MOLECULAR ANALYSIS OF THE DYSTROPHIN GENE

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C E R T I F I C A T E

The research work embodied in this thesis entitled **"Molecular analysis of the dystrophin gene"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full, for the award of any other degree or diploma of any University.

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Dedicated

To

Muscular Dystrophy Patients

"The disease is one of the most interesting and at the same time most sad, of all those with which we have to deal: interesting on account of its peculiar features and mysterious nature; sad on account of our powerlessness to influence its course.... it develops with the child's development, grows with his growth—so that every increase in stature means an increase in weakness, and each year takes him a step further on the road to a helpless infirmity, and in most cases to an early and inevitable death."

Sir William Gowers on DMD

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CONTENTS

INTRODUCTION

The muscular dystrophies are a group of inherited disorders characterized by progressive weakness of the skeletal muscle. They are classified on the basis of clinical features, severity of muscle weakness and patterns of inheritance. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked, recessive, allelic neuromuscular disorders. The severe form, DMD, is characterized by progressive proximal muscle weakness with onset at 2-5 years, leading to loss of ambulation by 10-12 years of age and death before 20 years. On the other hand, BMD is a milder form of muscular dystrophy where the pattern of muscle involvement is similar to DMD. The onset is at 5-15 years. The rate of progression of the disease is very slow and a wheelchair may never be needed. A normal life span is frequently maintained. Limb girdle muscular dystrophy (LGMD) usually has an autosomal recessive inheritance pattern although an autosomal dominant inheritance has occasionally been observed. The patients show preferential involvement of shoulder and/or pelvic girdle and most of them remain ambulatory until middle age (Sheilds, 1986; Emery, 1988). The similarity in clinical features of BMD and LGMD makes differential diagnosis of an isolated male case difficult. Spinal muscular atrophies (SMAs) constitute yet another group of neuromuscular disorders with severe, intermediate or mild phenotypes (Cicero et al., 1994). Most patients with SMA experience clinical onset in childhood and demonstrate autosomal recessive inheritance. A smaller number show autosomal dominant or X-linked inheritance, and onset begins later in life (Munsat, 1994).

DMD and BMD are caused by mutations in the dystrophin gene at Xp21 (Hoffman and Kunkel, 1989). The gene consists of 79 exons distributed over 2.4 Mbp (den Dunnen et al., 1989; Roberts et al., 1992a). The gene is transcribed into a 14 kb mRNA which codes for a 427 kDa protein called dystrophin, located at the plasma membrane of all muscles and some neurons. Dystrophin protein is speculated to play an important role in maintaining the integrity of the muscle cell membrane during muscle contraction (Hoffman et al. 1987a; Monaco, 1989). It is absent in the muscle cells of DMD patients and a partially functional protein is present in the BMD patients.

Cloning of the dystrophin gene and the identification of its protein (Koenig et al., 1987, 1988) has resulted in the development of a number of techniques which make possible the identification of mutations in DMD/BMD patients (Beggs and Kunkel, 1990). Southern analysis with cDNA probes (Kunkel et al., 1986; Koenig et al., 1987; Darras et al., 1988; Read et al., 1988; Baumbach et al., 1989; Liechti-Gallati et al., 1989) and polymerase chain reaction (PCR) (Beggs et al., 1990; Chamberlain et al., 1990) have been used successfully to identify about 70% of the mutations in the dystrophin gene.

Because of the extremely large size of this gene, the majority of the mutations are intragenic deletions (~65%) or duplications (~5%) (Koenig et al., 1987; Forrest et al., 1988; Hu et al., 1988). The deletions in the dystrophin gene are non-randomly distributed in two regions, at the 5′ terminus (minor "hot spot") and the distal half of the central rod domain around exons 44-53 (major "hot spot") (Koenig et al., 1987; Forrest et al., 1988). According to the "reading frame rule" (Monaco et al.,

1988) mutations that disrupt the open reading frame (ORF) cause DMD, whereas those which maintain it result in BMD. Overall, about 92% of deletions conform to the "reading frame rule" (Koenig et al., 1989).

About 30% of the mutations are undetectable by Southern hybridization or PCR. These include point mutations in the coding region of the gene which can be easily identified by single strand conformation polymorphism (SSCP) (Roberts et al., 1992a).

Studies have shown that abnormalities of the dystrophin gene are also present in neuromuscular syndromes other than "classic" Duchenne or Becker muscular dystrophies. Patients with disorders consistent with denervation and with normal creatine kinase levels in the serum (Kugelberg Welander syndrome), scapulo-humeral dystrophy, limb girdle muscular dystrophy and the "stick-man" type of congenital muscular dystrophy have shown deletions of the DMD gene (Kakulas, 1992). Abnormalities of the dystrophin are also present in patients with fascioscapulohumeral syndrome, quadriceps myopathy, the Fukuyama type of congenital muscular dystrophy and limb girdle muscular dystrophy, thus widely extending the range of phenotypic expression of the dystrophin gene (Arikawa et al., 1992). According to Lunt et al. (1989), Norman et al. (1989), Sunohara et al. (1990) and Arikawa et al. (1991), all male patients with progressive muscular dystrophy of the kind of LGMD/QM/SMA should be routinely screened for dystrophin abnormalities at the gene and protein level as a useful adjunct to their clinical diagnosis since the results have important implications for genetic counseling of affected families.

The molecular analysis of the dystrophin gene has been undertaken in muscular dystrophy (DMD/BMD/QM/LGMD/SMA) patients to understand the relationship between a given deletion and the resulting phenotype. An attempt has been made to study:

1. Intragenic deletions:

    a) Its frequency and distribution in patients and family members.

    b) A comparison of the deletion frequency in isolated and familial cases.

    c) To look for the possible differences in deletion patterns of DMD and BMD patients.

    d) Correlation between the size and distribution of the deletions in boys with mental subnormality and those with normal intelligence.

2. Reading frame hypothesis: to evaluate the concordance between the clinical phenotype and the reading frame hypothesis propounded by Monaco et al. (1988).

3. Intrafamilial variability in the severity of the disease.

4. Carrier status of female relatives of the DMD/BMD patients

REVIEW OF LITERATURE

CLINICAL FEATURES

Duchenne Muscular Dystrophy (DMD) was first described by Meryon in 1852 followed by Guillaume Duchenne de Boulogne and the inheritance pattern recognized by William Gowers in 1879 (Lidov and Kunkel, 1993). It is the most common and devastating of the muscular dystrophies. The disease is characterized by progressive muscular weakness and severe degeneration of skeletal muscles. The first symptoms are observed at the age of about 2-3 years. Somewhat later, patients develop a waddling gait, have difficulty in climbing stairs and are subject to frequent falls. Cardiac involvement is common. Intellectual levels are low in some patients. The skeletal muscle weakness is progressive and symmetrical, affecting first the proximal muscle of the lower extremities. Patients are generally wheelchair bound by the age of 10 years and death results around the age of twenty (Worton and Burghes, 1988).

Becker Muscular Dystrophy (BMD), a milder myopathic disorder described by Becker and Keiner (1955) shows very similar clinical features to DMD. It is distinguished from DMD by its slower rate of progression, resulting in a less severe phenotype. Onset is usually between 5 and 15 years and the disabling characteristics ensue much more slowly. Some of the patients have near normal routine up to 40 or 50 years of age. Cardiomyopathy is also a feature of BMD and some degree of cognitive impairment appears likely. Clinical severity and time course vary greatly, ranging from cases almost as severe as DMD to much milder forms; thus, the clinical

distinction between DMD and BMD in some cases is rather arbitrary (Lidov and Kunkel, 1993). There are also cases of intermediate severity that are difficult to assign to either the Becker or Duchenne categories (Rideau, 1977; Brooke et al.,1983; Spielgler et al., 1987).

Limb girdle muscular dystrophy (LGMD) usually has an autosomal recessive inheritance pattern although an autosomal dominant inheritance has occasionally been observed. The patients show preferential involvement of shoulder and/or pelvic girdle and most of them remain ambulatory until middle age (Sheilds, 1986; Emery, 1988). Patients with mild and slowly progressive muscle wasting and weakness limited to the quadriceps femoris muscle have been diagnosed as quadriceps myopathy (QM) (Arikawa et al., 1992).

The DMD/BMD patients have increased serum activity (10-100 folds) for the enzyme creatine kinase (CK), pyruvate kinase and aldolase. In 70-80% of female carriers, moderate increase in the serum enzyme activities can be detected. Although unrelated to the basic defect these biochemical markers are still useful as diagnostic tests for the disease.

On muscle biopsy, both DMD and BMD present features typical of myopathy. As early as one year there is excessive variation in fiber size and a subtle increase in the thickness of connective tissue septae between myofibrils. With the passage of years large rounded hypertrophic fibers, fiber splitting, central nucleation, fiber necrosis and basophilic regenerating fibers are prominent. There is marked increase in endomesial connective tissue, which manifests as pseudohypertrophy, and cellular infiltrates composed of lymphocytes and macrophages, as well as,

fibroblasts, and myocyte nuclei. In the end stage biopsy, muscle fibers are sparse and atrophic and contractile elements are replaced by sheets of dense connective tissue and fat (Lidov and Kunkel, 1993).

Both DMD and BMD are inherited as X-linked recessive disorders. As such, the vast majority of affected individuals are male hemizygotes. Though most cases are transmitted via an unaffected carrier (heterozygote) mother, 30% of cases exhibit no previous family history and are considered "spontaneous" cases involving a de novo mutation in the germ line of either the mother or her parents.

Duchenne muscular dystrophy is amongst the most common genetic diseases, occurring with a frequency of approximately one in every 3,500 live male births in European population (Emery, 1991). The incidence of BMD at birth is reported to be approximately 1 in 30,000 males (Garden-Medwin, 1980) and 1 in 18,348 males (Hoffman et al., 1989). However, Mostacciuolo et al. (1993) recalculated the incidence rate of DMD and BMD in a given sample of the Italian population using the results of DNA and dystrophin analysis. While the incidence rate of DMD remained unchanged, the new figure for the incidence of BMD (1 per 13,888 male live births) was much higher than previously reported, since molecular diagnosis revealed additional cryptic cases. In a study on the racial distribution of DMD in west Midlands region of Britain, Roddie and Bundey (1992) found that the incidence of DMD among patients of Indian origin was one in 1388 and no other data on the incidence of DMD in Indians was available. Although DMD/BMD manifests predominantly in males, a small number of females with DMD/BMD have been reported. Translocations between the X-chromosome and autosomes are found to

be the major alterations observed in the karyotypes of these patients. The breakpoints lie in the Xp21 region and disrupt the dystrophin gene. This along with the non-random inactivation of the X-chromosome leads to the expression of the diseased phenotype (Boyd et al. 1986, 1988). These cases vary from mild (in manifesting carriers) to severe (in which the patient has a translocation breakpoint in dystrophin gene). The major translocations can be visualized by various chromosome banding techniques. The parental origin of the translocations have been studied using the probe M27b which detects the methylation pattern in active and inactive chromosome. In eight females investigated the translocations were found to be paternal in origin (Bodrug et al. 1990; Boyd and Fraser, 1990).

MOLECULAR GENETICS OF DMD/BMD

Mapping the gene to chromosome band Xp21

The discovery of the gene locus for DMD at Xp21 came, almost simultaneously, from three sources. New techniques of molecular genetics were applied to isolate DNA sequences from cloned fragments, derived from the human X-chromosome, for determining their exact location (Davies et al., 1981). In fact, Duchenne muscular dystrophy was the first disease to be localized in this way by linkage with these restriction fragment length polymorphism (RFLPs) (Murray et al., 1982). Secondly, the exchange point in a number of young girls with muscle disease, who had balanced X:autosome translocations, was shown to consistently reside on the short arm of the X chromosome at Xp21. There are more than 20 examples reported in the literature (Rowland, 1988). The final evidence to support the gene locus for DMD was the presence of microscopic deletions in some DMD boys with complex

phenotypes. The first such patient had five different X-linked conditions including DMD, chronic granulomatous disease, retinitis pigmentosa, the McLeod syndrome and mental retardation (Francke et al., 1985). High resolution cytogenetic analysis of DNA from a similar patient mapped the locus to within sub-bands Xp21.2-Xp21.3 (Bartley et al., 1986). Linkage studies in cases of BMD pointed to the same locus, confirming that the two conditions are allelic and due to the abnormalities of the same gene (Kingston et al., 1983, 1984; Bertelson et al., 1986).

Isolation and cloning of the segments from the DMD locus

The DMD patient BB described by Francke et al. (1985), played a crucial role in further defining the DMD gene. Kunkel and his colleagues (1985) used a method (phenol enhanced reassociation technique or pERT) to enrich the DNA sequences that lay within the deleted region DMD gene in this patient. The method involved the hybridization of sheared DNA from the BB patient with trace amounts of restriction enzyme digested DNA from a male with four X chromosomes. The conditions of annealing were such as to enhance the reassociation of unique sequences. Seven clones were isolated, which mapped within the BB deletion. Only one clone of 200 bp, called pERT 87, was a candidate for DMD locus because it failed to hybridize in five of 57 unrelated DMD males (Monaco et al., 1985). One of the five also had a brother with the same deletion. Analysis of this pedigree demonstrated that the DMD deletion had been inherited from the maternal great grandmother, by the mother of the affected boy (Bartlett et al., 1985).

A second independent approach was used to isolate sequences within the DMD locus by isolating a DNA fragment from a female with DMD who had a

balanced X:21 translocation (Verellen-Dumoulin et al., 1984). The site of the translocation in the autosome was found to be within genes encoding for ribosomal RNA (Worton et al., 1984). The identification of ribosomal DNA sequences on both translation-derived chromosomes led to the isolation of an 11 kb DNA fragment, XJ1, which contained the translocation junction from the X-derived chromosome (Ray et al., 1985).

DYSTROPHIN - THE PROTEIN PRODUCT OF THE DMD LOCUS

Dystrophin is the protein product of the Xp21 gene which is defective in patients with DMD/BMD (Koenig et al., 1988). The cDNA of the gene has been cloned and sequenced (Koenig et al., 1987). Dystrophin is encoded by an mRNA of 14 kb derived from at least 79 exons spread over 2400 kb of the X-chromosome (den dunnen et al., 1989; Roberts et al., 1992a). The 427 kDa protein has 3,685 amino acids (Hoffman et al., 1987a).

Dystrophin structure

Based on its deduced primary structure, dystrophin was originally predicted to consist of four distinct regions, dominated by a large rod-shaped domain composed of 24 spectrin-like repeats with an overall length of 125 nm (Koenig et al., 1988). The large rod domain is flanked on its amino terminus by 240 amino acids with high homology to the actin-binding domains of alpha-actinin, spectrin and Dictyostelium actin-binding protein 120 (Koenig et al., 1988; Bresmick et al., 1990; Karinch et al., 1990). Towards the carboxy-terminal of the rod domain of dystrophin is a cysteine-rich region with significant homology to a domain of

Dictyostelium alpha-actinin that contains two Ca^{2+} binding sites. The Ca^{2+} binding domain is thought to be non-functional in skeletal muscle dystrophin (Anderson and Kunkel, 1992). The last carboxy-terminal 420 amino acids comprise the fourth distinct domain of dystrophin, which was supposed to have no relation to any previously characterized proteins until the discovery of "B" gene product, which has homology to this region. It is found in muscle and is autosomal (Love et al., 1989; Ervasti and Campbell, 1993).

Dystrophin abnormalities

Dystrophin protein is generally absent in DMD whereas in BMD, this protein is altered in molecular size or amount (Hoffman et al.,1988a). However, the presence of a few muscle fibers with dystrophin-positive labeling has been observed in majority of DMD patients (Nicholson et al., 1989; Hoffman et al., 1990; Vainzof et al., 1990; 1991). These isolated normal-appearing fibers have been called "revertants". Vainzof et al. (1991) also found a relatively high proportion of partially stained "Becker-like" fibers in the majority of their DMD biopsies (18/22) relative to previous studies. They observed a higher percentage of fibers labeled with the amino-terminus antibody compared to the carboxy-terminal one. Their data, thus, supported the hypothesis that DMD patients may produce low levels of truncated dystrophin molecules.

Majority of the patients with other muscular dystrophies have normal dystrophin (Hoffman et al., 1988a; Vainzof et al., 1990). However, four male patients with QM were reported in a study by Sunohara et al. (1990) who had clear abnormalities of dystrophin protein. They concluded that the syndrome called QM

includes a group of forme fruste of BMD. Arikawa et al. (1991) found that 20% of patients with clinical diagnosis of LGMD showed abnormalities of dystrophin. Verma et al. (1992) described two cases of childhood muscular dystrophy who had clinical features suggestive of Emery-Dreifus muscular dystrophy and Prader-Willi syndrome respectively. Muscle biopsy from both cases revealed a clear abnormality of dystrophin. They were thus, diagnosed as DMD by immunofluorescence examination.

