein product of the DMD gene, dystrophin, is a 427 kd protein that localizes to the sarcolemma of muscle fibers (Zubrzycka-Gaarn, et al., 1988; Ahn and Kunkel, 1993) The sequence of the protein shows overall similarity to the cytoskeletal proteins β-spectrin and α-actinin (Koenig, et al., 1988; Davison, et al., 1989, Hammonds, 1987) which perform a structural role in the cell (Dubreuil, 1991; Bennett, 1990; Coleman, et al., 1989). Because of the similarity of dystrophin to these structural proteins and because part of the pathology of disease includes loss of membrane integrity, it has been proposed that dystrophin may also play a structural role. Localized separation of the plasma membrane from the basal lamina, also called a Δ lesion, has been suggested to be an indication of myofiber membrane weakness and therefore has been proposed to be the primary defect in dystrophic muscle (Carpenter and Karpati, 1979). By analogy to other structural proteins and the observation of the Δ lesion,
dystrophin is thought to be cytoskeletal protein which mediates the binding of the cytoskeleton to the basal lamina and structurally supports the plasma membrane during muscle contraction. However, the exact functional role of dystrophin is not yet known and the role of dystrophin in non-muscle tissue is unclear.

By analogy to spectrin, dystrophin is thought to associate in a dimer with itself in an antiparallel orientation. The actin binding domain at the amino-terminal is thought to link the intracellular domain to the membrane cytoskeleton (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya, et al., 1992; Suzuki, et al., 1992). It has been proposed that the carboxyl-terminus of dystrophin may interact with the dystrophin-associated glycoprotein complex (DAGC), see page 12 (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya, et al., 1992; Suzuki, et al., 1992). Additional evidence comes from analysis of the Dystrophin Associated Proteins (DAP) in the dystrophin-associated glycoprotein complex (DAG) in DMD patients. It was found that DAPs are absent in patients with carboxyl-terminal deletions or who produce no dystrophin. This suggests that the carboxyl-terminal end of dystrophin is involved in the binding activity of DAG complex to the sarcolemma (Matsumura, et al., in press).
Dystrophin is expressed in alternatively spliced forms in many tissues, including cardiac muscle, smooth muscle, brain, nerve, and liver (Ahn and Kunkel, 1993). Smooth muscle that has been longitudinally sectioned stains continuously around the membrane, while transverse sections (same orientation as the skeletal muscle sections shown in Chapters II, III, and IV) show a patchy staining (Byers, et al., 1991). In smooth muscle, unlike in skeletal muscle, dystrophin and vinculin segregate into complementary domains along the membrane (North, et al., 1993) and exclude dystrophin from regions associated with force transduction. Therefore, dystrophin may have a different role in smooth muscle than in skeletal muscle. This may also be true for dystrophin in cardiac muscle, where dystrophin is localized to the membrane except for the region between the cardiocytes that is associated with force transduction (Byers, et al., 1991). Dystrophin is also expressed in cardiac Purkinje fibers (Bies, et al., 1992). This may account for the conduction defects found in DMD and BMD patients. The muscle form of dystrophin is expressed at low levels in neurons in the brain (Lidov, et al., 1990; Chelly, et al., 1990). The brain form of dystrophin is expressed at higher levels and uses a different promoter and first exon than the muscle form (Boyce, et al., 1991). By immunofluorescence, dystrophin
is present in the soma and dendrites of all Purkinje cells of the cerebellum, the pyramidal cells of the hippocampus and some neurons in the cortex. Another form of dystrophin can be found in peripheral nerve. The nerve form of dystrophin has a unique short first exon and that then continues with exon 56 of the muscle form. Therefore, the nerve form of dystrophin is similar to only the carboxyl end of dystrophin and produces a 116 kD protein (Byers, et al., 1993). This protein is localized to the nerve submembrane of the external layer of each Schwann cell and at the nodes of Ranvier. The full-length form of dystrophin accumulates in muscle and neurons, but not in liver and Schwann cells, where the forms containing just the carboxyl-terminal end predominate (Ahn and Kunkel, 1993). Although all of these forms of dystrophin are associated with the membrane, they have differences in distribution. They also contain various portions of the full-length form of dystrophin and may have sequences not found in the muscle form of dystrophin. This suggests that although all these forms may have some role in membrane structure or maintenance, they likely serve slightly different functions from one another, the exact nature of which is still unknown.
DYSTROPHIN-ASSOCIATED PROTEINS

A protein complex composed of four glycoproteins can be co-purified with dystrophin (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; and Ervasti, et al., 1990). These proteins have been named dystrophin-associated proteins (DAPs). One of the proteins (156 kD) is extracellular, three (50 kD, 43 kD, and 35 kD) are integral membrane proteins and one is a cytoplasmic protein (Ervasti and Campbell, 1991). The 156 kD protein and 43 kD protein are translated from a single mRNA which is posttranslationally modified to yield the two different sized proteins. The 156 kD protein was shown to bind laminin, a major component of the basement membrane (Ibraghimoz-Beskrovnaya, et al., 1992). The DAPs are virtually absent in both patients who produce dystrophin with carboxyl-terminal deletions and patients who produce no dystrophin. In patients who produce dystrophin with amino-terminal or rod domain deletions, the level of DAPs is the same as the level of dystrophin, regardless of the patient’s phenotype. These results support the role of dystrophin as a linking protein in mediating the binding of the cytoskeletal lamina (Carpenter and Karpati, 1979; Matsumura, et al., in press).
DYSTROPHIN-RELATED PROTEIN

Dystrophin-related protein (DRP) is encoded by the DMDL (DMD-like) gene localized to chromosome 6q24. The 3' end of DMDL shares high homology to the 3' end of dystrophin. The DMDL transcript is expressed in adult and fetal tissues, including, liver, intestine, skeletal muscle, testis and kidney (Love, et al., 1991). Within human or mouse skeletal muscle, DRP is found to localize to the post synaptic membrane of the neuromuscular junction. In DMD or mdx mouse muscle, DRP localizes to the postsynaptic membrane of the neuromuscular junction and to the sarcolemma. The function of the DMDL gene is not known and no disease-causing mutations have yet been found.

PROSPECTS FOR THERAPY

To date, there is no successful means for treating DMD. Several drug trials for the treatment of DMD have been reported. However only prednisone has been shown to provide any beneficial affect (Mendell, et al., 1989). Prednisone treatment was shown to cause improvement of muscle strength as compared with the control group. However, further studies need to be done to see if this improvement can be maintained over an extended period of time. It does not seem that prednisone will be suitable for long term treatment. Various surgical procedures and physical therapies are known to delay the loss of
ambulation and reduce the degree of scoliosis (Brooke, et al., 1989). They may offer improvement in some of the symptoms of the disease, but none of these treatments or therapies halt the disease process. As an alternative, strategies for both gene replacement (Acsadi, et al., 1991; Ragot, et al., 1993) and myoblast transfer (Partridge, et al., 1989) are being attempted.

One method of gene replacement was developed because it has been shown that dystrophin expression can be detected following direct injection of DNA constructs into muscle (Wolf, et al., 1990). However, the low efficiency of uptake and technical difficulties associated with the necessity of multiple injection sites, makes this approach arduous.

Several vectors have been constructed in order to allow more efficient delivery of dystrophin into muscle tissue. One difficulty lies in the large size of the dystrophin cDNA, which most currently available vectors can not accommodate. Because dystrophin is absent in severe patients and can be expressed in a truncated form and at low levels in milder patients, it has been proposed that it may be beneficial to introduce a truncated form of dystrophin into DMD muscle (based on mild patients with large deletions). As a result, many of the constructs are truncated forms of dystrophin based on BMD deletions in

In addition to size, other obstacles are associated with using vectors for gene replacement and involve the control of tissue-specific uptake in a terminally differentiated tissue. Efficient retroviral-mediated gene transfer into differentiated muscle in vivo is inhibited by the dependence of retroviral transduction on host cell division (Miller, et al., 1990). However, it is possible to get retroviral transduction of the satellite cells which are fusing in response to muscle damage (Dunckley, et al., 1992; Thomason and Booth, 1990). Adenoviral vectors containing truncated forms of dystrophin have also been constructed (Ragot, et al., 1993). The advantages of the adenoviral system are that they can potentially carry large segments of DNA (36 kb genome), they can reach a very high titer \(10^{11} \text{ ml}^{-1}\), they can infect nonreplicating cells, and they are suitable for infecting tissue in situ, such as in the case of CFTR (Rosenfeld, et al., 1992). However, although transfection of the dystrophin-containing adenovirus construct is relatively efficient at producing dystrophin-positive staining fibers, therapeutic levels of dystrophin are not detectable by western blot analysis.
At the current time, a separate approach is being undertaken for the introduction of functional dystrophin into DMD muscle. Myoblast transfer is a cell transplantation procedure which consists of injecting suspensions of viable myoblasts from a healthy donor into the muscle tissue of a DMD patient. Some of the injected myoblasts may ultimately fuse with some of the host muscle fibers and thereby the transferred myoblast become functional myonuclei of certain muscle fibers of the host. This fusion can occur during development or during the process of regeneration. It has been demonstrated that dystrophin can successfully be introduced to muscle fibers in the \textit{mdx} mouse via myoblast injections (Partridge, et al., 1989; Karpati, 1989). Several technical challenges present when attempting to do myoblast transfer, such as the ability to culture large numbers of myoblasts, the need for multiple injections, continuous reinjection, and accessibility of the various affected tissues. Some of these problems are being addressed by using a well matched familial donor and by first testing the effectiveness of myoblast transfer in a limited muscle group. Initial data from four separate groups is inconclusive (Engel, 1993). Only one of the four studies claims that the procedure has therapeutic merit, however, not all of the studies were well designed and none of the studies control for the
occurrence of revertant fibers. Important controls for the effectiveness of the expression of dystrophin are described in previous work from this laboratory (Klein, et al., 1992) and in Chapter V of this thesis.

EXCEPTION PATIENTS

Since the cloning of the gene, several laboratories have analyzed their patient populations for deletions using Southern blot analysis and multiplex PCR. Approximately 65% of patients show a detectable deletion and a correlation was drawn between the type of deletion and the clinical presentation. Monaco et al. (1988) predicted that deletions leading to a translational frameshift could not produce functional dystrophin and would display a severe phenotype (DMD) while those with in-frame deletions would produce a truncated partially functional dystrophin and a mild phenotype (BMD). This observation has been referred to as the reading frame rule. Hundreds of DMD patients in our laboratory and others were analyzed for their adherence to the reading frame rule (Koenig, M., et al., 1989; Den Dunnen, J., et al., 1989; Gillard, E., et al., 1989). In approximately 92% of cases this holds true, but there are several notable exceptions to this rule. These patients are termed exception patients.
TRANSLATIONAL FRAME EXCEPTION PATIENTS

A number of exceptions to the reading frame rule have been reported (Koenig, M., et al., 1989; Den Dunnen, J., 1989; Bambauch, L., et al., 1989; Gilgenkrantz, H., 1989; Malhotra, S., et al., 1988). These patients either have an out-of-frame mutation and produce dystrophin, have an in-frame mutation and do not produce dystrophin, or have an out-of-frame mutation, produce no dystrophin but have a milder phenotype. The deletions in exception patients can occur in any part of the gene and may vary in size from one exon to over 30 exons. The most common type of exception patient deletion is a deletion of exons 3-7. In this work, we present several types of exception patients and show analysis at the DNA, RNA and protein levels. A discussion of related work previously reported in the literature for each type of patient is discussed in Chapters II and III as each set of patients is introduced.

REVERTANT FIBERS

DMD patients are characteristically deficient for dystrophin, however, approximately 50% of patients show clusters of rare positive dystrophin staining fibers, termed revertants (Shimizu, T., et al., 1988; Nicholson, L., 1989, Hoffman, E., et al., 1990; Burrow, K, et al., 1991; Klein, C., et al., 1992). Revertant fibers have also been observed in the mdx mouse. The mdx mouse (Bulfield,
G. et al., 1984) is an animal model for DMD and is dystrophin deficient due to a translational stop codon (Sicinske, P., et al., 1989). The murine fibers stain with a panel of antibodies spanning the dystrophin molecule. These dystrophin-positive cells occur in heart and skeletal muscle.

Several possible mechanisms for the occurrence of revertant fibers have been proposed; artifactual staining, somatic mosaicism, a second site mutation, or a suppressor mutation. The fact that revertant fibers in both the mdx mouse and in DMD patients (see table 1) stain with a panel of antibodies indicate that they are not due to artifactual staining. It is unlikely that a cross-reactive protein would be recognized by every dystrophin antibody. Additional evidence that demonstrates that the fibers are not due to artifactual staining is presented in the work from Klein, et al., 1992 and is discussed below. In addition, the frequency of dystrophin-positive fibers increases with mutagenic doses of x-rays (Hoffman, E., et al., 1990). The idea that an increase in DNA mutations due to irradiation would increase that chance for a secondary reversion mutation is consistent with this observation. (Hoffman, E., et al., 1990). Hoffman et al. suggest that the most likely mechanism is a second site reversion mutation or a tRNA suppressor mutation.
Evidence for the mechanism of revertant fibers in humans came from analysis by Klein et al. (1992) from our laboratory. We observed revertant fibers in deletion and non-deletion DMD patients and in patients with a family history of DMD. Since the DMD mutation in a somatic mosaic must occur after fertilization in the later stages of cell division, somatic mosaicism cannot account for revertant fibers in patients with a family history. C.J. Klein stained several DMD patients who had defined genomic deletions and revertant fibers with a panel of antibodies that span dystrophin (Table 1, Klein et al., 1992). In every case, the revertant fibers were absent for antibody staining in the region corresponding to their genomic deletion, but stained positively with amino- and carboxyl-terminal antibodies. Somatic mosaicism cannot account for the dystrophin in these cases. The most likely explanation is that a secondary deletion or mutation deletes the original mutation and restores the reading frame of dystrophin. Evidence for this mechanism is presented in this work (Chapter IV) from the analysis of a unique non-deleted patient with revertant fibers.

**IMMUNOCYTOCHEMICAL STAINING OF CARRIERS OF DMD MUTATIONS**

Females who have one allele with a mutation in the DMD gene are called carriers. Carriers may or may not show symptomatic features of muscular dystrophy. One indicator
that a female is a carrier is high serum creatine kinase (CK) level (normal range is from 18 to 86). Carriers may develop some muscle weakness or present with cramping. Often electromyographic changes can be seen. However, some carriers remain asymptomatic. These patients are often diagnosed as carriers because of a family history, such as having high CK levels and having an affected brother or by having an affected son (obligate carrier). If the mutation in the family is a known deletion, females can be tested for the mutation by dosage analysis on Southern blots or by quantitative PCR. If the mutation is a point mutation, they can be tested by allele specific oligonucleotide (ASO) analysis.

Immunocytochemical staining of muscle tissue from symptomatic carriers does not show a reduced level of dystrophin staining around all fibers, like in a Becker patient, but rather they show fibers that are either positive or negative for staining (Arahata, K., et al., 1989). The staining pattern is very similar to that seen in DMD patients with revertant fibers, but there are some differences. The percentage of positive and negative fibers in some carriers is approximately equal and are found intermixed next to one another. Only a small percentage, approximately 2-8% show patchy staining. It appears that the dystrophic myoblasts and normal myoblasts
were fused to each other during myogenesis. The positive and negative dystrophin staining pattern appears throughout the biopsy. DMD patients with revertant fibers generally have one cluster of positively staining fibers, whereas the staining in a carrier is more abundant and has a 'checker board' type appearance. However, with increasing age, the 'checker board' like appearance is replaced by a more uniform staining of all fibers, most likely due to retention of dystrophin containing fibers and loss of dystrophin deficient fibers (unpublished data; Karpati, et al., 1991). As presented in Chapter IV, it is possible to have two revertant fiber clusters arising from different secondary mutations. In DMD patients, the revertant fibers appear to arise from only one or two groups of fibers, whereas staining in carriers is more abundant and does not seem to arise from only one myoblast.

CONCLUSION

The focus of this research has been to understand how dystrophin is produced in these translational frame exception patients and the patients with revertant fibers and to understand how the production or absence of dystrophin relates to the patient phenotype. The approach used was to analyze the muscle tissue in these patients at the DNA, RNA, and protein levels.
Table 1

Staining of Revertant Fibers in DMD Patients with a Panel of Dystrophin Antibodies

Summary of the data obtained from staining revertant fibers in DMD patients with a panel of antibodies that span dystrophin. The top line (hatched boxes) indicates the region of dystrophin used to generate the antibodies. Dys-2 was raised against the last 17 amino acids at the carboxyl-terminus of dystrophin and therefore has no exon designation. The line below dystrophin is a diagram of dystrophin, indicating the exon number and domains of dystrophin. Patients are listed vertically on the left, along with the exons that are deleted or duplicated. The carrier status of the mother is indicated to the right. OBL=obligate carrier (defined as either a mother with a high creatine kinase (CK) on three separate assays, the presence of the deletion in the mother by dosage analysis, or direct indication from other affected individuals in the pedigree). Elevated CK levels are indicators of muscle membrane breakdown. N.D. = not determined; N.C.K. = normal CK levels in mother; and D.E.F. = definite carrier (defined as affected boy with a sister with high CK). The deletion in each patient is denoted by a black bar. The duplication in patient 67 is not indicated diagrammatically. The staining status of a revertant fiber or cluster is indicated by a plus (+) sign or minus (-) sign. Ab = antibody staining results. Del. = deletion result. All immunocytochemistry results were obtained on serial sections that were stained from amino- to carboxyl-terminus with the antibody panel.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Mother's Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>1-44</td>
<td>N.C.K.</td>
</tr>
<tr>
<td>24</td>
<td>1-44</td>
<td>OBL</td>
</tr>
<tr>
<td>25</td>
<td>1-44</td>
<td>N.D.</td>
</tr>
<tr>
<td>26</td>
<td>1-44</td>
<td>D.E.F.</td>
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![Diagram of Dystrophin](image)
# Table 1

## Staining of Revertant Fibers in DMD Patients with a Panel of Dystrophin Antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Exon #</th>
<th>9219</th>
<th>1377</th>
<th>9218</th>
<th>DYS 2</th>
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<tbody>
<tr>
<td><strong>Dystrophin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Deleted DMD's</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>81 (12-41)</strong></td>
<td>Del.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>17 (45)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>14 (45-54)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>92 (45-54)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>169 (46-47)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>1 (46-52)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>21 (46-56)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>85.1 (48-54)</strong></td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>85.2 (48-54)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Non-Deleted DMD's</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>71</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>13</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>49.1</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>67 DUP (5-7)</strong></td>
<td>Ab.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
CHAPTER II

Translational Frame Exception Patients:
RNA and Sequence Analysis

INTRODUCTION

The clinical progression of Duchenne muscular dystrophy (DMD) patients with deletions can be predicted in 93% of cases by whether the deletion maintains or disrupts the translational reading frame (frameshift hypothesis). We have identified and studied a number of patients who have deletions that do not conform to the translational frame hypothesis.

These patients were studied at both the DNA and RNA levels. First, their genomic deletions were defined and the maintenance of reading frame was predicted for the resulting mRNA. Deletions in DMD/BMD patients were identified and characterized by multiplex PCR (Chamberlain, et al., 1988) and Southern blot analysis. Muscle tissue was analyzed for dystrophin from 44 DMD patients (24 had deletions), 13 BMD patients (9 had deletions) and 15 intermediates (9 had deletions). The presence or absence of dystrophin was determined by
western blot analysis and/or immunocytochemistry (work done by C.J.Klein, D.D.Coovert and A.V.Winnard). All Duchenne patients, except two (218 and 113) had no detectable dystrophin on western blots with antibodies directed against the carboxyl-terminus. The patients for whom the clinical presentation or production of dystrophin were exceptions to the translational frame hypothesis are described below and their clinical features are listed in table 2. One possible explanation for non-adherence to the frame rule is that aberrant/cryptic splicing changes the frame of the mRNA from that predicted by the genomic deletion. In order to investigate this, RNA was isolated from patient muscle tissue for analysis by reverse transcription (RT)-PCR.

MATERIALS AND METHODS

Patient Population

One hundred and thirty-four DMD, 24 BMD, and 20 intermediate patients participated in this study. The following definitions were used to classify the patients. Patients who were not ambulatory at the age of 12 were classified as Duchenne (severe). Intermediate patients were ambulatory after the age of 12 but not after the age of 16. Becker (mild) patients were ambulatory past the age of 16. All of the exception patients presented here met
the above criteria. In our experience it is difficult to distinguish intermediates from Beckers when the patients are younger than 12. Therefore, milder patients younger than 12 were defined as intermediate and are distinguished from DMD by the clinical course as reported previously (Brooke, M., et al., 1983; Mendell, J., et al., 1987; Mendell, J., et al., 1989). Patients 93,32,1,59 were in a previous prednisone drug trial (Mendell, J., et al., 1989; Burrow, K., et al., 1991). Their clinical classification is given prior to the start of prednisone treatment.

**Deletion/Duplication Analysis**

Genomic DNA was isolated from leukocytes, as previously described (Ray, P., et al., 1985). Genomic DNA isolated from patients was assayed for deletions by multiplex PCR (Chamberlain, J. et al., 1990; Chamberlain, J., et al., 1988; Beggs, A., et al., 1990). The PCR was done under the following conditions: 150ng of each primer, 2.5 units of Amplitaq polymerase (Perkin Elmer/Cetus, Norwalk, CT) in a final volume of 100μl (Chamberlain, J., et al., 1990). The PCR buffer contained 0.5mM of dATP, cCTP, dGTP, dTTP, 3mM of MgCl₂, 67mM of Tris (pH 8.8), 16.6mM (NH₄)₂SO₄, 7.6mM EDTA and 10mM of 2-β-mercaptoethanol. The amplification was performed using standard conditions by denaturing at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extending at 72°C for
3 minutes for 35 cycles in a thermal cycler (Ericomp, San Diego, CA).

In conjunction with PCR analysis, Southern blots were also used to assay for deletions or duplications. The various cDNA clones used have been reported previously (Monaco, A., et al., 1986; Burghes, A., et al., 1987; Koenig, M., et al., 1987). Patient DNA samples were digested with Hind III, Bgl II or EcoRI according to manufacturer's instruction. The digested DNA sample was run on a 0.8% w/v agarose gel using standard procedures (Sambrook, J., et al., 1989). The DNA was transferred to Hybond N+ (Amersham) using a standard Southern blot protocol, except that 2X SSC was used instead of 10X SSC for the transfer. The cDNA probes were labelled by the random primer method (Feinberg, A. and Vogelstein B., 1983). Hybridization conditions were as described previously (Ray, P., et al., 1985). In all identified exceptions, RT-PCR analysis of mRNA was used to confirm the deletion boundaries.

**PCR mRNA Analysis**

Approximately 1 μg-30 mg of muscle tissue from patients was homogenized in guanidine buffer (4M guanidine isothiocyanate 25mM sodium citrate, pH 7; 0.5% w/v Sarkosyl, 0.1% v/v 2-β-mercaptoethanol). When total RNA yields were expected to be very low due to limited
availability of tissue, 0.1 μg tRNA (Pharmacia, Piscataway, NJ.) per ml of guanidine buffer was added to act as a carrier during isolation. Approximately 1 μg of glycogen (Bethesda Research Labs Bethesda, MD.) was used as a carrier in the final precipitation. The homogenized tissue was loaded onto a 5.7 M CsCl cushion and centrifuged in a Beckman table top untracentrifuge at 55K rpm, 15°C for 3 hours (Chirgwin, J., et al., 1979). The RNA pellet was recovered and total RNA was reverse transcribed into cDNA using 5-10 units of AMV-reverse transcriptase (United States Biochemical, Cleveland, Ohio), 10 units of RNAsin, 50 mM Tris-HCL, pH 8.5 (pH 8.3 at 42°C) for 2-12 hours. Approximately 1/25 volume of the cDNA reaction was used as the template for PCR amplification in 50mM KCl, 10 mM (pH 8.3), 2.5 mM MgCl₂, 0.01% v/v Triton X-100, 0.1% v/v Tween 20, 0.1% w/v gelatin, 0.2mM dNTPs, 0.125 nmoles of each primer and 1 unit of Amplitaq (Perkin Elmer Cetus, Norwalk, CT.).

The primers used in each case, their positions, and the resulting fragment size are indicated in table 3. The reactions were initially denatured at 94°C for 3 min., followed by 40 to 45 cycles of 94°C, 30s; annealing at 45°C to 58°C, 30s; extension at 72°C, 3 min., 30s to 4 min., 30s. A final extension at 72°C was performed for 7
minutes. Approximately 1/10 to 1/30 of each PCR reaction was analyzed by agarose gel electrophoresis.

For sequencing, the PCR products were either subcloned into a T-tailed vector (InVitrogen, SanDiego, CA.) or sequenced directly. In all cases the DNA was purified using a Magic Mini Prep Column (Promega, Madison, WI). The subclones or PCR products were sequenced using a cycle sequencing kit according to the manufacturer's instructions (Promega, Madison, WI).

Western Blot Analysis of Proteins

The western blot procedure was essentially the same as that described previously (Burrow, K., et al., 1991). One hundred micrograms of muscle protein was loaded onto a 6% SDS polyacrylamide gel and then the proteins were transferred to nitrocellulose. One section of the nitrocellulose was stained with colloidal gold to ensure equal levels of myosin were present between patients. The other half was immunostained with the antibody 9219 (Bulman, D., et al., 1991) or Dys2 (NovaCastra Labs, Newcastle-upon-Tyne, UK). The antibodies were used at 1:3000 and 1:200 dilutions, respectively and the blots were developed with donkey anti-sheep conjugated to alkaline phosphatase or rabbit anti-mouse conjugated to alkaline phosphatase (Jackson Immunoresearch Labs, West Grove, PA).
Quantitative western analysis was performed essentially as described previously (Bulman, D., et al., 1991). All samples were run on a mini polyacrylamide gel and myosin levels were quantitated. The levels of dystrophin were determined by scanning the western blots on a Shimizu densitometer. The levels reported were normalized to myosin levels. In addition, western blots were stained with an antibody directed against nebulin (Sigma, St. Louis, MO). The dystrophin levels were determined relative to myosin to maintain consistency with the literature.

RESULTS
In-frame DMD and Becker Patients

One patient, (32), had an in-frame deletion of exons 4-18 at the DNA and RNA levels that removed the majority of the actin-binding domain (Figure 1D). There was no evidence for dystrophin staining on western blots (Figure 1B) or by immunocytochemistry (data not shown) with amino and carboxyl-terminal antibodies.

Two patients with large in-frame deletions of greater than 30 exons were identified. Patient 113, a definite DMD (table 2), had an out-of-frame deletion of exons 4-41 at the DNA level but an exon 3-41 in-frame deletion at the RNA level (figure 1D, table 3). This patient, although a
DMD, produced truncated dystrophin of approximately 200kD (predicted size 202 kD) (Figure 1A). The second patient, 302, was a BMD patient (table 2) who had an in-frame deletion of exons 13-48 at the DNA and RNA levels (figure 1C). This BMD patient produced a truncated protein of approximately 200 kd (predicted size 209 kd) (figure 1A). Quantitative western analysis of patients 113 and 302 showed that the dystrophin levels were approximately the same despite their different clinical phenotypes (table 3).

Muscle tissue from DMD patient 113 and Becker patient 302 were immunostained with a panel of antibodies spanning the dystrophin protein. In DMD patient 113, the antibodies 9219 and 1377 did not stain because their epitopes were deleted. Antibodies 9218 (against the rod domain) and Dys 2 (against the carboxyl-terminus) immunostained patient 113 and showed normal localization of this internally deleted dystrophin (figure 2). The BMD patient 302 showed immunostaining with the antibodies 9219, 9218, and the carboxyl-terminal antibody Dys2, but not with 1377.

Intermediate and Becker Patients

The dystrophin produced in BMD patients immunostained with antibodies directed against the amino- and carboxyl-terminus.
Nine intermediate patients had deletions which overlapped with those seen in Duchenne patients. All intermediate patients having out-of-frame deletions had no detectable level of dystrophin (table 2). One interesting patient (77) had an out-of-frame deletion of exon 45. This deletion was confirmed at the RNA level and was shown to have only 1 detectable message that was out-of-frame (figure 3C). We detected no in-frame mRNA due to aberrant splicing. These results are consistent with western blot data and immunocytochemistry and patient 77 which showed absence of dystrophin (table 1, figure 3A and 3B). Immunocytochemistry with the panel of antibodies 9219, 1377, 9218 (data not shown) and Dys 2 (figure 3A) also showed absence of dystrophin. Another intermediate patient (93) with a typical Becker in-frame deletion stained positively for dystrophin (table 2). Clinically, patient 77 is the mildest intermediate and he is still running at the age of 12. He is milder than patient 93 who produces dystrophin.

CONCLUSIONS

We identified two DMD patients with an in-frame mutation at the RNA level. Patient 32 produced no detectable dystrophin and was deleted for exons 4-18. A similar patient was reported by Chelly, et al, 1990. These
authors suggest that this deletion disrupts the ability of dystrophin to dimerize. A similar phenomenon has been reported for β-spectrin where a point mutation is thought to disrupt conformation and prevent αβ-dimers associating to form a tetramer (Tse, et al., 1990).

The second DMD patient, 113, had a deletion of exons 4-41 at the DNA level and 3-41 at the mRNA level. This patient produced dystrophin at a level comparable to Becker patients and stained positively with antibodies directed at the carboxyl-terminal. We have subsequently identified a second in-frame DMD patient with a 3-42 DNA deletion that immunostained with the carboxyl-terminal antibody (unpublished data). In addition, other DMD patients with similar deletions, exons 3-25 (Nicholson, in press; Vainzof, et al., 1993) and exons 3-31 (Vainzof, et al., 1993) show similar immunostaining patterns. Therefore, it is clear that some DMD patients with large in-frame deletions do produce dystrophin.