Function of dystrophin

The amino acid sequence of the 427 kDa protein closely resembles spectrin (a major erythrocyte membrane protein) (Koenig et al., 1988), and like spectrin, dystrophin binds actin filaments (Levine, et al.,1992). Dystrophin is part of the membrane cytoskeleton and may contribute to a physical link between the intracellular contractile myofibrils and the extracellular basal lamina (Hoffman et al., 1987b; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Dickson et al., 1992; Minetti et al., 1992a; Porter et al., 1992; Straub et al., 1992). Many of the proteins normally bound directly or indirectly to dystrophin show a secondary deficiency in DMD (Ohlendieck and Campbell, 1991; Minetti et al., 1992b). The binding of dystrophin to the membrane glycoprotein complex is thought to be mediated by the third domain (cystein-rich) and first half of the carboxy-terminal domain (Suzuki et al., 1992). In DMD the dystrophin protein is lost due to the mutations in the corresponding gene. Most of the associated proteins show secondary deficiency. The consequences to the muscle fiber is increased membrane fragility, leading to fissures of the plasma membrane. Leakage of cytoplasmic contents to the

extracellular space and extracellular components into the fiber (Ca^{2+}) is then thought to occur. This leakage is proposed to serve as a trigger for proliferation of the extracellular matrix, leading to failed regeneration of muscle (Hoffman and Wang, 1995). Ultrastructural studies suggest that the C-terminal end is membrane bound, and the recent description of glycoproteins associated with dystrophin may indicate part of the membrane binding mechanism. The overall functional appearance seems to be consistent with the role as a cytoskeletal component that links plasma membrane or integral membrane proteins to the actin scaffold (Lidov and Kunkel, 1993).

Animal models of Xp21 dystrophies

A number of animal models of the Xp21 dystrophy have been identified in recent years and have already attracted a lot of interest both from the investigative and therapeutic point of view. These include the mdx mouse which is genetically homologous to the human disease and lacks dystrophin, but has a practically normal clinical phenotype (Bulfield et al., 1984; Hoffman et al., 1987c); the xmd dog, an X-linked muscular dystrophy, which both clinically and pathologically is very similar to Duchenne dystrophy, and also lacks dystrophin (Cooper et al., 1988); and an xmd cat with deficiency of dystrophin but not clinically weak and showing marked prominence of the muscles (Carpenter et al., 1989).

The consequences of the lack of dystrophin from skeletal muscles of dog and man are devastating, and give rise directly or indirectly to the progressive degeneration of skeletal muscle fibers and possibly to the malfunction and/or degeneration of cardiac and smooth muscle as well. The early stages of regeneration

of the muscles of dystrophic patients do not appear to be affected by the absence of dystrophin, but the growth and maturation of the newly regenerated fibers is impaired, and it is generally accepted that the muscle fibers enter repeated cycles of degeneration and regeneration until the regenerative capacity of the tissue is exhausted (Webster and Blau, 1990). In contrast, the dystrophin-deficient muscles of mice compensate well for the loss of dystrophin, go through a limited cycles of degeneration and regeneration, grow to maturity and have apparently normal functional capacity.

Clearly, the widespread occurrence of dystrophin between species and in numerous cell types, both contractile and non-contractile, indicates that it is a protein of considerable importance to the architecture, development, survival and function of the cell. Its precise role in the life-cycle of the cell is, however, less clear (Harris and Cullen, 1992).

STRUCTURE OF DYSTROPHIN GENE

In a study describing the complete cloning of the dystrophin cDNA (Koenig et al., 1987), it was shown that hybridization of the full-length clone to human genomic DNA digested with HindIII yielded 65 distinguishable bands. As there are only five HindIII sites within the cDNA sequence, this implied a minimum of 60 exons. The approximate order of these fragments, with a number of ambiguities, was established by cDNA hybridization to deletion patient DNA and genomic contig DNA from the walks surrounding pERT87, pERT84, J-Bir, J-MD, and J-47 (Monaco et al.,1987). Large-scale mapping of the gene (van Ommen et al., 1986; Kenwrick et al., 1987; Burmeister et al., 1988; Meitinger et al., 1988; den Dunnen et al., 1989)

using pulsed-field gel electrophoresis (PFGE) has placed limits on the distances between a number of intragenic landmarks and has led to an estimate of 2.4 Mb for the total size of the gene. This is, to date, the largest gene ever characterized, and contains the largest intron known [brain intron 1 is 400 kb in size (Boyce et al., 1991)].

The dystrophin gene comprises at least 81 exons that span approximately 2.4 Mb of DNA in Xp21. The major muscle isoform of the gene is encoded on 79 exons and is transcribed from a promoter that is expressed predominantly in muscle (Coffey et al., 1992; den Dunnen et al., 1992; Roberts et al., 1992a). A second promoter is used predominantly in the brain , whereas a third promoter, near the 3' end of the locus, is active to varying degrees in virtually all tissues examined (Blake et al., 1992; Lederfin et al., 1992). Bies et al. (1992c) found that mouse and human tissues contain at least 11 alternatively spliced mRNA isoforms at the 3' end of the gene, and that these isoforms are expressed in both tissue-specific and developmentally regulated patterns. It remains unclear whether specific spliced forms of the mRNA are expressed only from a subset of the three promoters (Chamberlain, 1992).

The 3' end of the gene is of particular interest because it encodes the portion of dystrophin that is not structurally related to spectrin or alpha-actinin. That this region of the gene can be expressed separately from the 14 kb mRNA suggests a unique role for the 71 kDa isoform and implies that the dystrophin gene arose during evolution by fusion between a spectrin like gene and a gene corresponding to the 4.9 kb transcript. Hoffman et al. (1991) described an unusual case of DMD in which the entire C-terminus of the gene was deleted. The patient displayed

membrane bound dystrophin and had a phenotype that was more sever than typical DMD, providing further evidence that the C-terminus is functionally important (Chamberlain et al., 1992).

Expression of dystrophin gene

In normal individuals, dystrophin is found in muscle tissue (striated, cardiac, and smooth), and in lower amounts in many other electrically active cells (plexiform layer of retina, myoepithelial cells, some neuronal synapses, glia, and cardiac Purkinje fibers) (Hoffman et al., 1988b; Chelly et al., 1990a; Houzelstein et al., 1992; Miyatake et al., 1990; Gorecki et al., 1991; Bies et al., 1992b). Within expressing cells, dystrophin appears at plasma membranes and membrane specializations (neuromuscular junctions, myotendinous junctions, and sunsynaptic regions of neurons) (Lidov et al., 1990; Byers et al., 1991; Yeadon et al., 1991; Squarzani et al., 1992; Tamura et al., 1993). Many of these tissues express specific protein products (isoforms) that arise from alternative promoters, or splicing, or both (Feener et al., 1989; Ahn and Kunkel, 1993). Some of these isoforms consists of only the carboxy-terminal regions of dystrophin (Dp71; Dp116) and have been shown to be expressed in most tissues, except for muscle tissue (Bar et al., 1990; Hugnot et al., 1992; Byers et al., 1993). The pathologic consequences of deficiencies of dystrophin isoforms in non-muscle tissues is overshadowed by the clinical disease resulting from deficiency of full-length isoform in muscle tissue. Dystrophin deficiency in most non-muscle tissues does not cause any overt clinical disease (Hoffman and Wang, 1995).

Chelly et al. (1988) used *in vitro* co-amplifications of the mRNAs of the dystrophin gene and of a reporter gene aldolase A, and were able to obtain a quantitative estimate of the dystrophin gene transcript. They detected a processed, transcribed segment in 13 different human tissues. It ranged from 0.02-0.12% of total mRNA in skeletal muscle to 25,000 times less in lymphoblastoid cells.

Boyce et al. (1991) studied the expression of dystrophin in brain. They demonstrated that the 5' region of dystrophin found in brain contains an alternate promoter for dystrophin, which is located >90 kb upstream of DXS142 region containing the major muscle promoter (Klamut et al., 1990). This finding suggested that deletion mutations may disrupt expression of one tissue-selective promoter without affecting expression in other tissue, thus giving rise to novel phenotypes. They identified a BMD patient with a deletion of muscle promoter with low level of dystrophin expression in muscle; and suggested that the dystrophin transcription in this patient occurred from an alternative promoter.

NATURE OF THE MUTATIONS IN THE DYSTROPHIN GENE

Duchenne and Becker muscular dystrophies are caused by mutations in the dystrophin gene. Because of the extremely large size of this gene, the majority of the mutations are intragenic deletions (~65%) or duplications (~5%). Point mutations in the coding and regulatory regions of the gene and in the splice sites which affects the mRNA processing, are responsible for the disease in one-third of the DMD/BMD patients.

Deletions

Deletions of the dystrophin gene in 6%-10% of individuals with DMD or BMD were first detected with DNA probes pERT 87 (DXS164; Kunkel et al., 1985) and XJ (DXS206; Ray et al., 1985) (Monaco et al., 1985; Hart et al., 1986; Kunkel et al., 1986; Thomas et al., 1986). The use of genomic probes J-Bir (DXS270) and J66-H1 (DXS268; Monaco et al., 1987) increased the number of detectable gene deletions to 17% by Southern analysis and to more than 50% by field-inversion gel electrophoresis (den Dunnen et al., 1987). The later results indicated that a deletion "hot spot" existed in the 950 kb between probes J-Bir and J66-H1. The position of the "hot spot" was further defined by genomic clone p20 (DXS269) within this region, which revealed deletions in 16% of DMD/BMD patients (Wapenaar et al., 1988).

Koenig et al. (1987) found the overall deletion frequency for the DMD gene to be 50% by using a series of approximately 1 kb dystrophin cDNA subclones on HindIII digests of DNA samples from 104 patients. Deletions of the gene were found to occur frequently in two regions of the 14 kb cDNA. The first of these (cDNA 1b) correspond to the pERT 87/XJ region and accounted for 28% of deletions, while the second (cDNA 8) mapped between J-Bir and J66-H1 and accounted for 51% of deletions. Similar findings were reported by Darras et al. (1988a), who found an overall deletion frequency of 66% (21/33 patients) for the DMD gene. Of these 21 patients, 57% had deletions of exons detected by cDNA 8 and 29% had deletions of exons detected by cDNA 1-3. Deletions of the DMD gene were also detected in PstI digests of DNA samples from 59 of 107 DMD/BMD patients by using two cDNA clones, Cf23a and Cf56a (Forrest et al., 1987a, 1988). These two clones correspond

approximately to cDNA 7 and cDNA 8 of Koenig et al. (1987). Lindlöf et al.(1989) reported that the frequency of deletions increased from 16% to 41% when cDNA probes were used to analyze 49 patients who were previously studied with genomic markers.

Two deletion prone regions, corresponding to cDNA probe 1-2a and probes 7 and 8 have now been confirmed by several independent groups (Cooke et al., 1990; Blondon et al., 1991; Walker et al., 1992; Nicholson et al., 1993a). Combined use of probes from these two areas detects 86 to 99% of deletion mutations (Cooke et al., 1990; Blondon et al., 1991; Walker et al., 1992; Nicholson et al., 1993a). This has had considerable impact on the development of screening strategies for prenatal diagnosis (Love and Davies, 1989; Walker et al., 1992).

Norman et al. (1990) analyzed BMD patients from 58 separate families and reported that BMD deletions are homogeneous. They observed a common pattern of deleted exons in 49% of the families with deletion. Passos-Bueno et al. (1990) also reported that the deletions in their BMD patients were homogeneous and also found a higher frequency of deletions in sporadic (73%) compared with familial DMD (28%) and BMD cases (33%). However, the deletions in DMD patients are quite heterogeneous with respect to the size and location of deletions. There is considerable variation in the severity and progression of the disorder among affected individuals. An understanding of the relationship between a given deletion of the DMD gene and the resulting phenotype would be of considerable value for clinical prognosis and would provide further insights into the role of dystrophin in the disease.

The correlation between the pattern of deletion and the clinical severity has been studied (Malhotra et al., 1988; Monaco et al., 1988; Read et al., 1988; Gillard et al., 1989; Koenig et al., 1989; Liechti-Gallati et al., 1989; Upadhyaya et al., 1990). These studies have shown that the size of deletion and the clinical severity are not completely in parallel. On the basis of 3 BMD and 3 DMD patients with deletions in the DXS164 locus, it was proposed that the milder BMD phenotype resulted from DMD gene deletions that maintained the translational reading frame, while the more severe DMD phenotype resulted from DMD gene deletions that shifted the translational reading frame (Monaco et al., 1988).

To assess this hypothesis, HindIII genomic DNA fragments that correspond to successive exons were identified and their borders sequenced (Chamberlain et al., 1988; Malhotra et al., 1988; Monaco et al., 1988; Koenig et al., 1989; Roberts et al., 1992b, 1993), so that it is now possible in most cases to predict, on the basis of the pattern of deleted HindIII fragments detected by a cDNA probe, whether a patient has an "in-frame" or an "out-of-frame" deletion (Mandel, 1989). Exceptions to the "reading frame rule" were first reported by Malhotra et al. (1988) who showed that "out-of-frame" deletions of exons 3-7, which remove most of the amino-terminal domain, produce phenotypes that vary from a mild or severe Becker to a Duchenne type. They proposed that the transcription could begin on a methionine codon just downstream to the deletion, giving rise to a truncated protein. Other exceptions were described by Baumbach et al. (1989). In a large multicentre study, 258 independent deletions were analyzed and compared with clinical evaluation of patients (Koenig et al., 1989). The type of exon-intron borders of the remaining two exons that flanked the deletion were examined to analyze the effect of the deletion

on the reading frame. They classified the exon borders as 1, 2, or 3 if they were after the first, second, or third nucleotide of a coding triplet, respectively. The intron-exon borders were, thus, also classified as 1,2, or 3 if they were before the second, third, or the first nucleotide of a coding triplet, respectively. They postulated that a deletion that juxtaposes two exons with borders of the same type maintains the reading frame. In contrast a deletion that juxtaposes two exons with borders of a different type disrupts the reading frame, leading to early termination of the protein translation. Majority (92%) of the cases followed the reading frame model; 4% of the exceptions were either of the deletion of exon 3-7 (Malhotra et al., 1988), or were in-frame deletions of more than 30 exons leading to severe disease. Thus, only 4% of the exceptions could not be accounted for (Koenig et al., 1989). Alternative splicing mechanisms such as exon skipping could well provide an explanation for the latter cases (Chelly et al., 1990b).

A puzzling observation made by Koenig (1989) was the lack of Becker deletions in the region of exons 31-44 (within the spectrin like domain), whereas many Becker deletions were found that extended toward the 3' end of exon 44. The Duchenne deletions were found both on the 5'and 3' side of intron 44. This led Koenig to hypothesize that the deletions in the region of exons 31-44 that follow the reading frame might result in normal phenotype or in very mild muscular symptoms. In support of this, Gospe et al. (1989) described a large family with X-linked myalgia and cramps but without muscle weakness, and where a relatively large deletion of the dystrophin gene was found that encompasses exons 10-22. In another such family, where no deletion was found, the trait cosegregated with restriction-fragment length polymorphism (RFLP) markers within the dystrophin gene.

Based on the "reading frame" hypothesis Koenig et al. (1989) proposed that DMD patients with deletions beginning with exons 54, 55, or 58 could potentially synthesize the first three quarters of the dystrophin protein, including the N-terminal domain and the first 22 or 24 repeat units of the rod domain. These truncated molecules were predicted to lack only the last 800-1000 amino acids, including the cysteine-rich and C-terminal domains, and were either non-functional or unstable, because they resulted in DMD. Therefore, at least a part of the last two domains of dystrophin seemed to be essential for the function or stability of dystrophin. In contrast, the first two domains could be entirely missing (N-terminal domain) or partially missing (up to 1400 amino acids or 13 repeats of the rod domain) and still resulted in milder BMD. They did not observe any deletions in the regions encoding the cysteine-rich and C-terminal domains.

The use of genomic probes may identify some individuals with deletions, in the intronic DNA alone, yet do not show any clinical signs of DMD (Koh et al., 1987; Bartlett et al. 1989). This suggested that the diagnostic accuracy in prenatal screening of fetuses using such intronic probes might not be 100%. Diagnostic accuracy is higher using cDNA probes. However, one normal male with inherited deletion of one exon within the DMD gene has been reported (Nordenskjold et al., 1990).

Hart et al. (1989) described the pathogenic and non-pathogenic deletions in two families with DMD. They detected 3.5% non-pathogenic deletions in normal males from DMD families and 0% non-pathogenic deletions in normal males from non-DMD families. They speculated that these "silent" deletions in normal members

of DMD families have a role in the generation of further lethal deletions, possibly by unequal pairing at female meiosis. The deletions did not seem to affect the fitness of their carriers. Thus, they could be "premutations" predisposing to the development of a DMD/BMD gene in later generations, contributing to the very high mutation rate of this gene.

However, in all these studies the deletions were identified by Southern blot analysis of the genomic DNA. In almost all the cases, the mRNA is supposed to result from procession of the remaining exons based on an assumption in which the gene deletion would not affect splicing. Chelly et al. (1990) investigated PCR amplified dystrophin transcripts from muscle samples of DMD/BMD patients with known intragenic deletions. On sequencing these transcripts, they found some alternate splicing patterns that were not expected from their deletion patterns of the gene.