We identified one Becker patient (302) with a deletion of exons 13-48. This patient is still walking at age 24 and has a clinically mild course. This patient, however, is more severely affected than the Becker patient with a deletion of exons 17-48 reported by England, et al., (1990). To our knowledge, patient 302 has the largest deletion reported for a Becker patient. This patient
produces dystrophin at approximately the same level as the Duchenne patient 113. One notable difference between these two patients is the maintenance of the actin-binding domain (Hammonds, 1987) in the BMD case but not the DMD case. Therefore, if dystrophin is involved in stabilizing the membrane, as has been suggested (Zubrzycka-Gaarn, et al., 1988; Karpati and Carpenter, 1986), the loss of attachment to other cytoskeletal components through the actin binding domain could be detrimental. Alternatively, the conformation of dystrophin in DMD patient 113 may be such that although it is localized normally, it does not bind one (or more) of the dystrophin-associated proteins correctly and therefore, results in a severe phenotype. The significance of exceptional patients like Becker patient 302 and DMD patient 113 is that truncated dystrophin constructs based on the deletions in these patients can be made. Since one of these patients has a mild phenotype and both produce and localize dystrophin, truncated constructs based on them should produce partially functional dystrophin. This is significant because currently available vectors cannot accommodate full length dystrophin. These constructs can also be used to see if the reason for the variation of phenotype in these two is due to the binding affinity of the different truncated forms of dystrophin.
Deletion of exon 45, an out-of-frame deletion, has been associated with variable clinical phenotypes, ranging from DMD to BMD (Gillard, et al., 1989; Bambauch, et al., 1989; Hodgson, et al., 1989; Love, et al., 1990). We identified two intermediate patients, 77 and 110, with a deletion of exon 45, and in both cases, dystrophin was below detectable limits, except for revertant fibers commonly seen in DMD muscle (Klein, et al., 1992). Patient 77 has the mildest clinical phenotype of the intermediates studied and he is still running at age 12. Analysis of mRNA from this patient muscle showed the presence of one mRNA that was deleted for exon 45. Studies of mRNA isolated from peripheral blood leukocytes show that patients deleted for exon 45 can splice out exon 44 (Roberts, et al., 1991) which results in correction of the translational reading frame. However, all four of the patients analyzed by Roberts, et al., (1991), possessed this in-frame transcript in muscle, but all were severe. It is possible that these severe patients do not produce this in-frame transcript in muscle, whereas mild patients do. However, the patient we describe is mild and shows one mRNA species deleted for exon 45 and produces no detectable dystrophin in the skeletal muscle tested. The reason for the mild phenotype in this patient is not clear. The persistence of the dystrophin-associated
proteins at higher levels that normally found in DMD patients may result in a milder phenotype if these proteins are crucial in the phenotypic presentation of Duchenne (Campbell and Kahl, 1989; Ervasti, et al., 1990; Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya, et al., 1992). However, preliminary staining with antibodies directed against dystrophin-associated proteins in this patient indicates that they are not present at higher levels (unpublished data). Another possibility is that alternative splicing of dystrophin could occur in other muscle groups such as heart, diaphragm and smooth muscle and could potentially result in a mild phenotype.

This study shows that Duchenne and Becker patients can show variability in their clinical presentation which cannot be solely explained by the level of dystrophin or the presence of the carboxyl-terminus. Some of the variability is probably due to the domains retained and the conformation of the protein which affect the function of dystrophin, perhaps by inhibiting the ability to dimerize. In the future, it will be important to study these exceptional patients to determine whether variation in clinical severity relates to alterations in dystrophin associated proteins and/or disruptions of specific domains of dystrophin. A discussion of their relation to possible functional domains in dystrophin is discussed in Chapter V.
Figure 1

In-Frame DMD Patients

(IA) Western blot analysis of large in-frame deletion patients. Lanes 1 and 5 show normal muscle protein. Lane 2 shows patient 302. Lane 3 shows patient 113. Lane 4 shows a DMD patient. The top arrow indicates the normal position of dystrophin and the bottom arrow indicates the position of the truncated forms of dystrophin found in patients 113 and 302. The western blot was stained with the antibody Dys2.

(IB) Western blot analysis of an in-frame DMD patient. Lanes 1 and 4 show a DMD patient. Lane 2 patient 32 (expected molecular weight is 346 kD); Lane 3, patient 77 (expected molecular weight is 420 kD); Lane 5, a normal muscle. The arrow indicates the position of dystrophin.

(IC) PCR amplification of reverse transcribed RNA from muscle from patient 302. Lane 1 shows the λ-HindIII marker with the 2.3 and 2.0 kb shown. Lane 2 shows the PCR product obtained from amplification of the junction fragment from muscle cDNA isolated from patient 302. The primers and PCR conditions are listed in materials and methods.

(ID) PCR amplification of reverse transcribed RNA from muscle from patients 113 and 32. Lane 1 shows the φX-174, Hae III marker. The fragments are the following size from top to bottom: 1,350, 1,078, 872, 603, 310, (281, 271), 234, and 194 basepairs. Lane 2 shows the PCR product obtained from amplification of muscle cDNA from patient 113. Lane 3 shows the PCR product obtained from amplification of muscle cDNA from patient 32. The details of primers and PCR conditions used are given in materials and methods.
Figure 1
Figure 2

Immunocytochemical Analysis of Large In-frame Deletion Patients Producing Dystrophin.

A panel of antibodies which span dystrophin was used to stain muscle from patients 113 and 302. The region of dystrophin the antibodies were directed against is shown at the top of the figure. The staining obtained for each antibody is shown below the diagram of dystrophin. The antibodies used are 9219, 1377, 9218, and Dys2, shown from left to right.
Figure 2
Figure 3

Intermediate Patient with a Mild Phenotype and No Dystrophin.

(3A) Immunocytochemistry of patient 77 muscle using the antibody Dys2. One revertant fiber is shown to emphasize the dystrophin-negative background. Similar results were obtained with the antibody 9219.

(3B) Western blot analysis of normal (N), and intermediate patient 67 (INT), patient 77, and a BMD patient (BMD) was performed with antibody 9219 (amino-terminus). The position of dystrophin is indicated by an arrow.

(3C) PCR amplification of reverse transcribed mRNA isolated from muscle from patient 77. Lane 1 shows the DNA marker \( \Phi X \)-174. The fragments are the following sizes from top to bottom: 1,350, 1,078, 872, 603, 310, (281, 271) 234, and 194 bp. Lanes 2, 3, 4 and 5 show PCR products obtained from amplification of muscle cDNA from a normal individual and DMD patient with primers that do not flank exon 45. Lane 6 shows the PCR products obtained from amplification of muscle cDNA from a normal individual with primers that flank exon 45. Lane 7 shows the PCR product obtained from amplification of muscle cDNA isolated from patient 77 with primers that flank exon 45. The conditions for PCR and the primers used are given in materials and methods.
Figure 3
Table 2

Patient Phenotype Data on Intermediate and Exceptional Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Diagnosis</th>
<th>Age</th>
<th>Deletion Exon(s)</th>
<th>Frame Shift</th>
<th>Dysrophin Analysis</th>
<th>Amino Carboxyl</th>
<th>Age 8 L.F.</th>
<th>Neck</th>
<th>F.V.C.</th>
<th>Age 12 L.F.</th>
<th>Neck</th>
<th>F.V.C.</th>
<th>L.F. or Age at Loss of Ambulation</th>
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<td>DMD</td>
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<td>4–41</td>
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<td>+</td>
<td>1</td>
<td>3</td>
<td>73%</td>
<td>9</td>
<td>3</td>
<td>92%</td>
<td>12</td>
</tr>
<tr>
<td>32</td>
<td>DMD</td>
<td>9</td>
<td>4–18</td>
<td>no</td>
<td>–</td>
<td>–</td>
<td>2</td>
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* L.F. = Leg Function
Table 3

PCR Amplification Results and Dystrophin Quantitation

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<th>Patient #</th>
<th>Amplified between 5'bp/3'bp</th>
<th>Primers sense/antisense</th>
<th>Junction fragment size</th>
<th>Deleted exons at mRNA</th>
<th>Junction sequence 5'bp/3'bp</th>
<th>Dystrophin level: % of normala</th>
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<tr>
<td>300</td>
<td>1/2077</td>
<td>33/14</td>
<td>1518</td>
<td>3-7</td>
<td>AAG/ATG 301/837</td>
<td>18%</td>
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<td>63</td>
<td>1/2077</td>
<td>33/14</td>
<td>1518</td>
<td>3-7</td>
<td>AAG/ATG 301/837</td>
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<td>218</td>
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<td>3-7</td>
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<td>32</td>
<td>236/2940</td>
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<td>598</td>
<td>4-18</td>
<td>CTG/GCC 394/2301</td>
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<td>656</td>
<td>45</td>
<td>AAG/GCT 646/6823</td>
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<td>13/10</td>
<td>1947</td>
<td>13-48</td>
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* The error in measurement is ± 4%
Table 4
PCR Primers

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<td>Sense</td>
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<tr>
<td>2</td>
<td>Anti-sense</td>
<td>5'TTG TAG CCT CTT TTT TCT GTC TGA3'</td>
</tr>
<tr>
<td>4</td>
<td>Anti-sense</td>
<td>5'TCC TCC AGT TTC ATT TAA TGG TGG GAG3'</td>
</tr>
<tr>
<td>10</td>
<td>Anti-sense</td>
<td>5'TCT CAG GAT TIT TGG GCT GGT TCC ATG3'</td>
</tr>
<tr>
<td>11</td>
<td>Sense</td>
<td>5'TGT TAT GAA AGA GAA GAT GTT CAA AAG AAA3'</td>
</tr>
<tr>
<td>13</td>
<td>Sense</td>
<td>5'AGC ATT GAA GCC ATC CAG GAA ATG3'</td>
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<tr>
<td>14</td>
<td>Anti-sense</td>
<td>5'GGA GTG AAT ACA GGT TCC CCA TGG3'</td>
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<tr>
<td>16</td>
<td>Anti-sense</td>
<td>5'GAAA GTC TGC ATG CAG GAA CAT GGG3'</td>
</tr>
<tr>
<td>33</td>
<td>Sense</td>
<td>5'GGG ATT CCC TCA CTT TCC CCC TAC3'</td>
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CHAPTER III

Exon 3 to 7 Deletion Patients:

DNA, RNA, Sequence and Protein Analysis

INTRODUCTION

The most common type of translational frame exception patient has a deletion of exons 3 to 7. This deletion is out-of-frame and would not be predicted to produce dystrophin. However, western blot analysis and immunocytochemical analysis of muscle tissue using antibodies directed against the carboxyl-terminus of dystrophin (antibody Dys2) and an antibody recognizing exons 4 to 16 (antibody 9219) indicate that these patients produce dystrophin at about 10-15% of the normal level (Chelly, et al., 1990; Gangopadhyay, et al., 1992; Winnard, et al., 1993). Chelly, et al. (1990) performed RT-PCR analysis on two exon 3-7 deletion patients. They reported that the major mRNA species observed was one which contained an exon 3-7 deletion, but that there were two additional in-frame messages: one containing a deletion of exons 3-9 and one with a deletion of exons 2-7. However, these in-frame messages were present at only 1-2% of the
level of the out-of-frame mRNA and could be found in normal muscle samples. Chelly, et al., (1990) suggests that these minor in-frame transcripts are elevated in the BMD cases but not in DMD patients with this deletion, thus accounting for the phenotypic variation. However, as described later in this chapter, we have not observed these transcripts in normal samples or in DMD, BMD or intermediate patients deleted for exons 3-7. Likewise, Gangopadhyay, et al., (1992), have also not observed these in-frame transcripts in the exon 3-7 deletion patients they studied. In addition, patients with deletions that disrupt translational frame that are internal to exons 3 to 7 do not show a wide range of phenotypes and do not produce dystrophin with an intact carboxyl-terminus (Bulman, et al., 1991). Malhotra, et al., (1988), suggested other mechanisms that do not involve splicing which might account for the dystrophin production: use of a cryptic promoter, ribosomal frameshifting, and reinitiation of translation. Malhotra, et al., 1988, analyzed the deletion breakpoints in exon 7 and suggested that a new mRNA synthesized from a cryptic promoter in intron 7 seems highly unlikely because of the scattered distribution of deletion endpoints in the intron. However, they could not rule out the possibility of a cryptic promoter in exon 2 being used. The possibility of new initiation or
reinitiation was proposed because of the presence of an ATG that is embedded in a Kozak's consensus sequence that can be found in exon 8 (Malhotra, et al., 1988). This proposed ATG start site in exon 8 is only 68 base pairs downstream of the first stop codon and 26 base pairs downstream of the second stop codon produced by the exon 2/exon 8 junction.

Arahata, et al., 1991, used an antibody directed against amino acids 215-264 to immunostain the muscle of two BMD patients deleted for exons 3-7. The antibody did not stain the patients' muscle. Antibody staining with the single antibody reported cannot distinguish between any of the proposed mechanisms. Data presented in this chapter will present a strategy for distinguishing between all the proposed mechanisms and the results provide an explanation for dystrophin production in exon 3-7 deletion patients.

In order to distinguish between all the proposed mechanisms, a series of antibodies directed against the amino-terminus of dystrophin was produced. Figure 10 is a schematic of the epitopes recognized by these antibodies and the proposed mechanisms that would be indicated by the possible combinations of staining patterns. These antibodies were used both on immunocytochemistry and for western blot analysis.
We analyzed nine exon 3-7 deletion patients in this study, ranging in phenotype from DMD to BMD. Table 5 lists the patients, their phenotypes, the genomic deletion and the deletion present in the mRNA species detected.

MATERIALS AND METHODS

DNA Analysis

Genomic DNA was isolated from leukocytes as previously described (Ray, et al., 1985). Briefly, whole blood was collected in 15 ml vacutainers containing EDTA as an anti-coagulant. The tubes were centrifuged at 2500rpm for 15 min. and the white buffy coat was collected. The cells were washed 3 times in wash buffer (0.114 M NH₄Cl, 0.01 M NaHCO₃), pelleted, and resuspended. The final suspension was in 12 mls TNE (0.1M Tris, pH 8, 1mM EDTA, 0.2M NaCl), 1% SDS and 1.5 mg proteinase K and incubated at 65 °C overnight. This solution was extracted 3 times with phenol:chloroform (50%v/v). The DNA was precipitated with approximately 5 volumes of 95% ethanol and resuspended in TE (10 mM EDTA, pH 8, 1mM EDTA).

Genomic blots were done according to standard procedures (Sambrook, et al., 1989). Briefly, 5-8μg of genomic DNA was digested with Hind III or EcoRI according to manufacturer's instructions. The digested DNA samples were run on a 0.8% w/v agarose gel using standard
procedures (Sambrook, et al., 1989). The DNA was transferred to Hybond N+ (Amersham, Arlington, IL) using standard Southern blot protocol, except that 2X SSC was used instead of 10X SSC for transfer. The cDNA probes were labelled by the random prime method (Sambrook, et al, 1989). Hybridization conditions were as described previously (Ray, et al., 1985). Southern blotting of PCR products were done by separation on a 1-2% agarose followed by transfer to Hybond N+ using the same procedure as for the genomic transfer except that the acid nicking step with 0.125M HCL was omitted.

RNA Isolation

RNA isolation was done according the method of Chirgwin, et al,(1979), with certain modifications. Approximately 1μg-30mg of muscle tissue from patients was homogenized in guanidine buffer (4M guanidine isothiocyanate, 25mM Na Citrate, pH 7, 0.5% w/v Sarkosyl, 0.1% v/v 2-β-mercaptoethanol). When total RNA yields were expected to be very low due to limited availability of tissue, 0.1 μg tRNA (Pharmacia, Piscataway, NJ) per ml of guanidine buffer was added to act as a carrier during isolation. Approximately 1 μg of glycogen (Bethesda Research Labs, Bethesda, MD) was used as a carrier in the final precipitation. The homogenized tissue (in approximately 1.5 ml buffer) was loaded onto a 0.5 ml,
5.7M CsCl cushion and centrifuged in a Beckman table top ultracentrifuge at 55K rpm, 15°C for 3 hours. The RNA pellet was recovered and total RNA was reverse transcribed into cDNA using 5-10 units of AMV-reverse transcriptase (United States Biochemical, Cleveland, OH), 10 units of RNAsin (United States Biochemical, Cleveland, OH), 50mM Tris-HCL, pH 8.5 (pH 8.3 at 42°C), 40 mM KCl, 80 mM MgCl₂, 0.8 mM dNTPs, 500ng of pdN₆ and /or oligo dT (12-18) (Pharmacia, Piscataway, New Jersey) at 42°C for 2-12 hours.

PCR

Approximately 1/25 volume of the cDNA reaction was used as the template for PCR amplification in 50 mM KCL, 10mM Tris (pH 8.3), 2.5 mM MgCl₂, 0.01 % w/v gelatin, 0.2mM dNTPs, 0.125 nmoles of each primer and 1 unit Amplitaq (Perkin Elmer/Cetus, Norwalk, CT).

The primers used in each case and their positions are indicated in table 3. The primer sequences and orientation are listed in table 4. The reactions were initially denatured at 94°C for 3 min., followed by 40 to 45 cycles of 94°C, 30s; annealing at 45°C to 60°C, 30s; extension at 72°C, 3 min., 30s to 4 min., 30s. A final extension at 72°C was performed for 7 min. Approximately 1/10 to 1/30 of each PCR reaction was analyzed by agarose gel electrophoresis. When necessary, nested PCR reactions or
reamplifications were done using the same cycling conditions, the appropriate primers and approximately 1/100 of the original PCR reaction.

Multiplex PCR of genomic DNA for determination of genomic deletions was done using the primer sets and conditions described by Chamberlain, et al., (1988) (analysis done by Clinical Genetics Laboratory, OSU).

**Sequencing**

For sequencing, the PCR products were either subcloned into a T-tailed vector (In Vitrogen, San Diego, CA) or sequenced directly. When necessary, such as in the case of patient #OSU245 who produced three products with the same set of primers, the PCR were first separated by low melt gel electrophoresis and then band isolated. In all cases, the DNA was purified using a Magic Mini Prep column (Promega, Madison, WI). The subclones or PCR products were sequenced using a cycle sequencing kit according to the manufacturer’s instructions (Promega, Madison, WI).

**Antibody generation**

Antibodies to dystrophin were generated using synthetic peptides. The peptides were synthesized at the Peptide and Protein Engineering Laboratory at Ohio State University. The sequences of each of the peptides and the region they correspond to in dystrophin are listed in
Each of the peptides were synthesized with an additional 22 amino acids which correspond to a measles virus epitope. This sequence was added to enhance the antigenicity of the peptides (Partidos, CD and Steward, MW, 1990).

The procedures used for antibody injections, titering and isolation were based on procedures in Antibodies, A Laboratory Manual (Harlow and Lane, 1988). Specifically, 2 mg of dry peptide was dissolved in approximately 400 µl of 10% glacial acetic acid. As much 50% NH₄OH as possible (approximately 10-50 µl) was added to raise the pH while maintaining the peptide in solution. Solubility was determined by visual inspection. The pH was checked by "dotting" the solution on pH strips (VWR Scientific, Philadelphia, PA). If possible, the pH was raised to >pH5. One volume (500 µl) of 1X PBS was added. A 1:1 mixture was made with Complete Freunds Adjuvant (Sigma, St. Louis, MO). The mixture was passed through glass luer lock syringes on ice until both fractions were in solution together and formed a thick white solution that was able to float on water. As soon as possible, this solution was subcutaneously injected into rabbits, approximately 100 µl per injection. Pre-immune serum was collected before antibody injection by collecting 10-45 mls of blood from the rabbit ear using a small scalpel nick. The blood was
allowed to clot overnight and then centrifuged at 1000rpm for 20 min. The serum was collected using a pipette, aliquoted in 1ml fractions, and stored at -20°C. Subsequent 'boosts' were done by dissolving 1 mg of dry peptide in 250 µl 10% acetic acid and the pH raised as described above. One volume of 1X PBS was added and the solution was subcutaneously injected into the rabbits every one to two weeks. Serum was collected, as described above for collecting pre-immune serum, approximately three to four days after a boost and tested for titer and/or suitability for immunocytochemistry and/or western blot analysis. Final serum collections from a rabbit were done using cardiac puncture. Approximately, 120 ml of blood were collected and the serum isolated and stored as described above.

**Titering of Antibodies**

The ELISA protocol was performed in order to test for initial antibody reactivity and to estimate that the antibody titers were high enough to test by immunocytochemistry and western blot analysis. Immulon 2 ninety six well microtiter plates (Dynatec Laboratories, Chantilly, VA) were coated with 100 µl/well of 2 µg/ml antigen. The antigens used were the peptides the antibodies were raised against and fusion proteins containing cloned sections of the dystrophin gene
(isolation of fusion proteins is described below). The coated plates were incubated at 4°C, overnight. The plates were washed 2X with PBT/1% horse serum (1X PBS, 0.05% Tween-20, 1% horse serum). The wells were blocked with 200μl/well 1X PBS/1% BSA and incubated for 2 hours at room temperature. The wells were then washed 5X with PBT/1% horse serum. 200μl of serum diluted in PBT/1% horse serum at dilutions of 10⁻² to 10⁻⁶ were added to the wells. Control wells contained no primary antibody, no secondary, or no antigen. The plates were incubated at room temperature for two hours. The plates were washed 5 times in PBT/1% horse serum. 100 μl of a 1/1000 dilution of goat anti-rabbit conjugated to horseradish peroxidase, EIA grade (Jackson ImmunoResearch Laboratories, West Grove, PA) was added to each well and incubated for one hour followed by 5 washings. The substrate was developed by adding 100μl of 0.024 M citric acid, 0.05 M Sodium phosphate, 0.4mg/ml O-phenylenediamine and 20μl/50mls of 30% H₂O₂ (added fresh). After addition of the developer, the plates were incubated for exactly 10 min. in the dark. 100 μl of stop mix (2.5M H₂SO₄) was added. The absorbance at 490nm was read on a Minireader II (Dynatech Laboratories, Alexandria, VA) and the baseline set zeroed to the reading of the well with no antigen. Positive signals were those absorbances that read above the blank.
Serum that showed reactivity at dilutions greater than $10^{-4}$ were considered ready to test by immunocytochemistry or western blot analysis.

**Isolation of Fusion Proteins**

Fusion proteins containing various portions of the dystrophin gene were isolated from available vectors. The description of the following fusion protein constructs are in Bulman, et al. (1991), Zubrzycka-Gaarn, et al. (1988), and Klein, et al., 1992: Path 11-63 (a subclone of the 2.0 kb cDNA clone, Burghes, et al. (1987), placed into Path 11 at the Bam HI site of dystrophin) and Path C8 (6900bp-7800bp). The fusion proteins were isolated according to previously described protocols, Ausubel, et al, 1992; Harlow and Lane, 1988. Briefly, colony streaks were grown overnight at 37°C on LB/ampicillin plates. Single colonies were then grown in 25 mls of M9 media (1mM MgSO$_4$, 0.1mM CaCl$_2$, 0.2% glucose, 0.01 mg/ml thiamine, 0.5% casamino acids) and 0.02mg/ml tryptophan and 100 µg/ml ampicillin overnight at 37°C. This culture is added to 400 ml M9 media (no tryptophan) and grown until OD 600 = 0.5 (approximately 4 hrs.). Protein production is induced by adding 1.6 mls of 2.5 mg/ml (in 95% ethanol) indolacrylic acid (IAA). The culture is grown for 3-6 hours. The cells were then pelleted at 7000g for 15 min. and resuspended in 2-5 mls of Laemmli buffer (0.125 M Tris, pH 6.8, 5% SDS,
100mM DTT). The sample was then briefly sonicated to shear the DNA. The proteins are then boiled for approximately 5 min. and centrifuged to separate out the insoluble fraction. The supernatant was collected and fractions run on a 10% SDS polyacrylamide gel. First, a mini gel was run to estimate the concentration. The fusion proteins were then run on individual single-well trough gel (10% SDS polyacrylamide gel). After the run, the gel was placed into 0.25 M KCl in order to visualize the bands. The appropriate band was cut out and placed in a dialysis bag with buffer (0.2M Tris-acetate, pH 7.4, 1% SDS, 10mM DTT) and then electroeluted in a gel tank containing 50mM Tris-acetate, pH 7.4, 0.1% SDS at 4°C, 100V, for 3 hours. The proteins in the elution buffer were then placed in a fresh dialysis bag and concentrated by placing the bag in a dish filled with dry polyethylene glycol (PEG) for approximately 30 min. until the final volume was approximately 2 mls. A fraction of the purified proteins were then run on a 10% SDS polyacrylamide minigel to estimate the concentration. These purified proteins were used in the Elisa assays, as antibody controls for western analysis, and for antibody purification on nitrocellulose.

**Antibody Purification**

Dystrophin antibodies were purified from rabbit serum by two different methods. The first was adapted from
Pringle, et al., 1991. Specifically, a saturating concentration of fusion protein (approximately 10 mg) was layered on top of an 1-2 inch square piece of nitrocellulose and incubated overnight at 4°C. The filter was briefly dried (5 min.) in an oven. The filter is then blocked with 5% nonfat dry milk in 1X PBS for 50 min. at room temperature. The filter was washed three times in 1X PBS. The filter was secured with Parafilm to the bottom of a petri dish and 300-400 μl of rabbit serum containing antibodies that recognize that fusion protein was loaded onto the filter and incubated for 2-4 hours at room temperature. The filter was then washed 3 times in 1X PBS. The antibodies are eluted by layering 200 μl of elution buffer (0.2M glycine, 1mM EGTA, pH 2.3-2.7) and incubating for 10 min. The solution was then neutralized by adding 100 μl of 100 mM Tris-base. The pH was checked by "dotting" the solution on pH strips. To reduce antigenic activity due to the measles specific sequence, the purified antibody was layered onto a 1" square section of nitrocellose that was saturated with a peptide that contained the measles sequence, but not the peptide this antibody was raised against. The peptide filters were prepared the same as the fusion protein filters. The purified antibody was layered on this peptide filter and incubated for 1-2 hours. The fraction that did not bind to
the filter was collected and used as the purified antibody. The fraction bound to the peptide filter was assumed to consist of antibodies reactive to the measles specific sequence. In addition, some fusion purified antibodies and non-purified serum were incubated with filters saturated with homogenized total \( mdx \) mouse muscle bound to a nitocellulose filter in the same manner as the fusion proteins described above. \( Mdx \) mouse does not contain dystrophin and therefore the antibodies should not bind to the filter. The unbound fraction was the one considered to contain purified or enriched dystrophin antibodies and the bound fraction was eluted from the filter and discarded and assumed to have antibodies that crossreacted with other muscle proteins besides dystrophin.

**Immunocytochemistry**

Antibodies 9219, 1377 and 9218 were kindly provided by D. Bulman. The description of the development of these antibodies is described in Klein, et al., (1992). Antibody Dys2 is commercially available from Nova-Castra Labs, Newcastle-upon-Tyne, UK. Antibody 1, antibody 2, and antibody 3 were generated as described above. For initial patient analysis, antibody NT-Dys (which recognizes the same epitope as antibody 1 and was also generated in rabbit) was a gift from K. Campbell until antibody 1
reached a sufficient titer. NT-Dys antibody was used for immunocytochemistry, but was unable to be used for western analysis. Only antibody 1 was used for western analysis.

Immunostaining was carried out on 10μm cross sections of skeletal muscle from open-limb biopsies. Immediately after the biopsy, tissue was mounted on wooden chucks in gum tragacanth, and the muscle tissue was frozen in isopentane that was cooled in liquid nitrogen and stored at -70°C. Some samples were provided from other medical centers and were mounted according to this protocol. Fresh serial sections were placed on Super Frost Glass Plus slides (Fisher Scientific, Pittsburgh, PA), fixed in cold acetone for 7 min., air dried for 30 min., and then rehydrated in 1X PBS for 5 min. The sections were then incubated in a moist chamber with the primary antibodies for 1 hour at room temperature. Purified antibodies were diluted as follows: Ab2 (1:1), Ab 3 (1:1), NT-Dys (no dilution), Dys2 (1:3). The sections were then washed 3 times with 1X PBS and incubated with the secondary antibody for 1 hour at room temperature. The secondary antibody was biotinylated goat-anti-rabbit or anti-mouse (Jackson ImmunoResearch Labs, West Grove, PA) used at a dilution of 1:200. The sections were then washed three times in 1X PBS and incubated for 1 hour at room temperature with the tertiary antibody. The sections were
then incubated with ExtrAvidin-fluorescein-conjugated avidin (Sigma, St. Louis, MO) at a dilution of 1:50. The sections were then washed 3 times with 1X PBS and coverslipped with 90% glycerol, 10% PBS, pH 9.0. Immunostaining was visualized by fluorescence microscopy and photographed using Kodak Tmax 400 black and white film.

**Western blot analysis**

The western blot procedure was essentially the same as that described in detail previously (Burrow, K., et al., 1991). Skeletal muscle from patients was homogenized in blending buffer (0.5 M Tris, pH 6.8, 1% SDS, and 0.5 mM EDTA and 0.5 mM PMSF, added fresh). Samples were then boiled and centrifuged to pellet the insoluble fraction and the supernatant stored at -20°C. Approximately 5 μl of a 100-200 μl sample was run on a mini 6% SDS acrylamide gel and stained with Coomassie blue in order to estimate the sample concentration. One hundred micrograms of muscle protein was loaded onto a 6% polyacrylamide gel and then the proteins were transferred to nitrocellulose using electrophoresis at 0.34V, at 4°C for 7-9 hours. The filter was blocked with 3% gelatin, 1X TBS for 1 hour at room temperature. The filter was then incubated with the primary antibody diluted in 1X TTBS (TBS, 0.2% Tween-20) and 1% goat or donkey serum for 12-24 hours at room
temperature. The dilutions of the purified primary antibodies were as follows: Ab1 (1:50), Ab2 (1:50), Ab3 (1:100), Dys2 (1:500). The filters were washed with three changes of 1X TTBS buffer for 15 minutes each. The filters were then incubated with the appropriate secondary antibody. For development with the BCIP/NBT detection system, the secondary antibody was an alkaline phosphatase conjugated goat-anti-rabbit or -anti-mouse antibody (1:1000 dilution) incubated for 2 hours at room temperature. The filter was then washed 2 times with 1X TTBS and once with carbonate buffer (0.1 M NaHCO3, 1mM MgCl2, pH9.8). The filter was developed with NBT/BCIP color development system (BioRad, Richmond, VA) according to manufacturer’s instructions. Alternatively, the secondary antibody used was a horseradish peroxidase conjugated goat anti-rabbit or anti-mouse and the antibodies were detected using the light chemiluminescence ECL detection system (Amersham, Arlington Heights, IL) according to manufacturer’s instructions. The fluorescent signal was detected on autoradiographic film.

RESULTS

Patients were chosen for this analysis because they were determined to have genomic exon 3-7 deletions by either Southern blot analysis or multiplex PCR
(Chamberlain, et al., 1988). Nine exon 3-7 deletions patients were used in this study. These patients ranged in phenotype from DMD to BMD (Table 5). For patients too young to definitively classify, the phenotype that was determined from their current clinical progression was listed (see asterisks, table 5). Figure 4 shows an example of Southern blot analysis of the of the exon 3-7 deletion patients. Patient DNA isolated from lymphocytes was digested with a restriction enzyme and probed with a radioactively labeled cDNA probe (see Materials and Methods, page 51 for details). Bands missing from the patient lanes, as compared to the lanes containing normal male and female DNA, correspond to deleted exon regions. In these cases, the patients were deleted for exons 3, 4, 5, 6, and 7. Alternatively, multiplex PCR was performed using a set of previously described primers (Chamberlain, et al, 1988) to amplify individual exons from the lymphocyte DNA (analysis performed by the Clinical Genetics Laboratory, OSU). Failure to amplify a particular exon indicated a deletion of that exon.