Anderson et al. (1992) analyzed the dystrophin mRNA in lyophilized biopsy tissue from two BMD patients with previously identified gene deletions and one individual with no deletion in the dystrophin gene. RT-PCR analysis revealed the deletion of additional exons which were not identified by DNA analysis. This emphasized the importance of mRNA analysis and also that the biopsy sample can be transported in lyophilized form at room temperature thereby obviating the need to transport frozen samples.

Recent studies have shown that abnormalities of the dystrophin gene are also present in neuromuscular syndromes besides DMD/BMD. Deletions in the dystrophin gene have been identified in some patients with spinal muscular atrophy

(Lunt et al., 1989), limb-girdle muscular dystrophy (Norman et al., 1989; Arikawa et al., 1991), quadriceps myopathy (Sunohara et al., 1990; Arikawa et al., 1992; Vonmitzlaff et al., 1993) and also in patients with Kugelberg Welander syndrome, scapulo-humeral dystrophy and a boy with the "stick-man" type of congenital muscular dystrophy (Kakulas, 1992). There are also several reports of BMD patients with atypical presentations including severe muscle cramps and/or cardiomyopathy in the absence of significant weakness (Kuhn et al., 1979; Gospe et al., 1989; England et al., 1990; Bushby et al, 1991; Palmucci et al., 1992; Quinlivan et al., 1995).

Muntoni et al. (1993) found a deletion of first muscle exon containing the muscle promoter of the dystrophin gene in all affected members of the family with a severe form of X-linked dilated cardiomyopathy. On the basis of their results, they proposed that the dystrophin gene and protein studies should be performed in families with X-linked cardiomyopathy in order to investigate a possible dystrophinopathy.

Maeda et al. (1995) studied the immunostaining pattern of cardiac dystrophin in endomyocardial biopsy samples from 83 patients with heart disease. They concluded that the discontinuous immunostaining pattern of cardiac dystrophin is characteristic of BMD and that an absent pattern may be associated with more severe cardiac dysfunction. Because genetic analysis cannot determine the correct diagnosis in 35% of DMD/BMD cases, they recommended routine examination of immunostaining pattern of dystrophin in endomyocardial biopsy samples in BMD patients with cardiomyopathy.

Nigro et al. (1995) evaluated the features and the course of cardiomyopathy in 68 BMD patients. They observed that the severity of cardiac involvement can be unrelated to the severity of skeletal muscle damage, and also confirmed that cardiac dysfunction is a primary feature of BMD.

Yu et al. (1995) reported a BMD family who presented with cardiac involvement. A G-to-T transversion at the terminal nucleotide of exon 13 was found. They concluded that the mutated dystrophin gene may cause symptoms of cardiac involvement prior to skeletal muscle involvement.

Segments of the cDNA have been used as probes to specifically examine all of the exons by Southern blot analysis. This requires radioisotope analysis and is more time consuming. The PCR is a non-radioisotope method, is rapid and requires only small amounts of DNA. It is generally used for screening small gene deletions. Southern hybridization methods can be used as a back-up method in cases where a suspected DNA rearrangement is not detected by PCR methods (Darras 1990). Chamberlain et. al. (1988) used PCR for the first time in the diagnosis of DMD/ BMD and they detected deletions in 37% of patients. Beggs et. al.(1990) reported that about 98% of deletions in patients with DMD or BMD could be detected by using primers for nine additional exons in conjunction with those described by Chamberlain et al.(1988, 1990) in two multiplex PCRs.

"Ectopic" or "illegitimate" transcription (Chelly et. al., 1988, 1989; Sarker et. al.,1989) in peripheral blood lymphocytes can be exploited to circumvent muscle biopsies for RNA studies and pathological DMD/BMD transcripts resulting from a genomic deletion can be detected in both hemizygous patients and heterozygous

carriers (Roberts et al., 1990; Schloesser et al., 1990). Roberts et al.(1991) have already shown that this procedure can be used for a comprehensive analysis of the complete coding region without the use of radioactive substrates. Rininsland et al. (1992) reported a new DMD gene mutation, identified exclusively by means of ectopic lymphocyte cDNA analysis in a patient with no apparent deletion after extended multiplex DNA amplification.

Approximately 30% to 50% of DMD patients have a moderate or severe non-progressive mental retardation (Emery, 1987) with high intrafamilial correlation among affected relatives (Cohen et al., 1968; Rabbi-Bartolini and Zatz, 1986; Ogasawara, 1989). The association between mental retardation and DMD is still not understood. According to Van Ommen et al. (1989) two alternative hypotheses might explain the enigmatic association of mental retardation and DMD:

1) a secondary effect of the malfunctioning of the dystrophin gene in brain during embryonic development; and/or

2) the presence of other unknown genes within the DMD gene, which could be damaged by DNA deletions.

The degree of mental retardation in muscular dystrophy was found to be related to the severity of the disorder in a study by Hodgson et al. (1989). However, they found that the distribution of deletions in boys with mental retardation was similar to that seen in all patients and showed no unique abnormalities. Rapaport et al. (1991) analyzed 162 DMD patients and two girls with a DMD phenotype for deletions in the central region of the dystrophin gene in order to determine if there

was any correlation between mental retardation and the pattern of deletions. They found that 70% of patients with a deletion of exon 52 of dystrophin gene were mentally retarded whereas only 38% of mentally retarded patients had deletions not involving exon 52.

Duplications

Duplications in part of the DMD gene were identified by probing the patients' DNA with both genomic probes (Bertleson et al., 1986; den Dunnen et al., 1987; Hu et al., 1988) and cDNA probes (Greenberg et al., 1988; Hu et al., 1988). Hu et al. (1990) studied 72 unrelated DMD/BMD patients with no exonic deletions and identified 10 duplications. They also found that duplications causing a shift in the translational reading frame tend to lead a more severe clinical phenotype than do duplications that maintained the reading frame.

In 17% of patients with exon duplication or deletion the breakpoint is sufficiently close to a non-deleted exon that it lies within the restriction fragment detected by a cDNA probe on a Southern blot (den Dunnen et. al., 1989). Such a band of altered mobility can be detected in carrier women. The frequency of detection of such diagnostic junction fragment, which provide unequivocal identification of the mutation in carriers, can be greatly increased by the pulsed-field gel electrophoresis (Chen et al., 1988; den Dunnen, 1989). Such junction fragments may also be detected in mRNA. Most deletion and duplication breakpoints lie within introns, which constitute over 99% of the dystrophin gene, so it is likely that transcription and transcript splicing are unaffected by the mutation. Thus, a transcript from a defective gene would probably differ from the normal transcript

only in that it bears a duplication or deletion of a number of exons. Amplification across the region of the mRNA which is duplicated or deleted should enable generation of a PCR product of anomalous size which is diagnostic of the presence of defective gene (Roberts et. al.,1990).

Point mutations

In about 30% of the DMD/BMD patients, the gross gene rearrangements are not detected. In these patients, the disease results due to the presence of point mutations. A screening for point mutations in the coding regions, splice sites and other relevant regions is, however, frustrated by extremely large size of the gene. Nigro et al.(1992) used a new approach involving multiple single strand conformation polymorphism (SSCP) and reported a nonsense mutation in a non-deleted DMD patient. A number of point mutations have now been described in DMD/BMD patients (Bulman et al., 1991a; Roberts et al., 1992a; Lenk et al., 1993b; Prior et al., 1993a, 1993b; Knepper et al., 1995; Lasa et al., 1995; Tuffery et al., 1995; Yu et al., 1995).

Genetic recombination

Genetic mapping has indicated that meiotic recombination occurs about 4 times more frequently in the dystrophin gene than expected on the basis of its length (Chen et al., 1989; Abbs et al., 1990; Oudet et al., 1991). Oudet et al., (1992) obtained evidence for two "hot spots" of recombination in the DMD gene: a major one between STR44, in the proximal part of intron 44, and STR50 (intron 50), and a minor one between the muscle dystrophin promoter and exon 8. The similar

localization of "hot spots" of both meiotic recombination and deletions suggest that they may share a common mechanism of genome instability.

DIAGNOSIS AND CARRIER DETECTION OF Xp21 DYSTROPHIES

Importance of early diagnosis

The recent advances in the understanding of the molecular genetics of DMD have led to improvements in the determination of female carrier status and to the provision of reliable antenatal diagnosis for most pregnancies at high risk (Forrest et al., 1987b). These advances highlight the importance of early diagnosis of DMD. Prevention of secondary cases within a family is possible if early diagnosis is improved and genetic counseling offered. It also allows for early comprehensive assessment and management, and has been shown to be desired by parents (Firth and Wilkinson, 1983). Smith et al. (1989) suggested that by screening all boys with unexplained overall developmental delay, especially in locomotor and language abilities, early diagnosis for DMD could be improved.

Dystrophin diagnosis of DMD/BMD

Although in normal individuals, dystrophin is detected immunocytochemically as a homogeneous ring around the periphery of all muscle fibers (Arahata et al., 1988; Sugita et al., 1988), muscle from DMD patients has little or no detectable staining (Arahata et al., 1988; Bonilla et al., 1988; Sugita et al., 1988), while muscle from BMD patients show variable staining and occasionally patchy and/or faintly stained fibers (Hoffman et al., 1988a; Bulman et al., 1991b). By immunoblot analysis, dystrophin is generally undetectable in muscle samples

from DMD patients. But, muscle biopsies from BMD patients show abnormal dystrophin protein of either altered molecular weight, or amount, or both (Arahata et al., 1989a).

Since defects in the DMD/BMD gene cause dystrophin with abnormal characteristics to be synthesized by patients with Xp21 muscular dystrophy (Hoffman et al., 1988a) it is possible that truncated dystrophin molecules, like those of other cytoskeletal proteins, will be broken down or turned-over more rapidly than normal (Lazarides, 1987) in these patients. Information on the entire repertoire of dystrophin-related proteins present in biopsy samples might therefore be relevant when trying to relate the clinical symptoms shown by patients to the form of dystrophin (size, abundance of breakdown products) present in their muscle (Nicholson et al., 1989). They concluded from their study that the majority of DMD patients have muscle fibers which can synthesize dystrophin in a limited manner.

DNA based diagnosis for DMD/BMD

DNA-based deletion analysis and linkage analysis are currently performed for clinical diagnosis. Using 18 pairs of multiplex primers designed by Chamberlain et al. (1988, 1990) and Beggs et al. (1990), up to 98% of gene deletions observed by cDNA can be identified by polymerase chain reaction (PCR) analysis (Beggs et al., 1990). Thus, PCR analysis is becoming useful because it is capable of rapid deletion detection on small quantities of DNA. However, duplications are harder to detect by PCR, so patients with no detectable mutations should be analyzed further by the Southern blot test (Arikawa et al., 1992).

Carrier identification

In familial cases of DMD, the disease is transmitted by a female carrier of the mutated gene. Carriers are heterozygotes, with a normal gene on one X chromosome and a mutated gene on the other X chromosome. If the son of a carrier woman inherits the mutated X chromosome, DMD inevitably appears. More than 90% of female carriers are asymptomatic (Moser and Emery, 1974). However, some carriers show calf hypertrophy or proximal limb weakness; these women have been called "manifesting" or "symptomatic" carriers (Moser and Emery, 1974; Boyd et al., 1986). The traditional clinical criteria for identifying a manifesting carrier are a family history of DMD, proximal limb weakness, high serum CK levels, and myopathic changes on both muscle biopsy and EMG (Moser and Emery, 1974; Yoshioka, 1981).

DNA analysis can define manifesting carriers in 55% of patients (Hoffman et al., 1992). In families where a deletion has been identified as the causative mutation, carrier determination can be performed by dosage analysis of several approaches such as Southern analysis (Darras et al., 1988b; Mao and Cremer, 1989; Hu et al., 1990; Prior et al., 1990a) or multiplex PCR (Beggs et al., 1990; Chamberlain et al., 1990; Prior et al., 1990b; Abbs and Bobrow, 1992; Ioannou et al., 1992), both in combination with sensitive spectrophotometric densitometry and computer assisted quantification (Ishii et al., 1992). Owing to sometimes subjective results, technical problems and some lack of reproducibility (Speer et al., 1989) the quantitative techniques are not widely applied for accurate carrier determination, whereas the usage of the chromosomal *in situ suppression hybridization* (CISS) (Ried et al., 1990) is limited by the fact that deletions have to span at least 2 kb of the dystrophin

gene. Regarding smaller deletions or splice site mutations more reliable results were obtained by a RT-PCR approach that determines the carrier status by the detection of bands of altered size due to gross gene rearrangements (Roberts et al., 1990) or exon skipping events (Roberts et al., 1992a). However, because of the extreme sensitivity of the RT-PCR this approach has always the risk of amplifying rare, aberrantly spliced ectopic transcripts from the lymphocyte RNA, which may complicate the evaluation of the results (Lenk et al., 1993a).

In cases without an identifiable mutation carrier detection depends mostly on DNA linkage studies, requiring RFLP markers (Hodgson and Bobrow, 1989; Bakker et al., 1985), polymorphic dinucleotide sequences (CA-repeats) (Beggs and Kunkel, 1990; Clemens et al., 1991; Feener et al., 1991; Hugnot et al., 1991; Patino et al., 1995) or other sequence polymorphisms within or flanking the dystrophin gene. However, the utility and accuracy of linkage analysis is often affected by small family size, inadequate family-member cooperation or deceased DMD males (Ward et al., 1989) and also by the extraordinary size of dystrophin gene with at least 5% recombination between its extremities (Abbs et al., 1990). Thus, the detection of disease causing point mutations by an appropriate routine approach would also allow a more reliable carrier diagnosis. The large size and the complexity of the dystrophin gene requires an efficient assay for the identification of point mutations that are sufficient to cause the disease. Roberts and co-workers (1992a) described an RNA approach, based on ectopic transcripts of peripheral blood lymphocytes, that permits scanning of the complete coding sequence for comprehensive mutation. This technically demanding method involves the use of hazardous chemicals and radioactively labeled probes, which probably prevent a wider application in routine diagnosis (Lenk et al., 1994).

Yau et al. (1993) reported a PCR-based direct test specifically designed for each of six previously described point mutation (Roberts et al., 1992a). The assay allowed accurate carrier testing involving naturally occurring or artificially introduced restriction sites at the mutation site, and, therefore, it requires at least one novel PCR primer.

For DMD carriers, immunocytochemistry is the most useful method for detecting patches of negative fibers among positive ones (Arahata et al., 1989b). However, it should be noted that even if there are no negative fibers, the diagnosis of a DMD carrier cannot be ruled out (Arikawa et al., 1992).

Molecular diagnostics of isolated cases of dystrophinopathy in females

Carriers of DMD with a positive family history and high creatine kinase levels show both dystrophin-positive and dystrophin-negative fibers on muscle biopsy (Bonilla et al., 1988; Arahata et al., 1989b); however, dystrophin testing is of little diagnostic utility: the high CK levels in females with a positive family history for DMD through maternal lineages defines them as carriers, and carriers with normal CK levels (30% of carriers) generally do not show dystrophin-negative fibers in their biopsy specimens (false-negative results). DNA analysis is therefore, the method of choice for detecting carriers in X-linked Duchenne/Becker pedigrees.

A major contribution of molecular diagnostics in female carriers has been the definition of a class of isolated female myopathy patients that show quantitative reduction in dystrophin (mosaicism) as the cause of their disease (Norman et al., 1989). A subset shows only hypercreatine kinasemia and are clinically normal.

Others show manifestations of disease ranging from easy fatigue in middle age to early onset weakness similar to DMD in a boy (Hoffman et al., 1992). The clinical criteria for suspecting dystrophinopathy in an isolated female myopathy are persistently elevated CK levels and myopathic histopathologic findings. Of girls and women fulfilling these criteria, 10% can be shown by immunofluorescence testing of muscle biopsy specimens to be carriers of DMD. Correct diagnosis of these patients is critical for appropriate genetic counseling of the proband and the immediate family (Hoffman and Wang, 1995).

STATUS OF EXPERIMENTAL THERAPEUTICS FOR DMD/BMD

Orthopedic surgery (tendon lengthenings for contractures and spinal fusions for scoliosis) can promote patients' mobility and comfort to some extent, and respiratory management by ventilation can substantially extend the lifespan of boys with DMD. Goertzen et al. (1995) performed a modified release of the spina muscles, resection of tensor fasciae latae muscle and a lengthening of the tendo calcaneus in 32 DMD patients (mean age 6.1 years). Their results demonstrated that early selective surgery in DMD patients just at or better before the onset of contractures without performing an additional aponeurectomy of the iliotibial band and percutaneous tenotomy of the hamstrings according to the original Glorion-Rideau technique safely prevents severe contractures and thereby delays the progression of scoliosis. Prednisone (0.15 to 0.75 mg/kg) has been shown to slow the progression of the disease, and, if started relatively early in the disease process, can add approximately 3 years of mobility (Fenichel et al., 1991; Backman and Henriksson, 1995). A different steroid, deflazocort, may have more limited

side effects and has been used successfully in clinical trials in Italy (Angelini et al., 1991). Unfortunately, all these therapies treat symptoms rather than the cause.