The reading frame of the predicted mRNA from this deletion junction was determined by comparison to the published cDNA sequence (Koenig, et al., 1987; Malhotra, et al., 1988). The mRNA corresponding to the deleted DNA was predicted to be out-of-frame and would not be
predicted to produce dystrophin. However, western blot analysis of total skeletal muscle proteins isolated from patient tissue, demonstrated the presence of dystrophin. Figure 5 shows an example of western blot analysis on three exon 3-7 deletion patients (Winnard, et al., 1993). This analysis showed that dystrophin was present in a slightly truncated size (as predicted from the deletion) and at approximately 10-15% the normal level of dystrophin (Winnard, et al., 1993).

In order to investigate the possibility that alternative or cryptic splicing created an in-frame mRNA in these patients, RT-PCR was performed on these patients. First, total RNA was isolated from skeletal muscle tissue and then reverse transcribed into cDNA. The cDNA was amplified with dystrophin specific primers (table 4) around the region corresponding to the genomic deletion (see Materials and Methods, page 52 for details). Figure 6 shows examples of the PCR analysis of some of the exon 3-7 deletion patients. Amplification between exon 2 and 8 produced a truncated product in the patients compared to the normal. The size of the product corresponds to that predicted from the deletion of exons 3-7. Amplification of exons 2 to 16 also demonstrated the truncated product in the patients, but patient #OSU245 produced three bands. Amplification of exon 8-16 showed a normal size PCR
product, as would be predicted because there is no genomic deletion in that region. Patient #OSU245 again showed extra bands by agarose gel electrophoresis. Separation of these same PCR products by acrylamide gel electrophoresis caused one of the three bands to migrate much slower in the gel. This is indicative of heteroduplex migration, indicating that there are only two PCR products present and that the third band is a heteroduplex of the two bands. This was confirmed by separately isolating these three bands from an agarose gel, radioactively labeling them, and using them as probes on Southern blots (figure 8, see Materials and Methods, page 51). The Southern blots consisted of digested DNA from normals and patients with known deletions in the amino-terminus of dystrophin. From this Southern blot analysis, it was demonstrated that exon 9 is present in the high MW PCR product, absent in the low MW PCR, and present is approximately half the intensity in the middle MW heteroduplex band. Also, exon 8 and 9 specific probes isolated from cosmid XJ10 hybridized to a Southern blot transfer of the PCR products from amplification of exons 2 to 16 demonstrated that exon 8 is present in all three bands while exon 9 identifies only the high MW band and the middle MW band (data not shown). Restriction analysis (data not shown) and sequence analysis (figure 9, Materials and Methods, page 53) of
these PCR products confirmed that exon 9 is present in the high MW band and absent in the low band. Sequence analysis of the deletion junctions in the PCR of these patients showed no evidence for cryptic or aberrant splicing. The junction from exons 2 to exon 8 was that predicted from the published sequence (table 3). The sequence from exon 8 to exon 9 read CAA CAG/ATC ACG, as predicted. Also, the exon 8 to exon 10 junction in the lower MW product from patient OSU 245, which was deleted for exon 9, read CAA CAG/CAT TTG, as predicted. Therefore, all the mRNA species we were able to detect in our exon 3-7 deletion patients were out-of-frame. We were not able to detect any of the alternatively spliced forms described by Chelly, et al., 1990.

These patients produce detectable levels of dystrophin by immunocytochemistry and by western blot analysis, therefore, some other mechanism besides alternative splicing must account for the dystrophin production. Antibodies that recognize specific sections of the amino-terminus of dystrophin were generated (see materials and methods, page 53). The amino acid sequence of the peptides these antibodies were raised against and the corresponding region recognized in dystrophin are listed in table 6. These antibodies were used to analyze muscle tissue by immunocytochemistry (materials and
methods, page 60) and western blot analysis (materials and methods, page 62). Figure 10 describes how positive or negative staining with these antibodies indicates which proposed mechanism produces dystrophin in exon 3-7 deletion patients. Table 5 lists the results of staining with these antibodies. All exon 3-7 deletion patients stained negatively with antibodies 1 and NT-Dys (exon 2) and antibody 2 (exon 8, before the ATG) and stained positively with antibodies 3 (exon 8, after the ATG) and antibody Dys2 (commercially available carboxyl-terminal antibody, NovaCastra Labs, Newcastle-upon-Tyne, UK). Figure 11 shows an example of immunocytochemical staining of two exon 3 to 7 deletion patients. Figure 12 shows an example of western blot analysis of exon 3-7 deletion patients. In addition, a patient with an exon 2 to exon 7 deletion was analyzed by immunocytochemistry and western blot analysis using the same antibodies. This patient showed the same staining pattern as the exon 3-7 deletion patients and produces dystrophin at a higher level than the exon 3-7 deletion patients.

In collaboration with J. Chamberlain’s laboratory (Ann Arbor, MI), the antibodies described above were used to analyze constructs containing the amino-terminus of dystrophin by western blot analysis. Constructs containing either exons 1 to 11 of dystrophin cDNA sequence or exons
1 to 11, deleted for exons 3 to 7 were transfected into Cos cells. Total protein was isolated from Cos cells transfected with these constructs, along with an untransfected control. These were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose (these constructs and blots were prepared by K.C. in J. Chamberlain's laboratory). The blots were then stained with the two antibodies corresponding to exon 8. These results indicate that initiation at the ATG in exon 8 is occurring.

**CONCLUSIONS**

RT-PCR and sequence analysis indicate that no detectable in-frame mRNAs are produced in exon 3-7 deletion patients. A deletion of exon 3-7 can affect splicing upstream, as demonstrated by patient OSU245, which produces an additional mRNA which is deleted for exon 3-7 and exon 9. However, this mRNA is also out-of-frame and cannot account for the dystrophin production in these patients. Immunocytochemical staining with antibodies that recognize exons 2 and exon 8 indicate that the mechanism of dystrophin production in these patients is due to initiation by utilization of an ATG located in exon 8 in exon 3-7 deletion patients and in an exon 2-7 deletion patient. Implications of this mechanism of dystrophin production are discussed in Chapter V.
Figure 4

Genomic Southern Blot Analysis

Hind III (lanes 1-5) and EcoRI (lanes 6-10) genomic restriction digests probed with a cDNA exon 1 to 11 specific clone (1-2a). Lanes 1 and 6, normal male; Lanes 2 and 7, normal female; Lanes 3 and 8, patient #OSU 244; Lanes 4 and 9, patient #OSU 245; Lanes 5 and 10, patient #300. Numbers on the left indicate exons. All patients shown are deleted for exons 3-7.
Figure 4
Figure 5

**Western Blot Analysis; Carboxyl-terminal Staining**

Lanes 1 and 7 = normal muscle; Lanes 4 and 5 = DMD patients; Lane 2 = patient OSU#218; Lane 3 = patient #63; lane 6 = patient #300. Arrows indicate the positions of dystrophin (D) and myosin (M) on the gel. Western blot analysis of these patients shows that they produce dystrophin, but at a reduced level and slightly truncated size.
Figure 5
Figure 6

Agarose Gel Analysis of Exon 3-7 Deletion Patient PCR Products

Lanes 2 to 6 = amplification between exons 2-8
Lanes 7 to 11 = amplification of exons 2-16
Lanes 12 to 16 = amplification of exons 8-16
Lanes 2, 7, 12 = blank control
Lanes 3, 8, 13 = normal muscle control
Lanes 4, 9, 14 = patient #OSU244
Lanes 5, 10, 15 = patient #OSU245
Lanes 6, 11, 16 = patient #OSU300
M = molecular weight marker

All patients show the expected size PCR products for genomic exon 3-7 deletions. However, patient #OSU245 shows an extra band when amplified between exons 8-16 and 2-16 but not 2-8. The middle band is a heteroduplex formed between the two PCR products during amplification.
Figure 7

**Acrylamide Gel Analysis of Exon 3-7 Deletion Patient PCR Products**

Lanes 2-5 = amplification between exons 8-16
Lanes 6-9 = amplification between exon 2-16
Arrows = migration of heteroduplex band

Analysis of the PCR products by acrylamide gel electrophoresis demonstrates that the middle MW PCR product from patient #OSU245 shifts migration patterns. This is characteristic of heteroduplexes in acrylamide.
Figure 8

**Southern Blot Analysis Using PCR Specific Probes from Patient Amplification**

The PCR products from patient #OSU245, amplified between exons 2-16, were band isolated, radioactively labelled, and used to probe genomic Southern blots. The Southern blot contained EcoRI digested genomic DNA from: a normal female (lanes 1,4, and 7); a patient with a genomic 3-6 deletion (lanes 2,5, and 8); a patient with a genomic exon 8-11 deletion (lanes 3,6,9). Lanes 1-3 were probed with the 2-16 high MW PCR product; Lanes 4-6 were probed with the 2-16 middle MW PCR product (heteroduplex); Lanes 7-9 were probed with the 2-16 low MW PCR product. Arrow = exon 9. These blots demonstrate that exon 9 is present in the high MW product, absent in the low MW product, and present at half the intensity in the middle MW heteroduplex product.
In order to determine if cryptic splice sites were being used in these patients, sequence analysis was performed on the PCR products. Figure 9A shows the sequences flanking the exon 8 to 9 junction present in the high MW PCR product of exons 8-16 in patient #OSU245. Figure 9B shows the exon 8 to 10 junction present in the low MW PCR product of exons 8-16 in patient #OSU245. All of these patients showed normal splicing between exons 10 and 11, including the PCR product deleted for exon 9 from patient #OSU245.
Figure 9
Table 5

Listing of Patient Number, Phenotype, Deletion and Antibody Staining Data.

* Asterisks indicate that the patient was too young for a definitive classification. The phenotype listed is the one most consistent with the current clinical progression of the patient.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Deletion</th>
<th>Antibody Staining</th>
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</thead>
<tbody>
<tr>
<td>DMD</td>
<td>Int (P)</td>
<td>+</td>
</tr>
<tr>
<td>BMD</td>
<td>Int (S)</td>
<td>-</td>
</tr>
<tr>
<td>nd (P)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** This patient had two mRNA species; one deleted for exons 3-7 and one deleted for exons 3-7 and exon 9.

Ab1 = amino-terminal antibody, exon 2
Ab2 = exon 8 antibody, before ATG
Ab3 = exon 8 antibody, after ATG
Dy2 = carboxyl-terminal antibody
+ = positive antibody staining
- = negative antibody staining
Table 5
Listing of Patient Number, Phenotype, Deletion and Antibody Staining Data.

<table>
<thead>
<tr>
<th>Pt. Number</th>
<th>Phenotype</th>
<th>DNA DELETION</th>
<th>RNA DELETION</th>
<th>IMMUNOCYTOCHEMISTRY</th>
<th>WESTERN BLOT</th>
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<td></td>
<td></td>
<td></td>
<td>NT Ab 1 Ab 2 Dy 1</td>
<td>NT Ab 1 Ab 2 Dy 2</td>
</tr>
<tr>
<td>59.1</td>
<td>INT</td>
<td>3-7 (P)</td>
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<td>- - + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSU300</td>
<td>*INT</td>
<td>3-7 (S)</td>
<td>3-7</td>
<td>- - + +</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSU218</td>
<td>DMD</td>
<td>3-7 (S)</td>
<td>3-7</td>
<td>- - + +</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSU245</td>
<td>BMD</td>
<td>3-7 (S)</td>
<td>3-7,9**</td>
<td>nd nd nd nd</td>
<td>+</td>
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<tr>
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<tr>
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<td>+</td>
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<td>63</td>
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</tr>
<tr>
<td>TP1</td>
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<td>+</td>
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</tr>
<tr>
<td>B4</td>
<td>*INT</td>
<td>3-7 (S)</td>
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<td>- - + +</td>
<td>+</td>
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</tr>
<tr>
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<td>*INT</td>
<td>3-7 (S)</td>
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<td>- - + +</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>*BMD</td>
<td>2-7 (S)</td>
<td>ND</td>
<td>- - + +</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 10

Schematic of the Amino-terminal Antibody Staining

This figure illustrates how antibodies 1, 2, and 3 will demonstrate the mechanism of dystrophin production in exon 3-7 deletion patients. The boxes represent the mRNA exons. White boxes are in-frame exons. Shaded boxes are out-of-frame exons produced from the new deletion junction. The position of the stop codon created by this frameshift is indicated by an arrow. The position of the proposed new start site in exon 8 is indicated by an arrow. The solid circle, square and triangle indicate the position of the epitopes recognized by antibodies 1, 2, and 3, respectively. The table on the right lists the possible outcomes of antibody staining. A + indicates positive staining, while a - indicates no staining. Mechanisms of protein production list what the possible staining results would indicate as the mechanism of protein production. New initiation would indicate that only the ATG in exon 8 is used as the translation start site. Reinitiation or ribosomal frameshifting would suggest that the normal ATG is used but that the ribosomes read through the stop and stay on the mRNA long enough to regain the correct reading frame at the ATG in exon 8. Negative staining with antibodies 2 and 3 could result from aberrant splicing of exons 2 to 10 which would create an in-frame message. Negative staining with antibody 1 could result from aberrant splicing of exons 1 to 8 which is also an in-frame message.
Figure 10
Table 6

Amino Acid Sequence

Table 6 lists the amino acids sequence of the synthetic peptides used to raise antibodies 1, 2, 3, and 4. Each peptide was 40 amino acids long; the first 18 amino acids were specific to a unique dystrophin sequence while the last 22 amino acids were common to all the peptides and corresponded to a measles specific virus sequence. This 22 amino acid sequence was synthesized to the carboxyl terminal end of the peptide sequences and is believed to increase the antigenic presentation of the peptides in vivo. The corresponding exon region and the dystrophin amino acid number are listed for each peptide.
### Table 6

**Amino Acid Sequence**

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Dys Exon</th>
<th>Dys AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH-EREDVQKKTFTKMWNAQF-COOH</td>
<td>Exon 2</td>
<td>12-29</td>
</tr>
<tr>
<td>NH-SLFQVLPQQSIEIAQEV-COOH</td>
<td>Before ATG Exon II</td>
<td>235-252</td>
</tr>
<tr>
<td>NH-PKPPKTVKKEHHFQLHQM-COOH</td>
<td>After ATG Exon II</td>
<td>256-273</td>
</tr>
<tr>
<td>NH-DQVIKSQRVMVGLEDIN-COOH</td>
<td>Exon 5I</td>
<td>2490-2507</td>
</tr>
<tr>
<td>NH-GPSLKLSSLIKGVHRLEGVE-COOH</td>
<td>Measles specific sequence</td>
<td></td>
</tr>
</tbody>
</table>
Figure 11

Immunocytochemical Staining of Exon 3-7 Deletion Patients

This figure shows the results of immunocytochemical staining with a panel of amino-terminal antibodies. Vertical rows show staining with the same antibody. The amino-terminal antibody (antibody 1) recognizes exon 2 of dystrophin. Antibody 2 recognizes the portion of dystrophin in exon 8 that comes after the stop codon produced from the out-of-frame junction but before the ATG used for initiation. Antibody 3 recognizes the portion of dystrophin just after the ATG in exon 8 used for initiation. Dys2 is a commercially available antibody that recognizes the carboxyl-terminus of dystrophin. Horizontal rows show sections from the same tissue. Control is from normal muscle tissue. DMD is tissue from a typical DMD patient (note the varied fiber size and large increase in connective tissue, characteristic of DMD tissue). The third row show tissue from patient TP1. The fourth row shows tissue from patient #59.1. Positive staining is indicated by a distinctive fluorescent "rimming" around the entire muscle fiber. The two patients showed negative staining with the amino-terminal antibody and the antibody in exon 8 before the ATG. They stained positive with the antibody after the ATG in exon 8 and the carboxyl-terminal antibody. This staining pattern was consistent in all exon 3-7 deletion patients.
<table>
<thead>
<tr>
<th>AMINO TERMINAL EXON 2</th>
<th>BEFORE ATG EXON 8</th>
<th>AFTER ATG EXON 8</th>
<th>CARBOXYL TERMINAL</th>
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<tr>
<td>CONTROL</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PATIENT</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 11
Figure 12

Western Blot Analysis of Exon 3-7 Deletion Patients: Amino-terminal Staining

Total protein isolated from muscle tissue from exon 3-7 deletion patient #59.1 was loaded into four separate lanes (100 µg/lane) of an SDS polyacrylamide gel. 100µg of normal control muscle proteins (C) was loaded beside each lane of the patient. The proteins were transferred to PVDF membrane (Immobilon) and the filter cut into 4 sections. Each section was stained with a separate antibody: antibody 1 (amino-terminal); antibody 2 (exon 8, before the ATG); antibody 3 (exon 8, after the ATG); and Dys2 (carboxyl-terminal). The patient’s protein are recognized by the antibody in exon 8 after the ATG and the carboxyl-terminal antibody, but does not stain with the amino-terminal or exon 8, before the ATG antibody. This data is consistent with the immunocytochemical data. Crossreactive staining with myosin allows for a visual estimation of the total amount of protein loaded in each lane.

D = the position of dystrophin
M = the position of myosin
Figure 12
Figure 13

Western Blot Analysis of Exon 3-7 Deletion Constructs

This figure shows western blot analysis of total protein isolated from Cos cells which contained dystrophin constructs. Lane 1 = protein molecular weight marker; Lane 2 = protein from untransfected Cos cells; Lane 3 = proteins from Cos cells transfected with a dystrophin construct containing cDNA sequence between exons 1 to 11; Lane 4 = protein from Cos cells transfected with a dystrophin construct containing cDNA sequence between exons 1 to 11 without exons 3 to 7. Panel A = staining with Poncaeu S.; Panel B = staining with the antibody before the ATG; Panel C = staining with the antibody after the ATG. The arrows indicate the position of the protein from the constructs. Bands present in lanes 2, 3, and 4 are crossreactive proteins and are not specific to the constructs. The expected size of the protein from the exon 1 to 11 construct is 50 kD. The expected size of the protein from exon 1 to 11, deletion of exons 3 to 7 construct is 24 kD.
Figure 13
CHAPTER IV

Revertant Fibers in DMD Patients:
DNA, RNA, Sequence, and Protein Analysis

INTRODUCTION

DMD patients are characteristically deficient for dystrophin. However, approximately 50% of patients show rare positive staining fibers termed revertants (Shimizu, et al., 1988; Nicholson, et al., 1989; Hoffman, et al., 1990; Burrow, et al., 1991; Klein, et al., 1992). The precise etiology of these fibers is unknown, however, several mechanisms have been proposed for the occurrence of revertant fibers (see Introduction, page 18). The most likely explanation is that revertant fibers are due to a somatic reversion/suppression event which restores the reading frame of the dystrophin mRNA. In this chapter, three unique cases of revertant fibers are presented. The analysis of these patients presents strong evidence that a secondary mutation that restores the dystrophin reading frame can be responsible for the occurrence of revertant fibers.

Immunohistochemical staining of DMD patients reveals the presence of dystrophin positive fibers in deletion and non-deletion cases, see table 1 (Burrow, et al., 1991;
Revertant fibers have been seen in patients with and without a family history (Burrow, et al., 1991; Klein, et al., 1992). Somatic mosaicism arises when a mutation occurs after fertilization of an egg cell during the later stages of cell division. Which tissues are affected and to what extent depend on when the mutation arose. Since the mutation occurs after fertilization, somatic mosaicism cannot account for the occurrence of revertant fibers in patients with a family history. Klein, et al., (1992), from our laboratory, stained several DMD patients who had well defined genomic deletions and revertant fibers with a panel of antibodies that span dystrophin (Table 1). All of the revertant fibers in these patients stained positively with amino-terminal (9219) and carboxyl-terminal (Dys2) antibodies. However, the revertant fibers did not stain with antibodies whose epitopes corresponded to the regions that were genomically deleted in the patients. Somatic mosaicism cannot be the mechanism of production of revertant fibers in these patients. Dystrophin positive fibers due to somatic mosaicism would contain a normal copy of dystrophin and therefore the revertant fibers would stain positively with all dystrophin antibodies. In addition, positive staining with all four dystrophin antibodies indicates that the revertant fibers are
producing dystrophin of a restored reading frame, since a crossreactive protein would not be expected to be recognized by all four antibodies. In addition, the staining pattern was specific to the patient’s DMD gene deletion.

Klein, et al., (1992), also stained revertant fibers in DMD patients who had no detectable deletion by Southern blot analysis. Two of these patients (#71 and #2, table 1) had revertant fibers which showed a unique staining pattern. They were positive with the amino-terminal (9219), first rod domain antibody (1377) and the carboxyl-terminal antibody (Dys2), but were absent for staining with the second rod domain antibody (9218). We proposed that the mechanism responsible for the staining in the revertant fibers was due to a secondary mutation or deletion that removes the original mutation and restores the reading frame of dystrophin. If this hypothesis is correct, then the staining pattern of the revertant fibers in this patient indicates that the disease causing mutation must lie in or very near the region corresponding to the deleted antibody epitope in the patient’s revertant fibers. Evidence presented below supports this hypothesis.

Since this mechanism of revertant fibers indicates that the secondary mutation arises somatically, it suggests that different myoblasts could give rise to the
secondary mutations independently from one another. If the revertant fibers were due to somatic mosaicism, they would contain a normal copy of the DMD gene. Therefore, in this case, all of the revertant fibers would have the same staining pattern and be positive for all dystrophin antibodies. Analysis of revertant fibers in a DMD patient (#67) with an exon 5-7 duplication indicates that two or more independent mutations can give rise to revertant fibers in the same patient.

Revertant fibers can also be seen in BMD and INT patients. Revertant fibers are often difficult to identify in BMD and INT patients that produce dystrophin because it can be difficult to differentiate the level of dystrophin staining by immunocytochemistry. Analysis of patient #218 demonstrates that revertant fibers can also be seen in exon 3-7 deletion patients.

MATERIALS AND METHODS

DNA Isolation from Muscle Tissue

DNA was isolated from muscle tissue by placing 10-40 cryostat sections (10 microns thick) of muscle tissue into approximately 2 mls of high TE buffer (0.1M Tris, pH 8, 40 mM EDTA). The tissue that did not dissociate in buffer was homogenized gently by passage through a 1 cc syringe. Five mls of lysis buffer was added (0.1M Tris, pH 8, 40 mM
EDTA, 0.2% SDS). 200 μl of proteinase K (10 mg/ml) was added and the sample was incubated 12-24 hours at 37°C. The sample was extracted 3 times with phenol:chloroform (50% v/v). The DNA was precipitated from the aqueous solution by addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol (95%). The samples were spun at 10,000 rpm for 15 min. The pellet was recovered, dried, and resuspended in approximately 100 μl of TE buffer (10 mM Tris, pH 8, and 1 mM EDTA).

Multiplex PCR

Multiplex PCR amplification was performed according to the method of Chamberlain, et al., 1988, in collaboration with the Clinical Diagnostic Laboratory at OSU. Approximately 1 to 2μl of DNA isolated from muscle tissue was amplified in 0.5 mmol/L deoxynucleotide triphosphates, 3 mmol/L MgCl₂, 67 mmol/L Tris (pH 8.8), 16.6 mmol/L ammonium sulfate, 6.7 μl EDTA, 10 mmol/L 2-mercaptoethanol, 150 ng of each oligonucleotide primer, and 2.5 units of Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT) in a final volume of 100 μl. The sequence of the primers are described by Chamberlain, J., et al. (1988) and Beggs, A., et al., (1990). Amplification was performed using 55°C, annealing; 72°C, extension and 94°C, denaturation.
Direct Automated Fluorescent Sequencing

Direct automated fluorescent sequencing was performed according to the method of Gibbs, et al., (1990). Approximately 1% of the band isolated PCR shown in figure 15 was taken to initiate a second PCR reaction for the introduction of both a biotinylated residue (sense primer) and a universal M13 DNA priming sequence (reverse primer) onto the DNA fragment. The reaction was carried out with 30 ng of each primer, 2 mM each dNTP, 1 unit Taq polymerase in 16.5 mM (NH₄)₂SO₄, 66 mM Tris-HCL, pH 8.8, 6.6 mM MgCl₂, 10 mM 2-mercaptoethanol, 6.6 mM EDTA, in a final volume of 100 μl. The reactions were cycled 25 times at 94°C, 30 sec.; 52°C, 30 sec., 72°C, 2 min. The sequence of the primers are listed in the legend of figure 16. One of the primers corresponded to the region in the beginning of exon 51 that was deleted in the cryptically spliced mRNA and therefore only one PCR product was amplified. The biotinylated strands were captured by mixing the PCR products with 40 μg of prewashed streptavidin-coated magnetic beads (Dynal, Great Neck, NY) at room temperature for 30 min. in a total volume of 80 μl with mild agitation. The supernatant was removed and the unbound strand eluted by treatment with 150 μl of 0.15 M NaOH for 5 min. at room temperature. The beads were washed twice in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and resuspended in 24 μl.
of H₂O. The fluorescent DNA sequencing reactions were performed using the Sequenase 2.0 sequencing kit reagents (United States Biochemical, Cleveland, OH). 4 μl of the beads, 1 ml of dye-labeled primer (Applied Biosystems Foster, City, CA), 4 μl ddNTP mix, and 0.25 μl Sequenase (United States Biochemical, Cleveland, OH) were added for each A and C reaction mix and 8 μl beads, 3 μl dye primer, 12 μl ddNTP mix, and 0.5 units Sequenase for each G and T reaction mix were used. The reaction was incubated at 37°C for 5 min. and the reaction was stopped by adding 50 μl H₂O. The sequencing reaction products were eluted from the beads in 3 μl of formamide at 37°C for 30 min. and the sample was loaded directly on an 8% denaturing acrylamide gel on an ABI 373A fluorescent sequencer and run according to manufacturer’s instructions.

**ASO Analysis**

Allele specific oligonucleotide analysis was performed using genomic specific primers to amplify exon 51. The primers are listed in the legend for figure 18. The buffer conditions were 50 mM KCL, 10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 0.01% w/v gelatin, 0.2 mM dNTPs, 0.125 nmoles of each primer. The cycling conditions were 94°C, 30 sec.; 52°C, 30 sec.; 72°C, 3min. for 25-28 cycles. The PCR products were either separated by agarose gel electrophoresis and transferred to Hybond N+ by Southern
transfer or dot blotted onto Hybond N+ membrane. The membrane was incubated with a $^{32}$P end labeled primer corresponding to either the normal allele (5' TTG ATG TTG GAG GTA C 3') or the mutant allele (5' TTG ATG TTA TAG GAT C 3') at 40°C for 12-24 hours. The blots were washed gently at 42°C in 2X SSC, 0.1% SDS for 5 min. and 1X SSC, 0.1% SDS for 30 sec. and then exposed to X-ray film.

RESULTS

Two DMD patients #2 and #71 (table 1) showed a unique pattern of revertant fiber staining. These patients showed no genomic deletion by Southern blot analysis, yet their revertant fibers stained positively with the amino-terminal antibody (9219), the first rod domain antibody (1377) and the carboxyl-terminal antibody (Dys2), but not with the second rod domain antibody (9218) (Klein, et al., 1992). To ensure that these patients did not have a deletion that was undetectable by Southern blot analysis and that the lymphocyte DNA analysis corresponded to the immunocytochemistry analysis, DNA was isolated from muscle tissue taken from the same tissue sample as that used for immunocytochemistry (Materials and Methods, page 97). Multiplex PCR was performed on the muscle DNA with dystrophin specific primers (Chamberlain, et al., 1990) that that included the region corresponding to the
antibody epitope that stained negatively, exons 47-52 (figure 14). No deletion was detectable, which was consistent with the Southern blot data.

In order to investigate the possibility of alternative splicing, a very small deletion, or a point mutation, RT-PCR analysis was done on mRNA isolated from one of these patients (#71). Total RNA was isolated from the same muscle tissue as that used for immunocytochemistry (Materials and Methods, page 51, Chapter III). The mRNA was reverse transcribed into cDNA and amplified between exons 47-52 (figure 15, Materials and Methods, page 52). Two PCR bands were produced from this amplification, indicating that two mRNA species were present. One of the PCR products amplified the same size PCR product as normal muscle tissue. The second PCR band migrated lower on the gel, indicating the presence of a smaller mRNA species. The PCR products were band isolated from a low melt agarose gel. The PCR products were re-amplified with internal primers which allowed for amplification of only the higher molecular PCR product (Materials and Methods, page 52). The PCR products were then directly sequenced by the automated fluorescent method (Materials and Methods, page 99). Sequence analysis of this PCR product, in comparison to the normal sample and the published sequence of dystrophin (Koenig, et al.,
indicated the presence of a 2 bp GG→AT mutation at base pairs 7609 and 7610 in exon 51 (figure 16) which creates a nonsense mutation. It changes the codon sequence from TTG GAG → TTA TAG: Leu Glu → Leu STOP. The presence of this 2 base pair mutation was confirmed at the DNA from both lymphocyte DNA (figure 17B) and DNA isolated from muscle tissue (data not shown). Lymphocyte DNA from the patient’s mother (#491) was analyzed using PCR, allele specific oligonucleotide (ASO) analysis, and direct sequencing (figure 17A). Direct sequencing was done according to the methods described Materials and Methods, Chapter III, page 53. The mother was not a carrier of either a 1 bp polymorphism or a 1 or 2 bp mutation, but germline mosaicism cannot be excluded.

In order to see if this mutation was common in our DMD population, ASO analysis was used to screen patients. Using PCR amplification of exon 51, followed by ASO analysis, 92 patients who had no detectable deletion were screened for the two base pair mutation found in patient #71 (figure 18). None of these patients were positive for the mutation, indicating that it is not a common mutation.

Direct sequencing (Materials and Methods, page 53) of the lower molecular weight PCR produced from amplification of exons 47-52 from patient #71 (figure 15, figure 19) showed a cryptically spliced mRNA species. The mutation
alters the sequence from ATG TTG GAG GTA → ATG TTA TAG/GTA. The new sequence conforms to the 3’ acceptor sequence (Py)n N Py AG/G. The cryptically spliced mRNA has the end of exon 50 spliced into the middle of exon 51. This new cryptic splice site is located 2 base pairs 3’ of the two base pair point mutation in this patient. This allowed for removal of the point mutation from this mRNA species, however, the resulting mRNA is out-of-frame and cannot account for the dystrophin production in this patient. A schematic of the mRNA species produced in this patient is illustrated in figure 20. Analysis of the neighboring exon/intron boundaries indicated that a cryptic splice from the end of exon 49 into the new cryptic splice site could produce an in-frame mRNA that was deleted for the nonsense mutation. Since the number of revertant fibers in this patient is very low in comparison to the total number of fibers, it would be predicted that the level mRNA specific to dystrophin produced in the revertant fibers would be very low. In order to amplify this mRNA species in the presence of the other mRNA species, a primer that was specific to the predicted exon 49/51 splice junction was designed. At the proper stringency, this primer should only amplify a product if a mRNA containing this sequence is present. Figure 21 shows amplification of mRNA from patient #71 and a normal muscle
mRNA control sample. A PCR product of the correct size is seen only in the patient sample and not in the control sample when the PCR products are analyzed by an ethidium bromide stained agarose gel (data not shown). A product specific to the patient sample is also seen even when the PCR products are Southern blot transferred (Materials and Methods, page 51) and probed with an end-labeled oligo that is specific to the exon 49/51 junction. The identity of this PCR product was confirmed by direct sequencing (data not shown, Materials and Methods, page 53) and shown to be a PCR product that contained sequence that from the end of exon 49 to the new cryptic splice (base pair 7613).