The identification and cloning of the gene responsible for DMD and characterization of the gene product, dystrophin, has led to major advances in the diagnostic and genetic counseling procedures for this inherited disorder (Kunkel and Hoffman, 1989; Hodgson and Bobrow, 1989; Dunne and Epstein, 1991). Due to its high mutation rate, however, individuals affected by DMD will continue to arise in large proportion by de novo mutations, and the search for direct therapy remains a high priority.

In this respect direct genetic correction of dystrophin deficiency via grafting of healthy myoblast stem cells (Partridge et al., 1989; Law et al., 1990; Karpati, 1991) or direct introduction of a functional DNA into diseased muscle tissue (Friedmann, 1989; England et al., 1990; Wolff et al. 1990; Felgner and Rhodes 1991; Lee et al. 1991) have both been proposed as potential therapeutic approaches.

Myoblast transplantation has received widespread media attention in the West, primarily due to the controversial nature of current human trials (Dubowitz, 1993; Hoffman, 1993). Biological constraints on efficiency of rescue by injected myoblasts have kept this method from approaching therapeutic efficacy: the effect of the injection is very localized (Patridge et al., 1989), the efficiency of "take" is very low (Gussoni et al., 1992), and immune barriers are likely to be present (Huard et al., 1992).

Direct injection of purified (naked) DNA with cellular uptake and expression seems possible only in muscle tissue (Wolff et al., 1990). Injection of the dystrophin

gene into dystrophin deficient mice has been shown to produce dystrophin in host muscle fibers (Ascadi et al., 1991). Again, both low efficiency and localized area of delivery surrounding the injection site limit the utility of this method as a full-fledged therapy for DMD.

Gene therapy offers hope in DMD and many other disorders. Most human clinical trials in gene therapy currently use an ex vivo approach: target cells are removed from a patient, infected (transduced) with a gene-carrying virus, and then reintroduced into the patient. This approach is not acceptable in DMD: one is limited to ex transduction of myoblasts that must then be delivered to muscle tissue via the same inefficient process used in myoblast transplantation. Therefore, in vivo delivery of the dystrophin gene must be accomplished in DMD. This may necessitate the use of truncated dystrophin mini-genes to accommodate the limited cloning capacity of current-generation viral delivery vectors. Phelps et al. (1995) constructed both murine and human mini-genes deleted for exons 17-48, and demonstrated that expression of either mini-gene can almost completely prevent the development of dystrophic symptoms in transgenic mdx mice. Similar results have also been reported by Wells et al. (1995) and Clemens et al. (1995). These studies suggested that viral-mediated expression of moderate levels of a truncated dystrophin could be an effective treatment for DMD.

MATERIAL

CLINICAL CASES

Patients with muscular dystrophy (Table 1 A-C) were included on the basis of clinical symptoms from the Out Patient Departments (Neurology) of A.I.I.M.S. and G. B. Pant hospital, New Delhi. Thirty two unrelated patients with proximal muscle weakness were tested for serum CK levels, EMG and ECG. Muscle biopsy was carried out in patients where consent was available. Of these, 8 boys were diagnosed as suffering from Duchenne muscular dystrophy (DMD), 10 males with Becker muscular dystrophy (BMD), 11 (7 male and 4 female) with limb girdle muscular dystrophy (LGMD), 1 male with quadriceps myopathy (QM) and 2 boys with spinal muscular atrophy (SMA).

Patients with Duchenne muscular dystrophy (Table 1A)

Eight male children (age range 4-10 years) presented to the clinic with a history of progressive difficulty in walking and getting up from squatting position since 2-6 years of age (Fig. 1A). On examination, muscle wasting, contractures, muscle hypertrophy, muscle power, Deep Tendon Reflexes, Gowers' sign and gait were recorded. **RS** had an elder brother suffering from the same illness who became wheelchair bound at the age of 6 years. The blood sample of the brother was not available. Another patient **HA** had a positive family history of the disease (details of family members not available). The CK in all patients was >1000 IU/L. Seven children showed an increase in the size of calf muscles over a time period. EMG done from quadriceps, gastrocnemius and deltoid revealed a myopathic pattern in

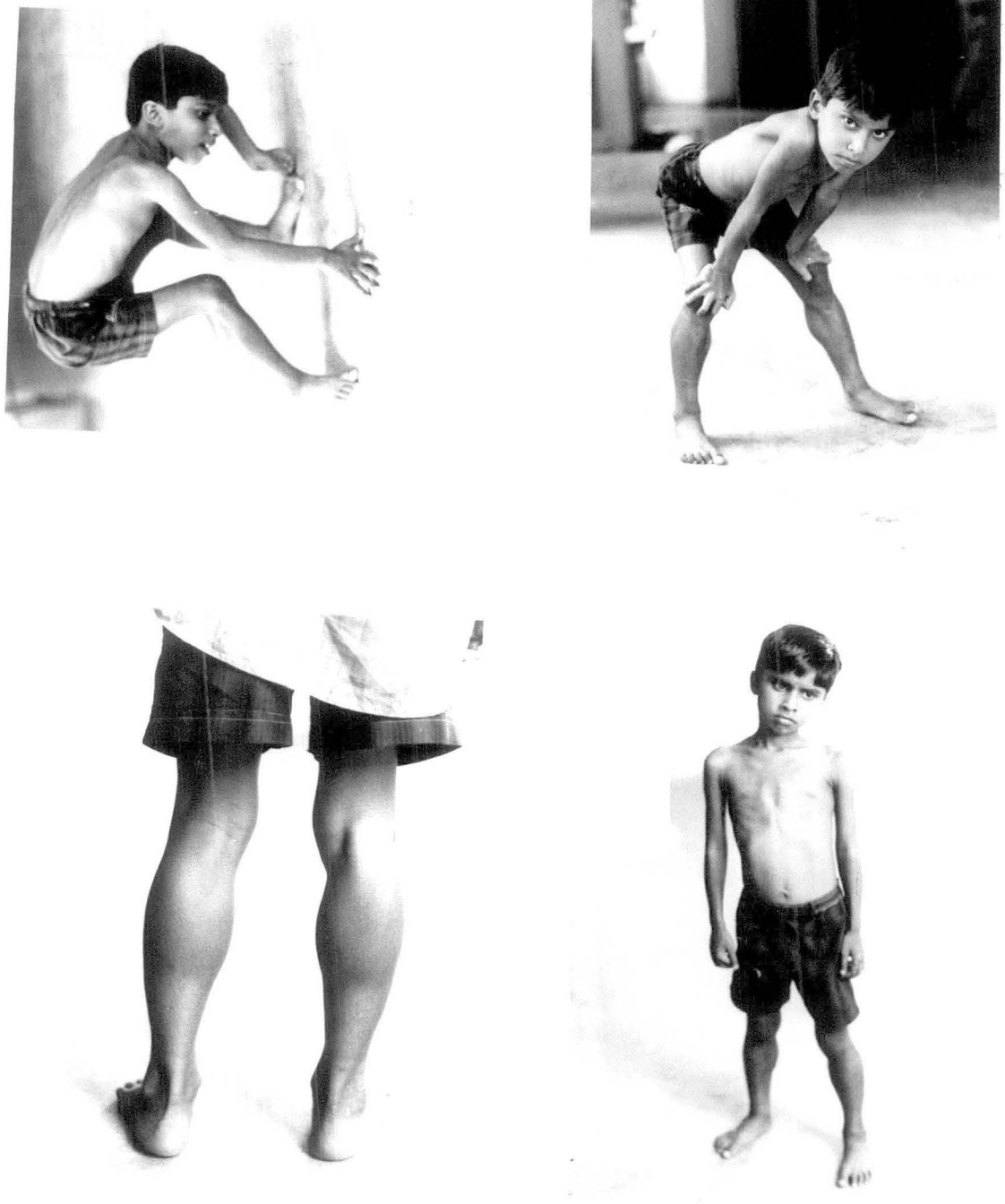


Fig. 1A: The Duchenne type muscular dystrophy: note the pseudo-hypertrophy of the calves and the characteristic method of rising from the floor.

3 patients (**AP**, **MS** and **SO**). In one patient (**RS**), EMG was normal. Investigations of 2 patients were not available (**NT** and **RH**), of which one (**NT**) died soon after hospitalization.

Patients with Becker muscular dystrophy (Table 1B)

Ten males (age range 9-22 years) presented to the clinic with a history of progressive proximal muscle weakness, pain in calves, muscle wasting in thighs and pseudohypertrophy of calf muscles (Fig. 1B). Four patients had a positive family history. **AR** had two elder brothers suffering from BMD (blood sample of only one brother was available). **PN** had a 16 year old cousin (maternal aunt's son) also afflicted with the same disease (blood sample not available). Maternal uncle of patient **DH** had the same disease (blood sample not available). **MO** had two elder brothers who became wheelchair bound at the age of 32 and 34 years, and a younger brother who was beginning to show the symptoms at the age of 14 years.

On examination, muscle wasting, contractures, muscle hypertrophy, muscle power, Deep Tendon Reflexes, Gowers' sign and gait were recorded. EMG mostly done from right quadriceps, gastrocnemius and deltoid revealed a myopathic pattern in all except one (**MO**) where it suggested a mixed pattern. CK was greater than 1000 IU/L in all the patients. Histopathology of muscle showed dystrophic changes.

Patient with quadriceps myopathy (Table 1C)

A 43-year-old male (**VK**) presented with the complaints of progressive buckling of the knees for the last five years, difficulty in going up and down the stairs and

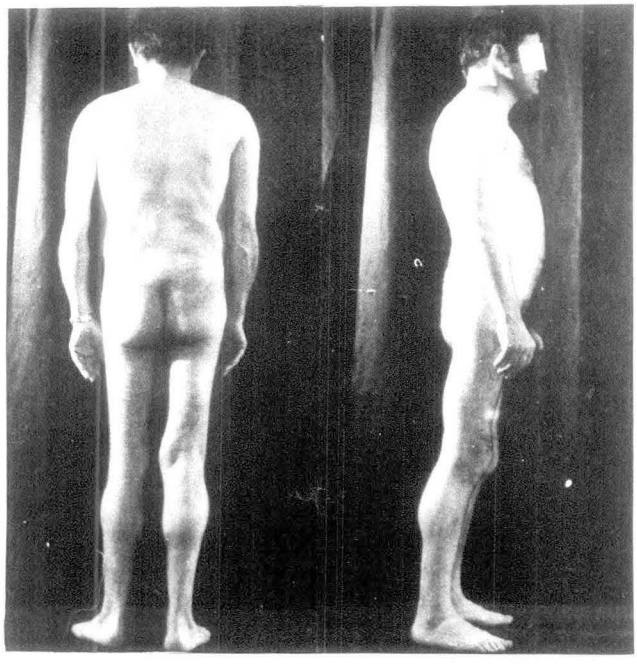


Fig. 1B: Becker muscular dystrophy: moderate hyperlordosis and pelvic anteversion, moderate muscular contractures in the lower limbs, pseudo-hypertrophy of calf muscles.

difficulty in getting up from the squatting position for 4 years. He also noticed the enlargement of calf muscles since adolescence. He underwent coronary artery bypass surgery three years back. On examination, he had hypertrophy of triceps, brachioradialis and calf muscles. There was thinning of both the thighs, right greater than left. Power in the upper limbs was 4/5, and in the lower limbs, around hip and knee 4/5 while, distally at the ankle 5/5. Gowers' sign was positive. Deep tendon reflexes were all elicitable with plantars down going. The gait was waddling in nature. EMG showed myopathic pattern and ECG was normal. The CK was 352 IU/L. Muscle biopsy was inconsistent. The patient after two years of follow up showed progressive difficulty in walking, climbing stairs and getting up from squatting position. He also had difficulty raising arms above the head for three years and mild weakness of the hand for the last two years.

Patients with limb girdle muscular dystrophy (Table 1C)

Seven males and four females with the clinical diagnosis of LGMD (age range 7-56 years) presented to the clinic with the complaints of progressive muscle weakness in lower limbs, pain in calf and thigh, and difficulty in getting up from squatting position. Calf hypertrophy was present in **PG, KK** and **HP** and was absent in **MN, PO** and **SC**. One patient (**HP**) had a younger brother with the symptoms of muscle disease. On examination, the EMG showed myopathic pattern in 5 patients. The CK of the patients was in the range of 157-1159 IU/L. In **MN**, the ECG showed right axis deviation along with sinus tachycardia, and was normal in **PG, NC** and **HP**. The histopathology of muscle in **MN** showed mild variation in the size of muscle fibers. There was no increase in fibrous or adipose tissue. The changes were

minimal and non-specific. In **NC**, **MP** and **HP**, a dystrophic pattern was revealed. The muscle biopsy was not informative in **RC**, **KK** and **PG**. All the patients were ambulatory. Investigations were not available for **SA**.

Patients with spinal muscular atrophy (Table 1C)

Two boys **AC** (6 years) and **VT** (3.6 years) reported to the clinic with the complaints of difficulty in getting up from squatting position and difficulty in walking and climbing stairs with frequent falls. On examination, there was proximal muscle weakness in both upper and lower limbs, no obvious calf hypertrophy and absent Deep Tendon Reflexes. The serum CK was marginally raised.

PERIPHERAL BLOOD SAMPLES

A total of 78 heparinised peripheral blood samples were collected from muscular dystrophy patients and their family members; DMD (8), BMD (10), LGMD (11), QM (1), SMA (2), mothers (14), fathers (9), brothers (5 symptomatic, and 8 normal), sisters (6), sons (2), daughter (1) and maternal uncle (1).

For the control study, heparinised peripheral blood samples were collected from 20 unrelated healthy individuals (11 males and 9 females) who had no family history of any muscle disease.

Table 1A: Clinical details of DMD patients

S.NO.	CODE	AGE (YEARS)			CK[a]	EMG[b]	ECG[c]	CH[d]	GS[e]	GAIT	PMW[f]	DTR[g]	DYSP[h]	MR[i]	MH[j]	FH[k]
		DIAG.	ONSET	WC												
1.	RS	4	3.6	7	>1000	N	N	+	+	TOE,W	LL,UL	+	-	-	ND	+
2.	AP	10	4	10.6	>1000	MP	ND	+	+	TOE,W	LL,UL	A+	NK	+	ND	-
3.	NT	12	DEAD													
4.	RH	8	3	-	>1000	ND	ND	+	+	TOE,W	LL,UL	ND	+	-	ND	-
5.	MS	6	2	-	>1000	MP	N	+	+	W	LL,UL	+	-	+	ND	-
6.	SO	6	2	-	>1000	MP	ST	+	+	W	LL,UL	+	NK	NK	DMD TYPE	-
7.	SD	9	7	-	15654	ND	ND	+	+	W	LL,UL	+	-	+	ND	-
8.	HA	8	5.6	-	5407	ND	ND	+	+	TOE,W	LL,UL	ND	NK	-	DMD TYPE	+

a = CREATINE PHOSPHO KINASE, b = ELECTROMYOGRAM, c = ELECTROCARDIOGRAM, d = CALF HYPERTROPHY,

e = GOWER'S SIGN, f = PROXIMAL MUSCLE WEAKNESS, g = DEEP TENDON REFLEXES, h = DYSPNEA, i = MENTAL RETARDATION BASED ON SCHOOL PERFORMANCE, j = MUSCLE HISTOLOGY, k = FAMILY HISTORY, DIAG. = DIAGNOSIS,

WC = WHEELCHAIR , N = NORMAL, LL = LOWER LIMBS, UL = UPPER LIMBS, ND = NOT DONE, NK = NOT KNOWN, A = ANKLE

MP = MYOPATHIC PATTERN, ST = SINUS TACHYCARDIA, W = WADDLING GAIT, '+' = PRESENT, '-' = ABSENT

Table 1B : Clinical details of BMD patients

S.NO.	CODE	AGE (YEARS)			CK[a]	EMG[b]	ECG[c]	CH[d]	GS[e]	GAIT	PMW[f]	DTR[g]	DYSP[h]	MR[i]	MH[j]	FH[k]
		DIAG.	ONSET	WC												
1.	AR	20	13	-	>1000	ND	ND	+	+	W	LL,UL	ND	NK	-	ND	+
2.	ARB	43	14	-	>1000	ND	ND	+	+	W	LL,UL	ND	NK	-	ND	+
3.	SR	19	17	-	932	MP	N	+	+	W	LL,UL	BTS+	NK	-	DYS	-
4.	RB	9	6	-	>1000	MP	N	+	+	TOE,W	LL,UL	+	+	-	DYS	-
5.	TR	20	14	-	>1000	ND	ND	+	+	TOE,W	LL,UL	K-	NK	NK	ND	-
6.	PN	12	10	-	5358	MP	ST	+	+	W	LL	-	NK	-	DYS	+
7.	JD	12	11	-	6200	MP	ND	+	+	W	LL	NK	NK	-	ND	-
8.	DH	14	13	-	4600	ND	AB	+	+	W	LL,UL	NK	+	+	DYS	+
9.	MO	16	12.6	-	>1000	MX	ND	+	-	W	LL,UL	1+	+	+	ND	+
10.	MOB1	40	14	34	INVESTIGATIONS NOT DONE											
11.	MOB2	35	14	32	INVESTIGATIONS NOT DONE											
12.	MOB3	14	14	-	INVESTIGATIONS NOT DONE											
13.	RL	22	18	-	>1000	ND	ND	+	+	W	LL,UL	NK	NK	NK	ND	NK
14.	MJ	10	2	-	>1000	MP	ND	+	+	TOE,W	LL,UL	+	NK	+	ND	-

B = BICEPS, T = TRICEPS, S = SPENCERS, K = KNEE, DYS = CONSISTENT WITH MUSCULAR DYSTROPHY, MX = MIXED PATTERN, AB = ABNORMAL OTHER ABBREVIATIONS AS IN Table 1 A

Table 1C : Clinical details of patients with QM/LGMD/SMA

S.NO.	CODE	AGE (YEARS)			CK[a]	EMG[b]	ECG[c]	CH[d]	GS[e]	GAIT	PMW[f]	DTR[g]	DYSP[h]	MR[i]	MH[j]	FH[k]
		DIAG.	ONSET	WC												
1.	VK(QM)	43	33	-	352	MP	N	+	+	W	LL,UL	+	-	-	INCONS	-
2.	MN(LG)	7	1	-	829	MP	ST	-	+	TOE,W	LL,UL	-	+	-	NS	-
3.	PG(LG)	36	34	-	1159	MP	N	+	-	KS,W	LL	K-	-	-	INCONS	-
4.	NC(LG)	30	20	-	>1000	MP	N	NK	+	W	LL,UL	RT+	-	-	DYS	-
5.	KK(LG)	13	12	-	349	MP	NK	+	+	W	LL	S,A+	NK	-	INCONS	-
6.	RC(LG)	56	50	-	293	MP	NK	NK	NK	NK	LL,UL	LT-	-	-	INCONS	-
7.	MP(LG)	18	-	-	157	MP	ND	NK	NK	W	LL,UL	+	NK	-	DYS	+
8.	SA(LG)	22	10	-	NK	NK	NK	NK	NK	NK	LL	NK	NK	NK	NK	-
9.	HP(LG)	20	12	-	4717	MP	N	RL+	+	W	LL	A+	-	-	DYS	+
10.	HPB(LG)	18	13.5	-	ND	ND	ND	+	+	W	LL	ND	-	-	ND	+
11.	PO (LG)	19	17	-	4042	N	AB	-	+	W	LL,UL	-	-	-	POLY	-
12.	SC (LG)	28	24	-	>1000	POLY	NK	-	-	W	LL	+	NK	NK	ND	-
13.	NG (LG)	34	24	-	NK	NK	NK	NK	NK	NK	LL	NK	NK	NK	NK	-
14.	AC(SMA)	6	3	-	108	ND	N	-	+	W	LL,UL	T+	NK	-	ND	-
15.	VT(SMA)	3.6	1	-	336	ND	ND	-	NK	N	LL,UL	-	NK	NK	ND	-

NS = NON-SPECIFIC, KS = KYPHOSCOLIOSIS, RT = RIGHT TRICEPS, A = ANKLE, POLY = CONSISTENT WITH POLYMYOSITIS, RL = RIGHT LEG, INCONS= INCONSISTENT OTHER ABBREVIATIONS AS IN Table 1 A AND 1 B

METHODS

ISOLATION OF GENOMIC DNA

The genomic DNA was isolated from peripheral blood drawn from the patients and their relatives. 6-7 ml of blood was suspended in 50 ml of lysis buffer and mixed gently on ice for 15 minutes. The mixture was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was discarded. The pellet obtained was resuspended in 4.5 ml of nucleus buffer. The nuclei were lysed by adding 250 µl of 10% SDS and 5 µl of Proteinase K (20 mg/ml) and mixed well by inverting the tubes upside down gently. The tubes were incubated at 37°C overnight. The solution was cooled to room temperature, equal volume of equilibrated phenol was added and the two phases were gently mixed by slowly turning the tube end to end for 15 minutes (till the two phases formed an emulsion). The tubes were centrifuged at 4000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a clean centrifuge tube using a thick bore pasteur pipette. The extraction was repeated once with phenol and twice with chloroform: isoamylalcohol (24:1) mixture. To the aqueous phase 0.1 volume 3M sodium acetate and 1.6 volume isopropanol were added. The DNA was allowed to precipitate at room temperature and transferred to an eppendorf tube using a wide mouth tip. The pellet was washed thrice with 70% ethanol at room temperature for 10 minutes each. The DNA pellet was allowed to dry at 37°C and was resuspended in 250 µl of TE buffer (pH 8.0). It was allowed to dissolve for a week at 4°C.

MULTIPLEX POLYMERASE CHAIN REACTION

To a 0.5 ml eppendorf tube following were added,

Template DNA (250 ng/µl)	1.5 µl
10 X Taq polymerase buffer	2.5 µl
10 mM dNTPs	0.5 µl
25 mM $MgCl_2$	4.0 µl
*Multiplex Primers	5.0 µl
Milli Q water	11.0 µl
**Taq polymerase (5U/µl) (Promega, USA)	0.5 µl

mixed well and centrifuged. 50 µl paraffin oil was added.

*0.5 nano moles in 400 µl

**The enzyme was added after initial denaturation at 94°C for 6 minutes. The reaction tubes were cycled in a thermocycler (MJ Research, Inc., USA) as follows:

Set I and Set II :

94°C for 30 seconds; 65°C for 4 minutes (30 times)

65°C for 10 minutes.

Set III :

94°C for 30 seconds; 50°C for 1 minute; 72°C for 1 minute (30 times)

72°C for 10 minutes.

The reaction was stopped by keeping the tubes in ice. 15 µl of the reaction products was run in a 2.5% agarose gel at 100 V in 1X TBE buffer. The gel was visualized under UV and photographed.

ISOLATION AND PURIFICATION OF INSERT

Rehydration of freeze-dried cultures

The freeze-dried cultures of *E. coli* containing plasmids with cDNA inserts (Table II, appendix) for DMD gene were obtained from ATCC (USA). For rehydration, the tip of the outer vial was heated in a flame and a few drops of water were squirted on the hot tip to crack the glass. The tip was removed by striking it with a file. Insulation and inner vial were also removed. The cotton plug was removed with a forcep and 0.3 to 0.4 ml of liquid medium (LB) was added aseptically to the freeze-dried material with a pasteur pipette. It was mixed well, and the mixture was transferred to a test tube containing 5-6 ml of LB. The tubes were incubated at 37°C overnight in a water bath shaker. Glycerol stocks of the cultures were aseptically prepared by mixing 300 µl of sterile glycerol with 600 µl of culture. The stocks were frozen in liquid nitrogen and stored at -70°C.

Plasmid DNA Isolation

The LB plates were streaked with glycerol stocks of the culture and incubated at 37°C. Single bacterial colony was transferred into 2 ml of LB medium containing ampicillin in a 15 ml glass tube. The cultures were incubated overnight at 37°C with vigorous shaking. 1 ml of this culture was inoculated to 250 ml of fresh LB

containing ampicillin in 500 ml flasks and incubated at 37°C overnight in a water bath shaker. The cells were pelleted down in GSA bottle at 4000 rpm for 15 minutes at 4°C and the supernatant was discarded. The open GSA bottle was left in an inverted position to allow all the supernatant to drain away.

The bacterial pellet was resuspended in 100 ml of ice cold STE buffer by mixing thoroughly with a sterile glass rod and the cells were pelleted down. The washed bacterial pellet was resuspended in 10 ml of solution I and 1 ml of freshly prepared lysozyme was added. To this, 20 ml of freshly prepared solution II was added and the contents were mixed thoroughly by gently inverting the bottle several times. The bottle was stored at room temperature for 5-10 minutes. 15 ml of ice cold solution III was added, mixed well and incubated at 4°C for 10 minutes. A white precipitate was formed. The bacterial lysate was centrifuged at 4000 rpm for 15 minutes at 4°C. The rotor was allowed to stop without brake. The supernatant was filtered through four layers of sterile cheese cloth into a 250 ml GSA bottle. To it 0.6 volume isopropanol was added and mixed well and stored at room temperature for 10 minutes. The nucleic acids were recovered by centrifugation at 5000 rpm for 10 minutes at room temperature. The pellet and the walls of the bottle were rinsed with 70% ethanol at room temperature. The pellet was dried and resuspended in 5 ml of water. Equal volume of equilibrated phenol was added and mixed by shaking for 5 minutes and centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was removed and one more phenol treatment was given. The supernatant was collected by centrifugation at 4,000 rpm for 5 minutes and equal volume of chloroform isoamylalcohol was added. It was mixed by shaking gently for 15

minutes and centrifuged at 4000 rpm for 10 minutes at 4°C. The chloroform-isoamylalcohol treatment was repeated. To the final supernatant 0.1 volume 3 M sodium acetate and twice the volume absolute ethanol was added. The DNA was allowed to precipitate at -70°C for 30 minutes, centrifuged in cold and collected. The pellet was washed twice with 70% ethanol, air dried and suspended in 500 µl of TE buffer (pH 8.0). The quality and quantity of plasmid DNA were checked by running on a 0.7% agarose gel.

Digestion of Plasmid DNA

The digestion was set as follows:

Plasmid DNA (500 ng/µl)	50.00 µl
10X buffer	40.00 µl
EcoRI (12 U/µl) (Promega, USA)	5.00 µl
Water	305.00 µl

and incubated at 37°C overnight. 2 µl of the digested sample was run on 0.7% agarose gel in 1X TAE buffer along with marker λ-DNA (HindIII/EcoRI double digest, Promega, USA). A preparative gel of the digested plasmid DNA was run and the insert band was cut with a sterile blade. 2-3 small pieces of agarose containing insert fragment were transferred to a 1.5 ml eppendorf tube. Approximately 1 ml of equilibrated phenol was added and incubated at -70°C overnight. The solution was centrifuged in cold at 12,000 rpm for 30 minutes to extract the nucleic acids from the pieces of agarose suspended in the phenol. To the supernatant 0.1 volume 3 M sodium acetate and twice the volume of absolute ethanol was added. The DNA was allowed to precipitate at -70°C for 30 minutes and centrifuged for 5 minutes.

The supernatant was discarded and the pellet was washed thrice with 70% ethanol. The pellet was air dried and dissolved in 20 µl of TE buffer (pH 8.0). The quantity and quality of the purified insert was checked by resolving it on a 0.7% agarose gel in 1X TAE buffer. The purified insert fragment was stored at -20°C.

NOTE :

1. For probe 1-2a the purified insert was digested with HindIII and the final insert 1-2a was purified as above.

2. The probe 9-14 was cut into 3 smaller fragments (9, 10, 11-14) with BamHI and purified as above.

Labelling the probe (By Random Priming)

2 µl (20 ng/µl) of insert DNA was mixed with 14 µl of nuclease free water, denatured by boiling at 100°C for 10 minutes and cooled on ice for 5 minutes. It was labeled using Random primer labelling kit (NEB, USA) as follows:

10X buffer (containing random primers)	2.50 µl
dATP	1.00 µl
dGTP	1.00 µl
α-P^{32} dCTP	3.00 µl
DNA polymerase Klenow	0.50 µl

- It was mixed well and incubated at room temperature overnight.

NOTE: λ-DNA digested with HindIII was labeled as above.

Quantitation and purification of probe

1 µl of labeled probe was spotted on GF/C paper (Whatman). The paper was dried and Cerenkov counts were taken. The paper was washed twice with wash solution I for 5 minutes each and once with absolute ethanol. It was air dried and the counts were taken again. The % incorporation was calculated as follows:

$$\frac{\text{Counts after washing}}{\text{Counts before washing}} \times 100 = \% \text{ Incorporation}$$

Preparation of sephadex G-50 column : The nozzle of a 1 ml syringe was plugged with autoclaved siliconized glass wool. The column was packed uniformly by adding sephadex G-50 solution, and centrifuging at 3000 rpm for 1 minute. The column was washed twice with 100 µl TE buffer (pH 8.0) by centrifuging at 3000 rpm for 3 minutes each.

50 µl of TE (pH 8.0) buffer was added to the 25 µl of labeled probe and loaded on to the sephadex G-50 column which was kept inside a centrifuge tube with a 0.5 ml eppendorf tube at the bottom to collect the purified probe. It was centrifuged at 3000 rpm for 3 minutes. The purified probe was transferred to a fresh eppendorf tube and the total counts were taken. The specific activity was calculated as follows:

Specific activity = Total counts X 25 X 2.5 cpm/µg

where, 25 = conversion factor for µg and 2.5 is the correction factor for Cerenkov counting.

The labeled probe was stored at -20°C till use.

SOUTHERN BLOTTING

Genomic DNA Digestion

For genomic DNA digestion following were mixed,

Genomic DNA (400 ng/µl)	45.00 µl
10X Labelling buffer	15.00 µl
Spermidine (10 mg/ml)	15.00 µl
BSA (10 mg/ml)	1.50 µl
HindIII (12 U/µl, Promega, USA)	8.00 µl
Milli Q Water	65.50 µl

and incubated at 37°C over night. 2 µl of the sample was run on 0.8% agarose in 1X TAE buffer to check if the DNA was completely digested. The digested DNA sample was run on 1% agarose (25 cm long) in 1X TAE buffer for 16-20 hours at 20-30 volts. The gel was stained in ethidium bromide for 10 minutes, and observed under UV. It was destained by keeping in excess of distilled water for 10-15 minutes.

Transfer of Digested DNA

The gel was *depurinated* in 0.25 N HCl by shaking on an orbital shaker at room temperature for 15 minutes till the dye became yellow. It was washed with distilled water to remove excess of acid. The gel was *denatured* by keeping in denaturation solution twice for 25 minutes each with gentle shaking at room temperature. The gel was *neutralized* by keeping in neutralization solution twice for 25 minutes each with gentle shaking at room temperature.

In the meantime, the apparatus was set for transfer by making wicks with 3MM Whatman paper with their ends dipping in 10X SSC. Three sheets of 3MM Whatman paper and blotting sheets of the size of the gel were cut. The Hybond N^+ (Amersham, U.K.) membrane of the size of the gel was cut and equilibrated with 2X SSC for 10 minutes.

After neutralization the gel was kept upside down on the 3MM Whatman paper and all the bubbles were removed by rolling a pipette gently. The equilibrated Hybond N^+ membrane was kept on the gel, avoiding any air bubbles. Two 3MM Whatman paper sheets, wet in 2X SSC, were placed on the membrane, avoiding air bubbles. The third 3 MM Whatman paper sheet (dried) and stack of paper towels just smaller than the 3MM paper were placed on top of the wet 3MM papers. A glass plate was kept on top of the stack and weighed down with a 500 gm weight. The sides of the tank were covered with cling film to prevent evaporation. The transfer was carried out for 16-20 hours, changing the blotting towels in between. The wet blotting sheets were removed from the nylon membrane and the wells were marked on it. The blot was dried at 42°C, auto crosslinked for 30 seconds in UV crosslinker (Stratagene, USA) and stored at room temperature till further use. The gel was stained with ethidium bromide and observed under UV to check for any residual DNA on the gel.

Prehybridization

For 5 ml of prehybridization solution 0.29 gm of NaCl was dissolved in 1 ml of water and the following were added :

50% Dextran sulfate	1.00 ml
10% SDS	0.50 ml
Formamide (Sigma, USA)	2.50 ml

The solution was incubated at 42°C for 15-30 min. The dried blot was transferred to a hybridization bottle and washed with 5X SSC at room temperature. Prehybridization was carried out at 42°C over night in 5 ml of prehybridization solution in the hybridization incubator (Robbin Scientific, USA).

Hybridization

To the labeled probe, the following were added,

Labeled λ-HindIII marker	5.00 µl
Herring sperm DNA (sheared, single stranded, Promega, USA)	50.00 µl

and mixed well. The probe was denatured in boiling water for 10 minutes, cooled on ice for 5 minutes and centrifuged in cold. The denatured probe was added to the prehybridization solution and hybridization was carried out at 42°C for 16-20 hours.

Stringency Washing

The hybridization solution was removed and stored at -20°C. The blot was washed with wash solution II at room temperature for 10 minutes with gentle shaking. The solution was discarded in a radioactive waste bottle. The blot was washed with wash solution III at 50°C for 15 minutes with gentle shaking and then with wash solution III at 55°C for 15 minutes with gentle shaking. The blot was

wrapped in Saran Wrap and an X-ray film was kept on it and exposed in a X-ray exposure cassette at -70°C for 5-7 days.

Developing and Fixing

The X-ray film was developed in developer for 5 minutes in a dark room. It was rinsed in water and fixed in fixer solution for 5 min. The film was washed in running tap water for 15 minutes and air dried.