It has been proposed that aberrant splicing of an entire exon containing a point mutation could be one mechanism responsible for the dystrophin production in revertant fibers. A deletion of exon 51 alone would not produce an in-frame mRNA, however, a deletion of exons 50 and 51 could produce an in-frame mRNA. In order to test whether the new cryptic splice site is being used in the dystrophin production in this patient or whether exons 50 and 51 are being spliced out, an antibody (antibody 4) was generated to the end of exon 51 (Materials and Methods, page 53; table 6; figure 20). This antibody does not recognize dystrophin in revertant fibers produced by a deletion of exons 50 and 51 but does recognize dystrophin
produced from utilization of the cryptic splice site. Staining of serially sectioned muscle tissue from this patient identified revertant fibers that stained with antibody 4 that recognizes the end of exon 51 and with the antibody that recognizes the carboxyl-terminus of dystrophin (figure 22). The dystrophin in these revertant fibers could be produced by the mRNA species identified by PCR amplification shown in figure 21.

Revertant fibers that appear in a cluster and demonstrate the same staining pattern most likely arise from a common myoblast in which the secondary reversion mutation occurs. It is possible, however, that revertant fibers arising from separate myoblast could be produced by a different secondary reversion mutation. Evidence for this comes from analysis of patient #67 (table 1).

Patient #67, who had revertant fibers, was shown to have a duplication of exons 5 to 7 by Southern blot analysis (figure 23). This duplication is predicted to produce an out-of-frame mRNA. RT-PCR was performed on mRNA isolated from this patient. Amplification between exons 2 and 8 indicated the presence of two mRNA species. One PCR product was larger than that produced from normal muscle RNA. The presence of this larger size PCR product was expected from the duplication and the size is consistent with the size of the duplication. The second PCR product
is the same size as that produced by the normal muscle mRNA. One possible explanation for the two mRNAs is that the larger PCR product corresponds to a mRNA which contains the duplication and to the fibers which stain negatively for dystrophin. Whereas, if the dystrophin-positive fibers in this patient were due to somatic mosaicism, the normal size PCR product could correspond to the normal mRNA sequence. Analysis of the patient’s mother’s DNA will determine if she is a carrier. If she is a carrier, then the revertant fibers in this patient could not be due to somatic mosaicism.

To further investigate this, the two PCR products were subcloned into a Bluescript vector using standard protocols (Sambrook, et al., 1989). Several single colony clones were purified for both the higher molecular weight PCR product and the lower molecular weight PCR product. The PCR amplification was performed with the purified clones using primers corresponding exons 2 and 8 of the dystrophin sequence (table 4). The clones containing the lower molecular PCR product amplified one band of the expected size (figure 25). Unexpectedly, the amplification using the same primers from purified clones containing the higher molecular weight PCR produced two bands, one of the expected size and one corresponding to the size of the normal PCR product (figure 25). Since the amplification
was done with purified clones, only one target sequence was present. The production of two PCR products could be due to either a migration artifact of the PCR created by a hairpin loop formation of the duplicated sequence or an error made by Taq polymerase amplifying from a target sequence that had formed a hairpin loop due to the duplication. Alternatively, if the lower molecular weight PCR product were a migration of the larger PCR product in a hairpin loop, it is possible that the loop was excised by the host bacteria during transfection and that is why it produces only one size PCR product during purification. In any case, it seems likely that this patient does not produce two detectable dystrophin mRNAs, but rather only one which contains a duplication of exons 5 to 7.

Additional evidence which indicates that the dystrophin positive fibers in patient #67 are not due to somatic mosaicism comes from immunocytochemical analysis. Serial sections of muscle tissue were taken from muscle tissue of patient #67 and stained sequentially with antibodies that span dystrophin (figure 26). Two small clusters of revertant fibers stained positively with antibodies that recognize exons 2, 8 and the carboxyl-terminal. However, a larger neighboring cluster of revertant fibers only stained with the carboxyl-terminal antibody. The serial sections were stained sequentially to
ensure that sectioning had not cut through the revertants. Additionally, the same cluster of fibers that stain only with the carboxyl-terminal antibody can easily be identified on sections stained with the other antibodies, even though they are not positive. This staining pattern indicates that two different mechanisms are responsible for the dystrophin staining in this patient. This would not be seen if they were due to somatic mosaicism.

Revertant fibers can also been seen in patients who produce dystrophin. A good example of this can be seen in patient #218, an exon 3-7 deletion patient. As illustrated in Chapter III, dystrophin is produced in exon 3-7 deletion patients due to utilization of an ATG in exon 8. They are therefore absent for the amino-terminus. Serial sections of muscle tissue from patient #218 was stained with the amino-terminal and carboxyl-terminal antibodies. Staining with the amino-terminal antibody shows a cluster of positively staining fibers against a background of negatively staining fibers. All of the fibers stain with the carboxyl-terminal antibody. The revertant fibers are identifiable because they stain more intensely than other fibers in the field.
CONCLUSIONS

Analysis of revertant fibers in patients #71 (a non-deletion DMD patient), patient #67 (a duplication patient), and patient #218 (an exon 3-7 deletion patient) indicate that revertant fibers can be produced by a secondary mutation that deletes the original mutation and restores the reading frame of dystrophin. Revertant fiber analysis of patient #67 indicates that a different secondary mutation that allows for the reversion of the same original mutation can arise independently in the same patient. Analysis of revertant fibers in patient #218 indicates that revertants are not unique to patients with the DMD phenotype, but they can be difficult to score in BMD and INT patients who produce dystrophin. With the understanding of how dystrophin is produced in exon 3-7 deletion patients, it is possible to first identify a revertant cluster in a negatively stained background and therefore confirm the identity of revertant fibers with an antibody that stains all of the patient fibers. Additionally, revertant fiber analysis of patient #71 indicates the position of the original mutation in that patient. The disease causing mutation was identified as a 2 base pair GG → AT nonsense mutation.
Figure 14

**Multiplex PCR**

C = control DNA; P = non-deleted control patient; B = blank control; 71 = patient #71; 2 = patient #2.

DNA isolated from the same tissue sample as that used for RNA isolation was analyzed by multiplex PCR using dystrophin primers specific for exons 1, 4, 6, 8, 13, 17, 19, 43, 44, 45, 47, 48, 50, 51, 52 and 53. The position of each exon is indicated by the arrow beside the exon number. Both patients #71 and #2 were positive for all exons tested. Southern blot analysis of lymphocyte DNA also indicated no deletions (data not shown).
Figure 15

**RT-PCR of Patient #71**

Lane 1 = molecular weight marker; Lane 2 = blank control; Lane 3 = normal control; Lane 4 = patient #71; Lane 5 = DMD patient.

RT-PCR was performed on muscle tissue mRNA reverse transcribed into cDNA between base pairs 6970 and 7834 (exons 47-52) using primers 5’ TTA CTG CTG GAA CAG TTG CCC CTG 3’ and 5’ GCT GGT CTT GTT TTT CAA ATT TTG GGC 3’. The PCR reaction conditions are listed in Materials and Methods, Chapter I. The PCR products were separated on a 1% low melt TBE agarose gel.
RNA/cDNA was amplified between base pairs 7534 and 7834 using primers 5′ TGT AAA CGA ACG GCC AGT ACT CTG GTG ACA CAA CCT CTG 3′ (5′ universal tail) and 5′ GCT GGT CTT GTT TTT CAA ATT TTG GGC 3′ (5′ biotinylation) according to methods described by Gibbs, et al., 1990). Panel A shows sequence from normal tissue and panel B shows sequence from patient #71. The position of the 2 base pair nonsense mutation is indicated by arrows.
Figure 17

Direct Sequencing of DNA from Patients #71 and #491

The presence of the 2 base pair mutation was confirmed at the DNA level by PCR amplification of DNA isolated from muscle tissue (data not shown) and from lymphocytes (panel B). Lymphocyte DNA from the patient’s mother (#491) was analyzed using PCR, ASO analysis, and direct sequencing (panel A). Direct sequencing of PCR products amplified with an M13 tailed primer corresponding to base pairs 7534-7572 in exon 51 and an intron primer between exons 51 and 52 (5’ TGT AAA CGA ACG GCC AGT CTG GTG ACA CAA CCT CTG 3’ and 5’ GGA GAG TAA AGT GAT TGG TGG AAA ATC 3’) was performed using the conditions listed Materials and Methods.

Asterisks (*) indicate the 2 base pair change.
Panel A = #491, patients mother.
Panel B = #71, DMD patient.
Figure 17
Using PCR amplification of exon 51 and ASO analysis, we screened 92 non-deleted DMD patients for the 2 base pair mutation found in patient #71. None of these patients were positive for the mutation, indicating that it is not a common mutation.

Genomic DNA isolated from lymphocytes of DMD patients with no known mutations was amplified with intron primers which flank exon 51 (forward - 5' GAA ATT GGC TCT TTA GCT TGT GTT TC 3' and reverse - 5' GGA GAG TAA AGT TGA TTG GTG GAA AAT C 3') and hybridized with either an oligo specific to the normal sequence (panel A) or to the mutant sequence (panel B). DNA samples and controls were loaded randomly. Positions A12, D7, and E6, correspond to patient #71.

Positions B7 and F9 had no DNA loaded. All the other lanes contained the other non-deleted patient DNA samples. Only patient #71 hybridized with the mutant sequence and not the normal sequence.
Figure 19

Direct Sequencing of PCR Products from Patient #71.

This figure shows direct sequencing of the PCR product from patient #71 shown in figure 15.  
A = the sequence of the PCR product from lane 3, figure 15.  
B = the sequence of the lower molecular weight band in lane 4, figure 15.  
To separate the two PCR products in lane 4, figure 15, the PCR products were purified twice over a 4.5% native acrylamide gel, band isolated and reamplified using the conditions listed for amplification of the products in figure 15. Sequencing was carried out as described in Materials and Methods using an internal sequencing primer corresponding to base pairs 7474-7497 (5' AAG CAG CCT GAC CTA GCT CCT GGA 3'). Arrows indicate the exon 50/51 boundary.
Figure 19
Figure 20

**Schematic of Cryptic Splice Products in Patient #71**

This schematic shows the type of splicing products seen in patient #71. **Line A** represents the intron/exon structure in that region. **Thick lines** represent introns and **boxes** indicate exons. **Lines B and C** represent the messages seen in figure 5. The position of the stop mutation and new splice site are indicated by **vertical arrows**. **Line D** indicates a splicing product that would produce an in-frame message. The **horizontal arrows** represent the position of PCR primers designed specifically to amplify this message (5' CCA GCC ACT CAG CCA GTG AAG GTA 3' and 5' CTG GTC TTG TTT TTC AAA TTT TGG GC 3'). These primers will not amplify a product from normal mRNA or from those messages represented by lines B and C. The **solid square** indicates the position of an antibody generated to detect this protein product in the revertant fibers. The fibers will stain positively with this antibody if the mechanism on line D is used (see figure 22). It will not stain the revertant fibers if some form of splicing which deletes all of exon 51 is used.
Figure 20
Amplification of mRNA/cDNA isolated from muscle tissue from patient #71 with primers specific to the exon 49 to exon 51 cryptic splice site junction described in the schematic in figure 20. Amplification was done using primers 5’ CCA GCC ACT CAG CCA GTG AAG GTA 3’ and 5’ CTG GTC TTG TTT TTC AAA TTT TGG GC 3’ . The PCR products were separated on a 2% agarose gel and transferred to Hybond N+ using Southern blot transfer (Materials and Methods, page 51). The PCR products were hybridized with a 32P-end-labeled primer specific to this junction (5’ CT CAG CCA GTG AAG/GTA CCT GCT CTG 3’: exon 49/exon51). Even on overexposure, this PCR product cannot be seen in the control lane but the expected 244 base pair fragment is present in the patient sample. Numbers listed vertically on the left of the figure indicate the position of the molecular weight marker. Blank = blank control; control = PCR amplified sample from normal muscle RNA; Patient = PCR amplified sample from patient #71.
Exon 49 to Exon 51 Cryptic Splice Site
Specific Amplification and Hybridization

Figure 21
Figure 22

**Immunocytochemical Staining of Muscle Tissue from Patient #71**

Serial sections of muscle tissue were stained with an antibody that recognizes exon 51 (antibody 4, table 6) and an antibody that recognizes the carboxyl-terminus (Dys2). Arrows indicate the position of the revertant fibers. The same fibers stain positively with both antibodies at approximately the same intensity.
Exon 51

Carboxyl-Terminal

Figure 22
Southern Blot Analysis of Genomic DNA from Patient #67

Lanes 1 and 4 = normal male; Lanes 2 and 5 = normal female; Lanes 3 and 6 = patient #67.
Genomic lymphocyte DNA was digested with either Hind III (lanes 1-3) or EcoRI (lanes 4-6). The bands were separated on a 0.8% TBE agarose gel and transferred to Hybond N+ using standard Southern blot procedure. The blot hybridized with probe 1-2a which recognizes exons 1-11. The number for the corresponding exons are labeled on the right sides. The intensity of a normal female is double that of a normal male when equal concentrations of total genomic DNA are loaded in each well. Duplicated bands are ones that show double intensity staining in a male. These results indicate that the exons marked by the arrows are duplicated (show double intensity) in the region of exons 5-7 in patient #67.
Figure 24

RT-PCR Analysis of Patient #67

Lane 1 = normal control; Lanes 2 and 3 = patient #67; Lane 4 = patient #15.

RT-PCR analysis was performed on mRNA/cDNA isolated from muscle tissue of patient #67. The samples were amplified between exons 2 and 8 using primers listed in table 3. These results show the presence of a higher molecular weight band and a normal band (band position indicated by arrows).
Figure 25

**Amplification of Subclones of PCR Products from Patient #67**

Lane 1 = molecular weight marker; Lane 2 = blank control; Lanes 3 and 4 = high molecular weight subclones; Lanes 5 and 6 = normal molecular weight subclones. Both the high molecular weight and normal molecular weight PCR products from patient #67 (see figure 24) were subcloned into Bluescript and single colony clones were purified. PCR amplification was done between exons 2 and exon 8 (primer sequence listed in table 4) using purified DNA from single colony preparations. The higher molecular weight subclone produced the expected higher molecular weight product and an unexpected normal molecular weight band.
Muscle tissue from patient #67 was serial sectioned. The number under each picture corresponds to order of the tissue section. Sections 1 and 5 were stained with the amino-terminal antibody. Sections 2 and 6 were stained with antibody 2 which recognizes the region before the ATG in exon 8 (table 6). Sections 3 and 7 were stained with antibody 3 which recognizes the region after the ATG in exon 8. Sections 4 and 8 were stained with the carboxyl-terminal antibody (Dys2). Solid arrows indicate the position of revertant fibers which stain positively with all the antibodies. Open arrows indicate the position of the revertant fiber cluster which only stains positively with the carboxyl-terminal antibody.
Figure 27

Immunocytochemical Staining of Revertant Fibers in Patient #218

Muscle tissue from patient #218 was serial sectioned. The two figures show adjacent muscle tissue sections. The top figure shows staining with the amino-terminal antibody. The bottom figure shows staining with the carboxyl-terminal antibody. Solid arrows indicate the position of the revertant fibers. Open arrow demonstrates positive staining with the carboxyl-terminal antibody in non-revertant fibers. Non-revertant fibers in the top panel are absent for staining with the amino-terminal antibody.
Amino-Terminal

Figure 27

Carboxyl-Terminal
CHAPTER V
SUMMARY

The clinical progression of Duchenne muscular dystrophy (DMD) patients with deletions can be predicted in 93% of cases by whether the deletion maintains or disrupts the translational reading frame (frameshift hypothesis). However, a number of exceptions to the reading frame rule model have been reported. The aim of the project had been to gain a better understanding of how these patients produce dystrophin in order to understand how their mutations relate to their phenotypes.