Deprobing

The blot was deprobed by boiling in deprobing solution for 30 minutes and cooled to room temperature. The blot was dried and stored at room temperature till further use.

QUANTITATIVE POLYMERASE CHAIN REACTION

To a 0.5 ml eppendorf tube, the following were added,

Template DNA (250 ng/µl)	1.50 µl
10X Taq polymerase buffer	2.50 µl
10 mM dNTPs	0.50 µl
25 mM $MgCl_2$	4.00 µl
*Multiplex Primers	5.00 µl
α-P^{32}dCTP	0.25 µl
Taq Polymerase (5U/µl)	0.50 µl
Milli Q water	11.00 µl

mixed well and centrifuged. 50 µl of paraffin oil was added to the tube. The reactions were cycled as follows in a thermal cycler (MJ Research, USA) :

94°C for 30 seconds

65°C for 4 minutes (18 times)

65°C for 10 minutes.

12 µl of the PCR product was run on 2.5% agarose in 1X TBE buffer at 100 V for 1-2 hours. The dye front was cut and the gel was washed in water for 5 minutes. It was fixed in 7% TCA + 5% glycerol for 15 minutes and dried in a gel drier. The dried gel was wrapped in Saran Wrap and exposed at -70°C over night. The film was developed, fixed and analyzed using the Collage software (Fotodyne Inc., USA).

*Multiplex primers is a mixture of selective primers of deleted and non-deleted exons in the patient.

RESULTS

DYSTROPHIN GENE ANALYSIS

The genomic DNA was isolated from the peripheral blood of muscular dystrophy patients and their family members. Of these patients, 8 were diagnosed as suffering from DMD, 10 BMD, 11 LGMD (7 males and 4 females), 1 QM and 2 SMA. The DNA was subjected to mPCR and Southern blot analysis.

MULTIPLEX POLYMERASE CHAIN REACTION

A total of 27 exons were analyzed by three different multiplex primer sets (Table 2). mPCR was carried out on 43 males (33 patients and 10 relatives). Table 3 shows the details of mPCR analysis with individual primer sets in 33 patients. No deletions were observed in the normal relatives.

Families with Duchenne muscular dystrophy

Eight DMD boys and two normal healthy sibs were analyzed by mPCR. Deletions were not observed in the normal sibs (**SOB** and **MSB**).

Patients with deletions

Multiplex PCR analysis with three different sets revealed deletions in 5/8 DMD patients. Four patients (**AP, MS, SO** and **SD**) had a deletion in the central region of the dystrophin gene localized to the major "hot spot" between introns 44-52. **AP** had a deletion of exons 50 and 51 (Figs. 2G AND 3B) and **MS** had a deletion of

Table 2: Target size of amplified fragments

SET I		SET II		SET III	
AMPLIFIED EXON	SIZE (bp)	AMPLIFIED EXON	SIZE (bp)	AMPLIFIED EXON	SIZE (bp)
45	547	Pm	535	49	439
48	506	3	410	16	290
19	459	43	357	41	270
17	416	50	271	32	253
51	388	13	238	42	195
8	360	6	202	2	174
12	331	47	181	79	137
44	268	60	131	25	113
4	196	52	113	66	68

Table 3: Summary of exonic deletions detected with different primer sets

PATIENT CODE	EXONIC DELETIONS DETECTED WITH		
	SET I	SET II	SET III
DMD Patients			
RS	NONE	NONE	NONE
AP	51	50	NONE
NT	NONE	NONE	NONE
RH	NONE	NONE	NONE
MS	45, 48	47, 50	49
SO	45, 48, 51	47, 50	49
SD	45	NONE	NONE
HA	4	6	NONE
BMD Patients			
AR	45, 48	47	NONE
ARB	45, 48	47	NONE
SR	45, 48	47	NONE
RB	NONE	NONE	NONE
TR	45	47	NONE
PN	NONE	NONE	NONE
JD	NONE	NONE	NONE
DH	45,48	47	NONE
MO	NONE	NONE	NONE
MOB1	NONE	NONE	NONE
MOB2	NONE	NONE	NONE
MOB3	NONE	NONE	NONE
MJ	51	NONE	NONE
RL	I7,19 44	43	16,41, 32

Continued on next page

Cont.

PATIENT CODE	EXONIC DELETIONS DETECTED WITH		
	SET I	SET II	SET III
QM Patient			
VK	45	47	NONE
LGMD Patients			
PG	NONE	NONE	NONE
NC	NONE	NONE	NONE
RC	NONE	NONE	NONE
MP	NONE	NONE	NONE
MN	NONE	NONE	NONE
KK	NONE	NONE	NONE
SA	NONE	NONE	NONE
HPB	NONE	NONE	NONE
SMA Patients			
AC	NONE	NONE	NONE
VT	NONE	NONE	NONE

exons 45, 47, 48, 49 and 50 (Figs. 2G; 3B and 4A). In **SO**, exons 45, 47, 48, 49, 50 and 51 (Figs. 2D; 3B and 4A) were deleted while in **SD**, exon 45 was deleted (Fig. 2A). One patient (**HA**) had deletion of exons 4 and 6 in the 5′ region of the gene localized to the minor "hot spot" between introns 2-8 (Figs. 2E and 3C).

Patients with no deletions

All the 27 fragments were amplified in **RS, NT** and **RH** as in the normal control (Figs. 2G; 3B and 4A).

Families with Becker muscular dystrophy

Ten BMD patients, seven sibs (4 symptomatic and 3 normal) and one nephew were analyzed by mPCR. No deletions were observed in the normal sibs (**RBB, TRB** and **JDB**) and the nephew (**MOSiS**) (Figs. 2A, F; 3C and 4B).

Patients with deletions

Multiplex PCR with three sets revealed deletions in six patients and one symptomatic sib. Five patients and one sib revealed deletions in the major "hot spot". **AR, ARB, SR** and **DH** had an identical deletion of exons 45, 47 and 48 (Figs. 2A, B, C, F and 3A, C) while in **TR** exons 45 and 47 were missing (Figs. 2C and 3A). In **MJ**, only exon 51 was deleted (Fig. 2A). One patient (**RL**) had a large deletion involving exons 16, 17, 19, 32, 41, 43 and 44 in the central region of the gene (Figs. 2E; 3D and 4B).

Patients with no deletions

Four patients (**JD, RB, PN** and **MO**) and three symptomatic sibs (**MOB1, MOB2** and **MOB3**) did not show any deletion (Figs. 2A, B; 3A, C, D and 4B).

Figs. 2-4: Analysis of dystrophin gene by multiplex polymerase chain reaction (mPCR) with primer sets I, II and III. The exon numbers represented by the respective PCR product are indicated on the left.

C = normal control

Arrow (◄) indicates patients (DMD/BMD/QM) and family members with various intragenic deletions.

Dot (.) indicates LGMD/SMA patients.

Fig. 2 A-G: PCR analysis by set I : exonic deletions.

A : **SD** (45), **MJ** (51), **DH** (45,48)

B : **SR** (45,48)

C : **TR** (45,48), **AR** (45,48)

D : **VK** (45), **SO** (45,48,51)

E : **HA** (4), **RL** (17,19,44)

F : **ARB** (45,48)

G : **MS** (45,48), **AP** (51)

EXON
C
JD
JDB
SD
MJ
DH
45
48
19
17
51
8
12
44
4
45
48
51
A

C
MP'
RB
SR
SA'
PN
45
48
B

C
TR
PG'
AR
45
48
C

C
NC'
VK
NGS
SO
45
48
51
D

C
HA
RL
19
17
44
4
E

C
ARB
TRB
MSB
45
48
F

C
MS
NT
RH
AP
MN'
RS
45
48
51
G

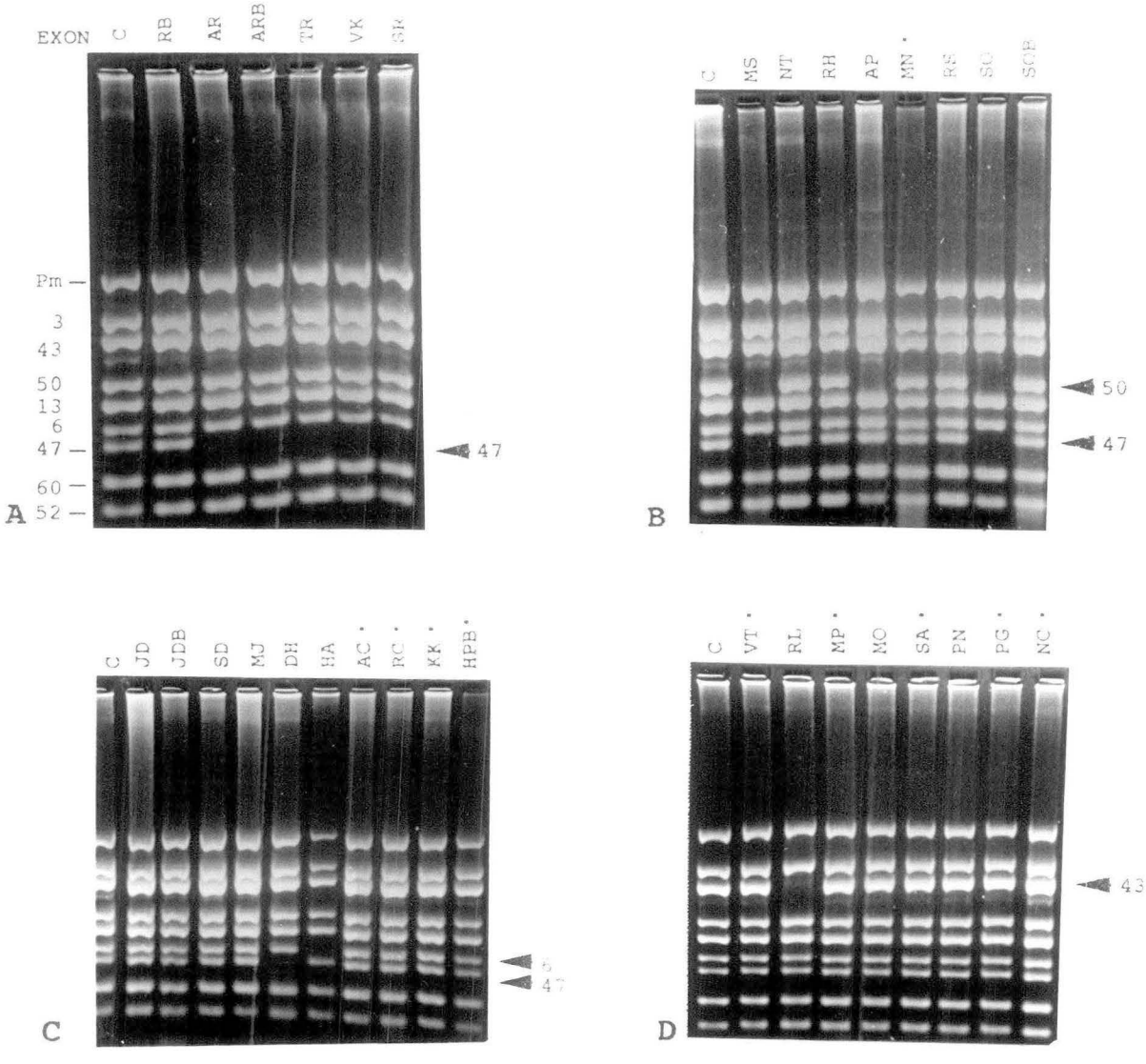


Fig. 3 A-D: PCR analysis by set II : exonic deletions

A : **AR, ARB , TR , VK, SR** (47)

B : **MS , SO** (47,50), **AP** (50)

C : **DH** (47), **HA** (6)

D : **RL** (43)

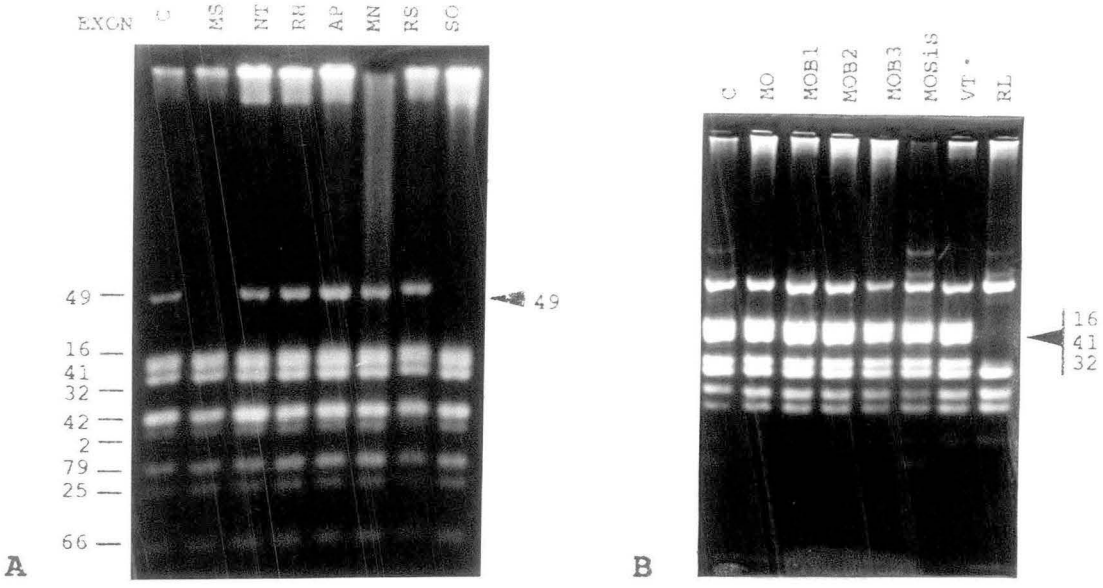


Fig. 4 A-B : PCR analysis by set III : exonic deletions

A : MS, SO (49)

B : RL (16,32,41)

Patient with quadriceps myopathy

One patient (**VK**), diagnosed as suffering from QM showed a deletion of two exons (45 and 47) in the major "hot spot" of the dystrophin gene (Figs. 2D and 3A). No deletion was observed in his healthy brother (**VKB**) .

Families with limb girdle muscular dystrophy

Seven male patients, one symptomatic sib and 3 normal male relatives (1 sib and 2 sons of female patients) were analyzed. No deletions were observed (Figs. 2B, C, D, G; 3B, C, D and 4A).

Patients with spinal muscular dystrophy

Two patients with SMA were analyzed by mPCR. No deletions were observed (Figs. 3C, D and 4B).

DELETION PATTERNS BY mPCR

The nine different deletion patterns observed in this study are summarized in Table 4. Two patients had a deletion affecting only one of the 27 amplified exons (pattern 3 and 9), 4 cases had deletion in two successive exons (pattern 1, 4 and 8) and 4 cases from three families had deletions in the three consecutive exons (pattern 5). One patient had deletions covering 5 of the exons (pattern 6). One patient had a deletion of six exons (pattern 7) and in another seven exons (pattern 2) were deleted. One patient (**RL**) had deletion pattern that could be attributed to multiple deletion events (pattern 2). This pattern was observed on repeat analysis.

Table 4 : Deletion patterns in DMD/BMD patients

FAMILY CODE	DELETION PATTERN	EXON NUMBERS																										
		Pm	2	3	4	6	8	12	13	16	17	19	25	32	41	42	43	44	45	47	48	49	50	51	52	60	66	79
HA	1	+	+	+	d	d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RL	2	+	+	+	+	+	+	+	+	d	d	d	+	d	d	+	d	d	+	+	+	+	+	+	+	+	+	+
SD	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+	+	+	+	+	+
TR	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	+	+	+	+	+	+	+	+
VK	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	+	+	+	+	+	+	+	+
AR	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	d	+	+	+	+	+	+	+
SR	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	d	+	+	+	+	+	+	+
DH	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	d	+	+	+	+	+	+	+
MS	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	d	d	d	+	+	+	+	+
SO	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	d	d	d	d	+	+	+	+
AP	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	d	+	+	+	+
MJ	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+

NOTE : '+' = normal amplification, 'd' = no amplification, Pm = muscle promoter

DELETION FREQUENCY

Deletions were identified in 63% of the DMD/BMD patients by mPCR. These were more frequent in the central region (12 out of 13) than in the 5′ terminal region (1 out of 13) of the gene. Fig. 5 shows the deletion frequency of various exons. Exon 45 was the most frequently deleted (eight patients and one sib).

SOUTHERN HYBRIDIZATION WITH cDNA PROBES

For the detection of molecular lesions in the DNA of the patients who had been subjected earlier to the mPCR analysis, eight contiguous segments constituting the entire region of the dystrophin cDNA were used. Genomic DNA samples from 37 patients (32 probands and 5 sibs) and 41 family members were digested with HindIII. The blots were probed initially with cDNA 5b-7 followed by cDNA probes 8, 1-2a, 2b-3, 4-5a, 9 , 10 and 11-14. The normal HindIII restriction fragments detected by these probes are summarized in Table 5. The cDNA probes identified both the deletions and the novel HindIII fragments in muscular dystrophy patients. The details of the novel fragments are given in Table 6.