MECHANISM OF DYSTROPHIN PRODUCTION IN EXON 3-7-DELETION PATIENTS

Several cases of translational frame exception patients were presented. The most common type of exception patients are those with exon 3-7 out-of-frame deletions. These patients produce dystrophin and can result in the clinical presentation of DMD, BMD, or Intermediate. One possible way that an out-of-frame deletion patient can produce dystrophin is by aberrant splicing that creates an in-frame mRNA. An example of this is seen in patient #113,
Chelly, et al., (1990), reported low levels of in-frame deleted mRNA species in exon 3-7 deletion patients. These in-frame mRNAs had either a deletion of exons 3-9 or 2-7. We performed RNA analysis of several exon 3-7 deletion patients and were unable to detect these in-frame mRNA species. Gangopadhyay, et al., (1992), were also unable to detect these mRNA species in exon 3-7 deletion patients. Our analysis indicates that some other mechanism besides splicing must account for the dystrophin production in these patients. Gangopadhyay, et al., (1992), suggested that ribosomal shifting or reinitiation could account for the dystrophin production in these patients.

Ribosomes in bacterial and retroviral systems have been shown to perform nonstandard decoding of mRNA, allowing for changes in the codon usage and resulting translational process (Atkins, et al., 1990). These nonstandard events involve ribosomal hopping, frameshifting, and readthrough of stop codons. Frameshifting can occur when the paired codon had anticodon disengage at certain sequences and allow the mRNA to "slip" with respect to the ribosome-peptidyl-tRNA complex. A string of four or more single base repeats constitutes a "slippery run" prone to frameshifting. An example of this is the E.coli polypeptide releasing factor
(RF2) in which a tRNA\textsubscript{leu} slips to a +1 reading frame (Craigen, et al., 1985). The context in which the slippery run lies can affect the direction of the frameshift, either +1 and -1. These surrounding sequences create what are called "hungry codons" because they influence the direction in which the slippage will occur (Lindsley and Gallant, 1993). Secondary signals, besides those in the slippery run, can affect the level of leaky frameshifting.

Ribosomal hopping involves a frameshift in which the ribosome disengages as in frameshifting. However, the ribosome repairs with several base pairs downstream with similar codon to the one that it disengaged from and protein synthesis is resumed. This occurs in the phage T4 topoisomerase subunit, gene 60. In this case, 50 base pairs are skipped between codons that translate to adjacent amino acids (Huang, et al., 1988). At least four distinct elements contribute significantly to the hopping: a stop codon at the 5' junction, a stem loop structure, a 50 base pair spacing, and the correct codons before and after the skip. Readthrough of a stop codon can occur by having either a normal amino acid, such as tryptophan, in response to a leaky UGA terminator in phage (Weiner and Weber, 1973) or selenocysteine in response to certain UGA codons in E. coli and several mammalian species (Zinoni, et al., 1986; Chambers, et al., 1986). In most cases,
these mechanisms of ribosomal nonstandard codon usage occur at relatively low efficiencies of only 1-3%, except in special situations (Atkins, et al., 1990). Except for the selenocysteine readthrough, these mechanisms of ribosomal decoding have only been observed in bacterial, viral and yeast systems. The 5′ sequence of the dystrophin gene in exon 3-7 deletion cases was inspected to see if any of the sequence features for these mechanisms could be found. No stretches of sequences contained all the proper features. It is possible, however, that the sequences for ribosomal decoding in mammals are different than those required for other systems and simply have not yet been identified.

Arahata, et al., (1991), used an antibody directed against amino acids 215-264 to immunostain the muscle of two BMD patients deleted for exons 3-7 (exon 8). The antibody did not stain the patients’ muscle. However, the antibody used by Arahata, et al., is directed against 60 amino acids, of which only 10 correspond to sequence after the ATG that could be used for reinitiation or ribosomal shifting or reinitiation. Thanh, et al., (1993), reported positive dystrophin staining in a BMD exon 3-7 deletion patient with an amino-terminal antibody that recognizes the first 11 amino acids of dystrophin. However, the staining was weak and very patchy and they were unable to
confirm the result by western blot analysis. It is possible that they were observing staining due to a truncated dystrophin translated from exon 1 to the stop codons produced in exon 8 due to the out-of-frame junction created. Bulman, et al., (1991), demonstrated that it is possible to detect truncated forms of dystrophin with amino-terminal antibodies. However, we have observed no amino-terminal staining that demonstrated consistent membrane staining and at the approximate levels as those seen with the antibody directed against exon 8 after the ATG. Amino-terminal staining on western blots also did not identify any bands consistent with the size of dystrophin produced in exon 3-7 deletion patients when stained with carboxyl-terminal antibodies. Neither results of Arahata, et al., (1991), or Thanh, et al., (1993), can distinguish between the mechanisms of aberrant splicing, ribosomal frameshifting, or reinitiation.

Our approach, schematically diagrammed in figure 10, allowed for distinction between all of these mechanisms. Our results indicate that dystrophin is produced in exon 3-7 deletion patients due to initiation at an ATG located in exon 8. The use of an alternative initiation site that restores reading frame in a mammalian system has not been previously reported, except in artificial constructs.
Thus, this represents the first example of use of an internal ATG to restore reading frame.

**MAINTENANCE OF PROPOSED AMINO-TERMINAL FUNCTIONAL DOMAINS**

Patient #32 had an in-frame deletion of exons 4-18, yet he produced no detectable level of dystrophin by either immunocytochemistry or western blot analysis. One possible explanation for why patient #32 does not produce dystrophin is that the mutation is too large or that it deletes a critical epitope of dystrophin. However, analysis of two other exception patients (#302 and #113) with large deletions of the same region of the gene as that deleted in patient #32 suggests that the explanation is not that simple. Patient #32 is similar to a patient reported by Chelly, et al., (1990). These authors suggest that this deletion disrupts the ability of dystrophin to dimerize. A similar phenomenon has been reported for β-spectrin where a point mutation is thought to disrupt conformation and prevent αβ-dimers associating to form a tetramer.

Patients #302 and #113 had with large deletions of over 30 exons at the amino-terminal end of dystrophin. Patient #113 had a genomic deletion of exons 4-41, which is out-of-frame, yet he produced dystrophin at approximately 84% of the normal level of dystrophin.
Analysis at the RNA level indicated that this patient produces an in-frame mRNA by cryptically splicing out exon 3 in addition to exons 4-41. A deletion of exons 3-41 is an in-frame mutation would be predicted to produce dystrophin. Therefore, dystrophin can be produced despite the large size of the deletion. However, despite the production of dystrophin at levels comparable to Becker patients, this patient was still severely affected. We have subsequently identified a second in-frame DMD patient with a 3-42 deletion who produces dystrophin and stains with the carboxyl-terminal antibody (unpublished data). In addition, other DMD patients with similar deletions, exons 3-25 (Nicholson, et al., 1993; Vainzof, et al., 1993) and exons 3-31 (Vainzof, et al., 1993) show similar immunostaining patterns. Therefore, it is clear that some large in-frame DMD patients do produce dystrophin.

However, the size of the deletion alone is not responsible for the severe phenotype because a Becker patient (#302) had an in-frame deletion of approximately the same size deletion, exons 13-48, as patient #113. Patient #302 produced approximately the same level of dystrophin as patient #113. It is then possible that it is not the size of the deletion but rather maintenance of a critical region that confers the milder phenotype. Patient #302 maintains exons 1-12 of dystrophin. It has been
proposed that the actin-binding sites present in exon 2 (amino acids 17-26) and exon 6 (amino acids 128-156) could be important in the function of muscle dystrophin. Figure 28 shows a schematic representation of the deletions in these exception patients and their resulting dystrophin levels and phenotypes. However, our results of exon 3-7 deletion patients indicate that they deleted both actin-binding domains and yet still produce and localize dystrophin, although at lower levels. Therefore, it is possible that maintenance of either both actin-binding domains or maintenance of at least one actin-binding domain and a critical portion of the rod domain located between exon 8-12 is important for the function of dystrophin.

In addition, exon 3-7 deletion patients range in phenotype from DMD to BMD. Evidence that other factors affect phenotype besides that conformation or abundance of dystrophin comes from analysis of patient #77, an exception patient with a one exon in-frame mutation. This patient produces no detectable dystrophin and yet he is an intermediate. Other patients with this same mutation reported in the literature range in phenotype from DMD to BMD. It was suggested by Roberts, et al., (1991), that an out-of-frame mRNA, which was detectable in lymphocytes due to illegitimate splicing, might be the mRNA species
present in muscle of these patients. However, we were able to analyze RNA muscle tissue patient #77 and found only one mRNA species, which was out-of-frame. This result was consistent with the antibody data. Therefore, other factors besides dystrophin can affect the phenotype in these patients.

REVERTANT FIBERS

Approximately 50% of DMD patients have rare positive dystrophin staining fibers termed revertants. Several mechanisms have been proposed for the occurrence of these fibers including artifactual staining and somatic mosaicism (see Introduction, page 18). Work by Klein, et al., from our laboratory demonstrated that the revertants were due to the presence of dystrophin of a restored reading frame and suggested that a reversion mutation produced the dystrophin. Analysis of the revertant fibers in a non-deleted DMD patients demonstrated that the revertant fibers in this patient were positive for all antibodies except a rod domain antibody, 9218. We proposed that this staining pattern was due to a secondary deletion or mutation that deleted the original mutation and created an in-frame mRNA. If this hypothesis were true, the original mutation should lie in or near the region corresponding to the deleted antibody epitope. RT-PCR
analysis and sequencing of the patient's mRNA in this region indicated that this was true. We were able to identify a 2 base pair nonsense mutation in exon 51. This mutation created a new cryptic splice site 2 base pairs 3' of the mutation that allowed for splicing products that would remove the original mutation. Using specifically designed primers, we were able to detect an in-frame mRNA that spliced the end of exon 49 into the middle of exon 51 and deleted the original mutation. In order to see if this mRNA species could be utilized in the revertant fibers, we generated an antibody to the end of exon 51, which could identify dystrophin produced from the exon 49/51 mRNA species but not from a mRNA that deleted the entire exon. We were able to detect revertant fibers that stained with the carboxyl-terminal antibody and the exon 51 antibody indicating that the in-frame mRNA species we detected is probably responsible for the dystrophin product.

In order to see how frequent the 2 base pair nonsense mutation was in the DMD population, we performed ASO analysis of 92 non-deletion DMD patients with oligos specific to the nonsense mutation. No other patient was positive for the mutation indicating that this is a rare mutation. This is consistent with other DMD point mutations reported. To date, approximately 12 point mutations have been reported in the literature. This is
because it is difficult to find point mutations in a gene as large as dystrophin. The first point mutation described in DMD was a nonsense mutation in exon 26 and was detected by immunological methods (Bulman, et al., 1991). The point mutations reported to date include nonsense mutation, splice site mutation, single base deletion, and a missense mutation (Winnard, et al., 1992; Prior, et al., 1993a, b,c; Kilimann, et al., 1992; Matsuo, et al., 1990; Nigro, et al., 1992; Roberts, et al., 1992; Saad, et al., 1992). Only one of these mutations is common to two patients (Prior, et al., 1993; Nigro, et al., 1992). Although the number of known point mutations so far identified is small, it seems that there are not high frequencies of common point mutations as seen in diseases such as Cystic Fibrosis. However, there are regions in certain exons that seem to have a clustering of point mutations (Prior, T., personal communication). Analysis of the missense mutations and their effect on the function of dystrophin will complement the deletion analysis and may provide a clearer indication of where the functional domains are in dystrophin and how they function.

Analysis of revertant fibers from a patient with a duplication of exons 5-7 (#67) indicates that more than one type of secondary mutation can occur in the same patient. Two separate groups of revertant fibers in the
same field showed different immunocytochemical staining patterns. This also supports the mechanism that secondary mutations arise independently, and in this case, at least one group of revertant fibers cannot be due to somatic mosaicism.

Analysis of revertant fibers in an exon 3-7 deletion patient (#218) demonstrate that the revertant fibers can occur in patients that produce dystrophin. It can be difficult to identify revertant fibers in patients that already produce dystrophin. However, with the understanding of how dystrophin is produced in exon 3-7 deletion patients, it is known that the dystrophin will not stain with amino-terminal antibodies, but will stain with carboxyl-terminal antibodies. This allows for identification of the revertant fibers first on a negative background, which when serially sectioned, can then be identified against a positive staining background. Although immunocytochemistry is not a quantitative method, the revertant fibers in this exon 3-7 patient stain with more intensity than the rest of the fibers. One interpretation of this is that since the revertant fibers stain with the amino-terminal antibody, dystrophin is produced from the normally used ATG in exon 1. The staining implies that utilization of this ATG is more efficient, even though the surrounding Kozak's consensus
is not as well conserved as that in exon 8. This is most likely related to the proximity of the start site to the promotor and may indicate why exon 3-7 patients produced such reduced levels of dystrophin as compared to many other BMD patients.

With this understanding of the mechanism of occurrence of revertant fibers, it is possible to generate antibodies which will control for the occurrence of revertant fibers in patients undergoing myoblast transfer. Myoblast transfer involves introducing normal full-length dystrophin into muscle tissue by injecting normal myoblasts into DMD muscle tissue. All dystrophin antibodies will recognize the newly introduced dystrophin, but not necessarily all revertant fibers. By choosing DMD patients with defined genomic deletions, it is possible to generate antibodies specific to the deletion which will only identify the newly introduced dystrophin and not the revertant fibers. These antibodies will serve as important controls during the initial stages of the myoblast transfer trials.

In conclusion, analysis of the translational frame rule exception patients presented in this study give further insight into the proposed functional domains at the amino-terminus of dystrophin and indicate that other factors besides dystrophin production influence phenotype.
In addition, results from the analysis of revertant fibers identified a 2 base pair point mutation in a DMD patient and supports the theory that these fibers can be produced by a secondary mutation that removes the original mutation. With this understanding of revertant fibers, we were able to design antibodies whose value is to distinguish between dystrophin in revertant fibers and newly introduced dystrophin. These antibodies will serve as useful controls, allowing for valid assessment of the efficiency of dystrophin expression during the initial stages of the myoblast transfer studies.
Figure 28

Schematic Diagram of Deletions in Exception Patients

This diagram is a schematic representation of the deletions in the exception patients. Open boxes indicate non-deleted regions the dystrophin gene. Solid boxes represent deleted regions in the dystrophin gene. The approximate level of dystrophin production and the phenotype of the patients are listed at the right.
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REFERENCES