Families with Duchenne muscular dystrophy

Eight DMD patients and 10 family members were subjected to dystrophin gene analysis by the above eight cDNA probes as per details below. The deletions were not observed in any of the healthy family members.

Patients with deletions

Deletions were observed by three probes in five patients which was manifested by the absence of exon containing HindIII fragments of the dystrophin gene.

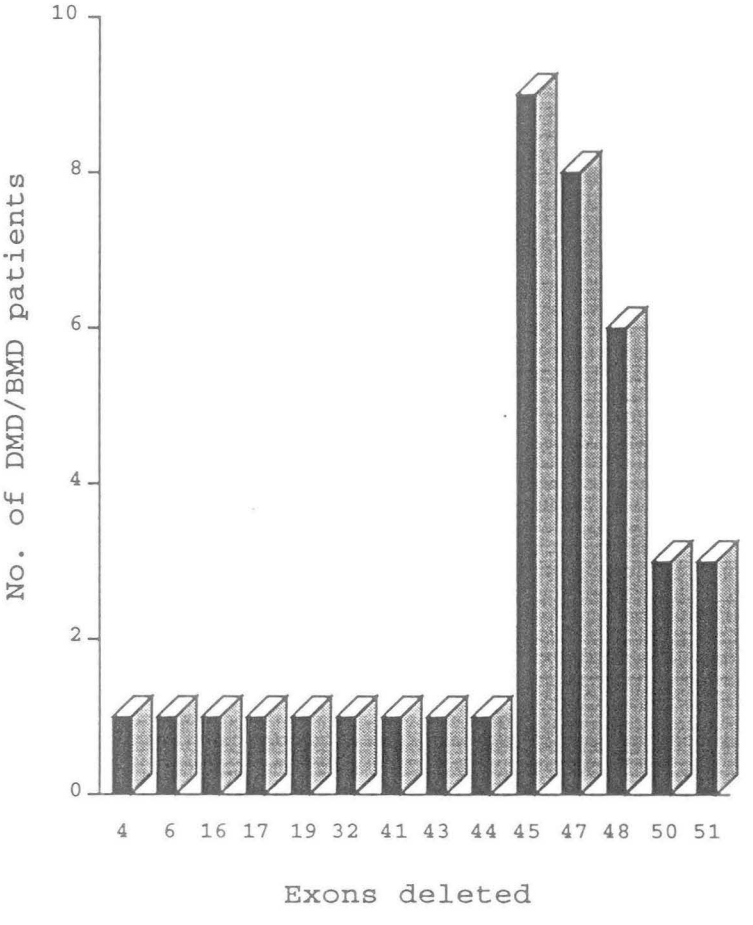


Fig. 5: Histogram showing the deletion frequency of various exons in DMD/BMD patients.

Table 5: Normal HindIII fragments detected by different cDNA probes

PROBE USED	EXON CONTAINING HINDIII FRAGMENTS* (kb)
1-2a	3.2; 3.25; 4.2; 8.5; 3.1; 8.0; 4.6; 7.5; 10.5
2b-3	10.5; 4.0; 6.6; 2.7; 6.0; 1.7; 12.0; 3.0; 7.3
4-5a	7.3; 11.0; 20.0; 5.2; 4.7; 12.0; 18.0
5b-7	18.0; 1.8; 0.4; 1.3; 1.5; 6.0; 6.2; 4.2; 11.0; 4.1; 0.5; 1.5; 10.0
8	10.0; 1.25; 3.8; 1.6; 3.7; 3.1; 7.0
9	7.8; 1.0; 8.3; 2.3; 1.0; 8.8; 6.0
10	6.0; 3.5; 6.6; 2.8; 12; 2.4; 2.55
11-14[a]	1.45; 6.8;1.5; 2.1; (6.0; 1.9); 10; 1.8; 5.9; 7.8
11-14[b]	1.45; 6.8; 1.5; 2.1; (2.4; 1.9); 10; 1.8; 3.4; 5.9; 7.8
11-14[c]	1.45; 6.8; 1.5; 2.1; (2.8; 6.0); 10; 5.9; 7.8

* : listed in 5'-3' order, bracket indicates the order not clear
a : Koenig et al. (1987)
b : Bies et al. (1992)
c : Present study

Probe 1-2a

One patient (**HA**) showed a deletion of 8.5, 3.1 and 8.0 kb HindIII fragments corresponding to exons 4-6 of the dystrophin gene (Fig. 6A).

Probe 5b-7

Three HindIII fragments of 10, 1.5 and 0.5 kb corresponding to exons 47, 46 and 45 of the dystrophin gene respectively, were found to be deleted in both **SO** and **MS** (Fig. 8E).

Probe 8

In **SO**, probe 8 identified the deletion of 10, 3.8/1.25, 1.6, 3.7, 3.1 and 7.0 kb HindIII fragments (exons 47-52). In **MS**, probe 8 detected the absence of 10, 3.8/ 1.25, 1.6 and 3.7 kb HindIII fragments corresponding to exons 47-50. In **AP**, 3.7, 3.1 and 7.0 kb HindIII fragments were not detected. This corresponds to a deletion of exons 50-52 (Fig. 9A, B).

No deletion was identified with probes 2b-3, 4-5a, 9, 10 and 11-14.

Patients with no deletion

Deletions were not observed in three patients **NT**, **RH** and **RS** with any of the eight cDNA probes.

Families with Becker muscular dystrophy

A total of 14 BMD patients belonging to 10 unrelated families, and eight relatives were studied by Southern blot analysis with eight cDNA probes as per details given below. None of the healthy relatives showed any deletions.

Patients with deletions

Seven out of 14 patients showed deletions with five cDNA probes.

Probes 2b-3 and 4-5a

A large deletion in the central region of the dystrophin gene was identified by both the probes in **RL**. With 2b-3, a deletion of 6.6, 2.7, 6.0, 1.7, 12, 3.0 and 7.3 kb HindIII fragments was revealed which corresponds to exon 13 to 20. With 4-5a, 6 HindIII fragments of 7.3, 12, 20, 4.7, 11 and 18 kb were found to be missing, corresponding to exons 20, 21, 22-25, 28, 29 and 30-33 respectively. The deletion of 5.2 kb fragment corresponding to exons 26-27 could not be confirmed because of the presence of an extra fragment (Fig. 7A and B).

Probe 5b-7

Deletion of 18, 1.8, 0.4, 1.3, 1.5, 6.1, 6.2, 4.2, 11 and 4.1 kb HindIII fragments corresponding to exons 30-44 was identified in **RL** by 5b-7. In **AR, ARB, TR, SR** and **DH**, it revealed the absence of 0.5, 1.5 and 10 kb HindIII fragments corresponding to exons 45-47 (Fig. 8A, B, C).

Probe 8

Probe 8 identified the deletion of the 10 kb HindIII fragment containing exon 47 in **TR**. In **AR, ARB, SR** and **DH**, 10 and 3.8/1.25 kb HindIII fragments (exons 47 and 48) were deleted. In **MJ**, the 3.1 and 7.0 kb HindIII fragments (exons 51 and 52) were deleted (Fig. 9B, C, D).

Probe 9

Only one patient **MJ** revealed the absence of 7.8/1.0 kb HindIII fragment containing exon 53 (Fig. 10B).

No deletion was identified with probes 1-2a, 10 and 11-14

Patients with no deletions

Four BMD patients (**MO, RB, PN** and **JD**) and three symptomatic sibs (**MOB1, MOB2, MOB3**) did not show any deletion with any of the eight cDNA probes.

Patient with quadriceps myopathy

Two out of eight probes identified deletions in **VK**. Probes 1-2a, 2b-3, 4-5a, 9, 10 and 11-14 did not reveal any deletion.

Probe 5b-7

A deletion of 0.5, 1.5 and 10 kb HindIII fragments corresponding to exons 45-47 of the dystrophin gene was observed (Fig. 8B).

Probe 8

The 10 kb HindIII fragment containing exon 47 of the dystrophin gene was found to be deleted (Fig. 9D).

Families with limb girdle muscular dystrophy and spinal muscular atrophy

A total of 14 patients (12 LGMD and 2 SMA) and 10 relatives were analyzed. No deletions were detected in any of these patients with any of the eight cDNA probes.

Figs. 6-11: Southern blot analysis. HindIII digests of DNA from patients with DMD/BMD/QM/LGMD/SMA and family members with eight cDNA probes (1-2a, 2b-3, 4-5a, 5b-7, 8, 9, 10 and 11-14).

M = marker (λ DNA HindIII digested)

C = control (normal male)

Arrow (◄) indicates patients with various intragenic deletions

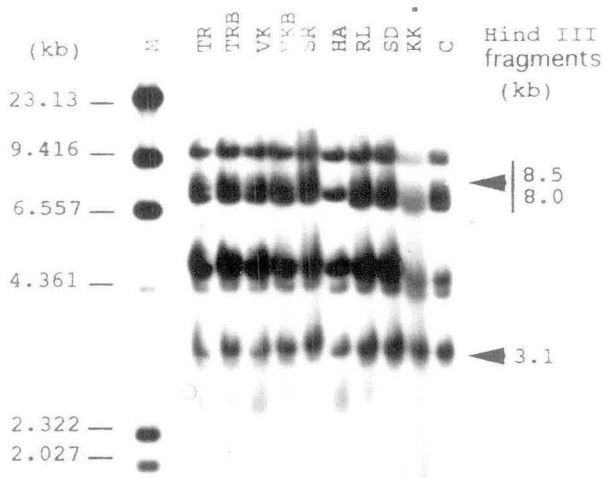
Arrow (◄) indicates extra fragments (novel) of various sizes

Dot (.) indicates LGMD/SMA patients

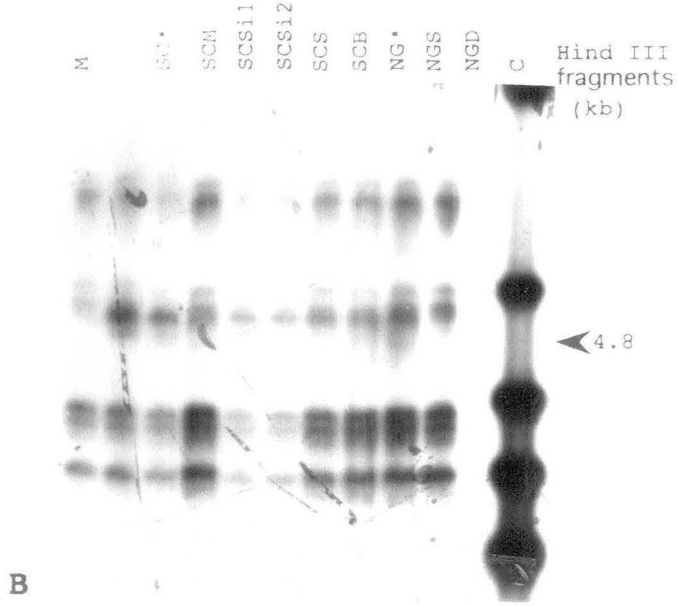
Fig. 6 A and **B**: Southern blot analysis showing intragenic deletions (◄) and extra fragment (◄) with probe 1-2a.

A : **HA**, deletions of 8.5, 8.0, and 3.1 kb corresponding to exon 4, 6 and 5 respectively.

B : Presence of ~ 4.8 kb extra fragment in **SC, SCM, SCSi1, SCSi2, SCS, SCB, NG, NGS** and **NGD**.


(kb)
M
TR
TRB
VK
VKB
SR
HA
RL
SD
KK
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
8.5
8.0
3.1

A

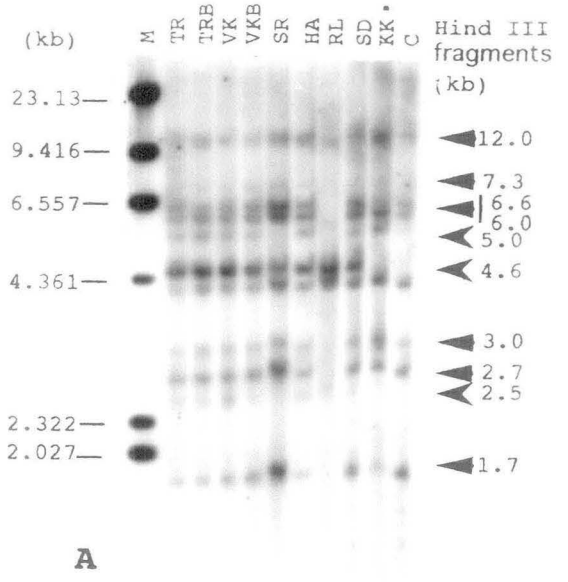

M
SCI
SCM
SCSi1
SCSi2
SCS
SCB
NG
NGS
NGD
C
Hind III fragments (kb)
4.8

B

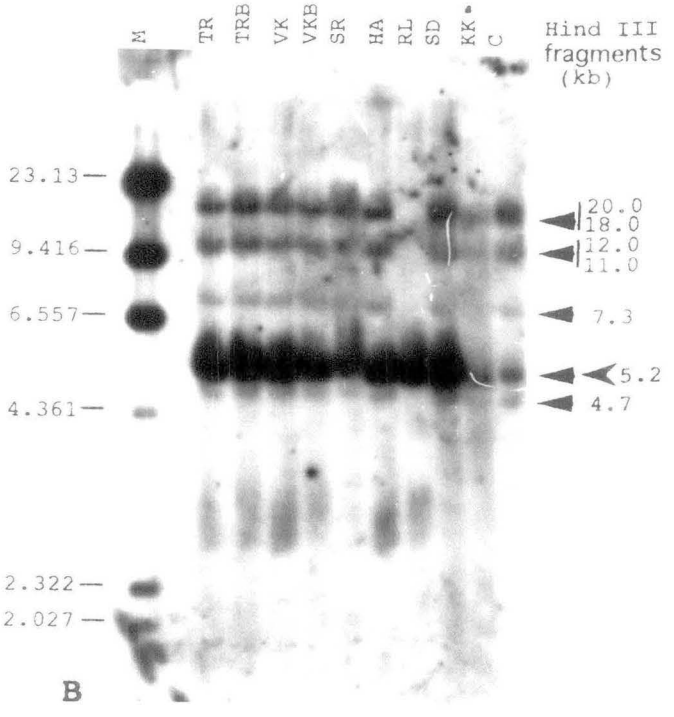
Fig. 7 A and **B**: Southern blot analysis showing intragenic deletions (◄) and extra fragments (≺) with probe 2b-3 and 4-5a respectively.

A: **RL**, deletion of 12, 7.3, 6.6, 6.0, 3.0, 2.7, and 1.7 kb corresponding to exon 18, 20, 13, 16, 19, 14, 15,and 17 respectively. Presence of extra fragments of ~4.6, ~5.0 and ~2.5 kb in **TR**, **VK**, **HA** and **SD**, ~4.6 kb in **SR**, ~2.5 and ~4.6 kb in **RL** and ~5.0 kb in **KK**.

B: **RL**, deletions of 20, 18, 12, 11, 7.3, and 4.7 kb corresponding to exons 23, 25; 30, 33; 28; 21; 20; and 29 respectively. Presence of ~5.2 kb fragment in **TR**, **TRB**, **VK**, **VKB**, **SR**, **HA**, **RL** and **SD**.


(kb)
M
TR
TRB
VK
VKB
SR
HA
RL
SD
KK
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
12.0
7.3
6.6
6.0
5.0
4.6
3.0
2.7
2.5
1.7

A


M
TR
TRB
VK
VKB
SR
HA
RL
SD
KK
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
20.0
18.0
12.0
11.0
7.3
5.2
4.7

B

Fig. 8 A-E: Southern blot analysis showing intragenic deletions (◄) and extra fragments (◄) with probe 5b-7.

A : **AR**, deletion of 10, 1.5, and 0.5 kb corresponding to exon 47, 46 and 45 respectively. Presence of ~5 kb extra fragment in **MO**, **MOB1**, **MOB2**, **MOB3**, **RB**, and **RBB**.

B : **TR**, **VK**, **SR**, deletion of 10, 1.5, and 0.5 kb corresponding to exon 47, 46 and 45. **SD**, deletion of 0.5 kb (exon 45). **RL**, deletion of 18, 11, 6.2, 6.0, 4.2, 4.1, 1.8, 1.5, 1.3, and 0.5 kb corresponding to exons 30,33; 43; 40,41; 38,39; 42; 44; 36; 37; 35; and 34 respectively. Presence of ~5 kb extra fragment in **TR**, **TRB**, **VK**, **VKB**, **HA**, **RL**, **SD**, and **KK**.

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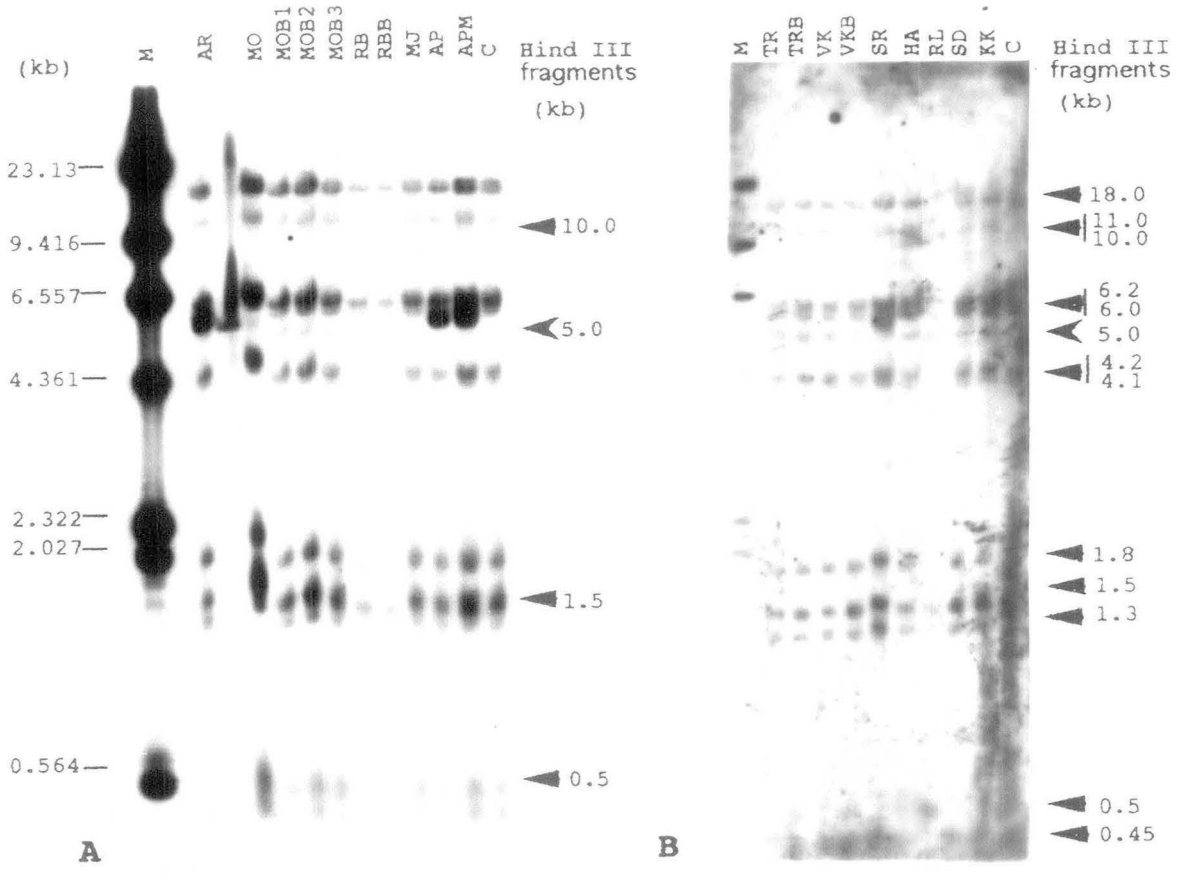

(kb)
M
AR
MO
MOB1
MOB2
MOB3
RB
RBB
MJ
AP
APM
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
0.564
10.0
5.0
1.5
0.5
A
M
TR
TRB
VK
VKB
SR
HA
RL
SD
KK
C
Hind III fragments (kb)
18.0
11.0
10.0
6.2
6.0
5.0
4.2
4.1
1.8
1.5
1.3
0.5
0.45
B

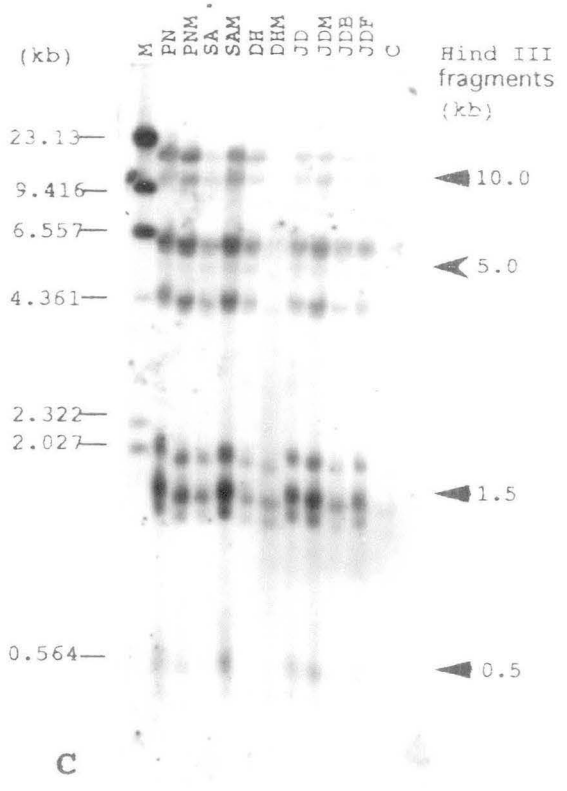
Cont.

Fig. 8:

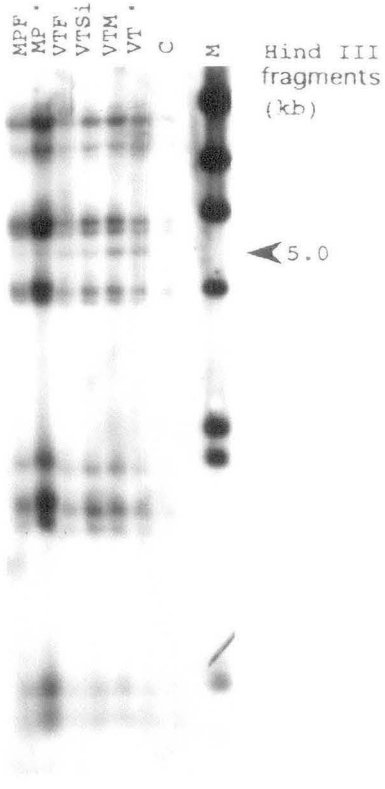
C: **DH**, deletion of 10, 1.5, and 0.5 kb corresponding to exon 47, 46 and 45 respectively. Presence of ~5.0 kb extra fragment in **PN**, **PNM**, **SA**, **SAM**, **DH**, **DHM**, **JD**, **JDM**, **JDB** and **JDF**.

D: Presence of ~5.0 kb extra fragment in **MPF**, **MP**, **VTF**, **VTSi**, **VTM**, and **VT**.

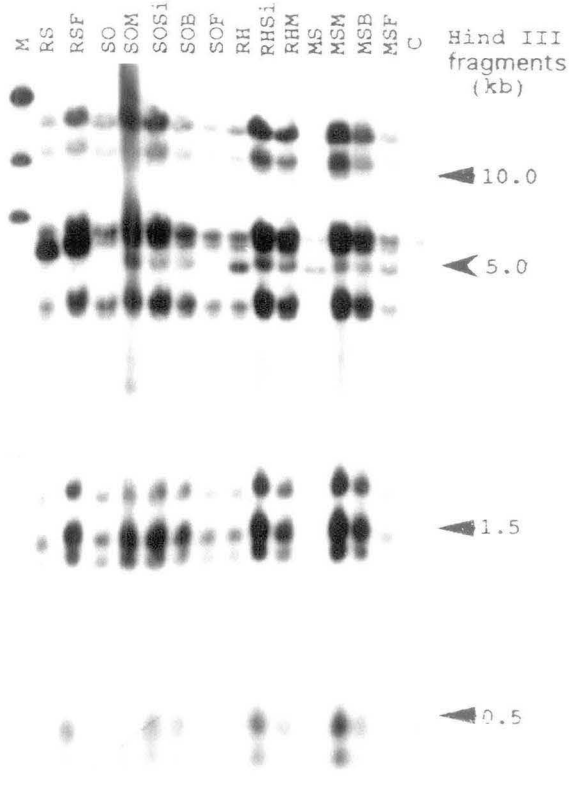
E: **SO**, **MS**, deletion of 10, 1.5, and 0.5 kb corresponding to exon 47, 46 and 45 respectively. Presence of ~5.0 kb extra fragment in **SOM**, **SOSi**, **SOB**, **RH**, **RHSi**, **RHM**, **MS**, **MSM**, **MSB**, and **MSF**.


(kb)
M
PN
PNM
SA
SAM
DH
DHM
JD
JDM
JDB
JDF
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
0.564
10.0
5.0
1.5
0.5

C


MPF
MP
VTF
VTSi
VTM
VT
C
M
Hind III fragments (kb)
5.0

D


M
RS
RSF
SO
SOM
SOSi
SOB
SOF
RH
RHSi
RHM
MS
MSM
MSB
MSF
C
Hind III fragments (kb)
10.0
5.0
1.5
0.5

E

Fig. 9 A-D: Southern blot analysis showing intragenic deletions (◄) and extra fragments (➤) with probe 8.

A: **SO**, deletion of 10, 7.0, 3.8/1.25, 3.7, 3.1, and 1.6 kb corresponding to exon 47, 52, 48, 50, 51, and 49 respectively. **MS**, deletion of 10, 3.8/1.25, 3.7 and 1.6 kb corresponding to exon 47, 48, 50, and 49 respectively. Presence of ~5.0 kb extra fragment in **SOM, SOSi, SOB, RH, RHSi, RHM, MS, MSM, MSB** and **MSF**; and ~0.75 kb extra fragment in **RS, RSF, SO, SOM, SOSi, SOB, SOF, RH, RHSi, RHM, MS, MSM, MSB,** and **MSF**.

B: **AR**, deletion of 10 and 3.8/1.25 kb corresponding to exon 47 and 48 respectively. **MJ**, deletion of 7 and 3.1 kb corresponding to exon 52 and 51 respectively. **AP**, deletion of 7, 3.7, and 3.1 kb corresponding to exon 52, 50 and 51 respectively. Presence of ~0.75 kb extra fragment in **AR, MO, MOB1, MOB2, MOB3, RB, RBB, MJ, AP,** and **APM**.

C: **DH**, deletion of 10 and 3.8/1.25 kb corresponding to exon 47 and 48 respectively. Presence of ~0.75 kb extra fragment in **PN, PNM, SA, SAM, DH, DHM, JD, JDM, JDB,** and **JDF**.

D: **TR, VK**, deletion of 10 kb (exon 47). **SR**, deletion of 10 and 3.8/1.25 kb corresponding to exon 47 and 48 respectively. Presence of ~5.0 kb extra fragment in **TR, TRB, VK, VKB, SR, HA, RL, SD,** and **KK**; and ~0.75 kb extra fragment in **TR, TRB, VK, VKB, SR, HA, RL,** and **SD**.

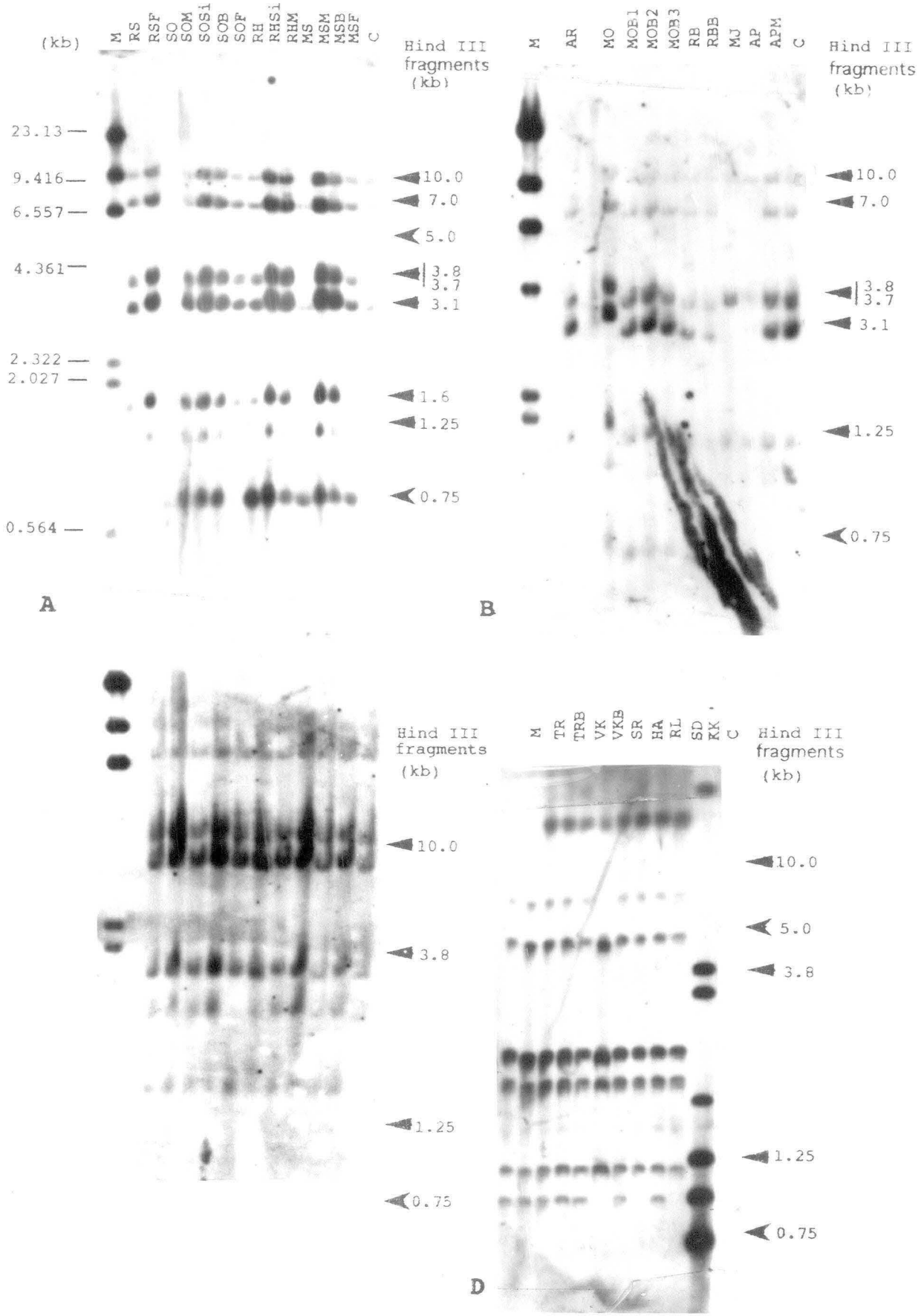
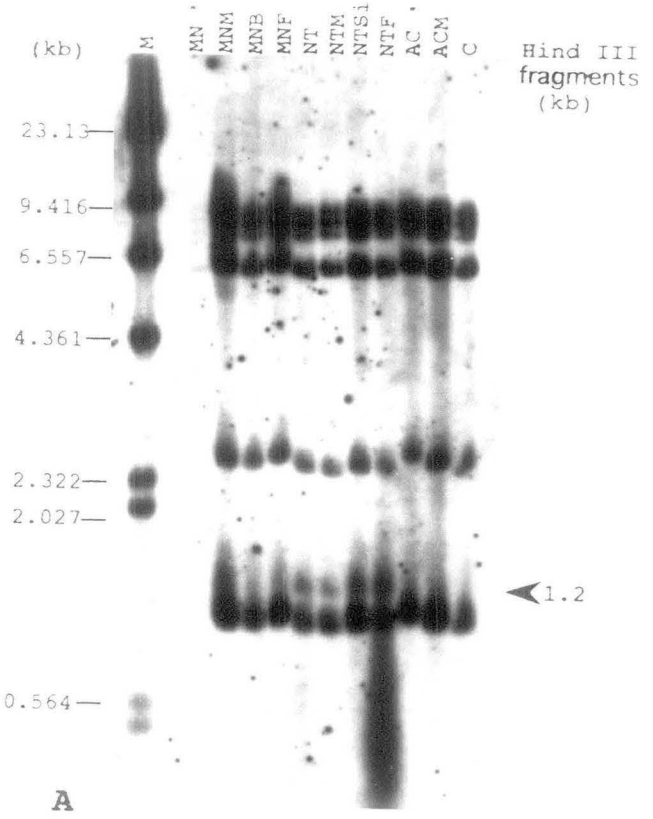

(kb)
M
RS
RSF
SO
SOM
SOSi
SOB
SOF
RH
RHSi
RHM
MS
MSM
MSB
MSF
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
0.564
10.0
7.0
5.0
3.8
3.7
3.1
1.6
1.25
0.75
A
M
AR
MO
MOB1
MOB2
MOB3
RB
RBB
MJ
AP
APM
C
Hind III fragments (kb)
10.0
7.0
3.8
3.7
3.1
1.25
0.75
B
Hind III fragments (kb)
10.0
3.8
1.25
0.75
M
TR
TRB
VK
VKB
SR
HA
RL
SD
KK
C
Hind III fragments (kb)
10.0
5.0
3.8
1.25
0.75
D

Fig. 10 A-B : Southern blot analysis showing intragenic deletions (◄) and extra fragments (➤) with probe 9.

A: Presence of 1.2 kb extra fragment in **NT, NTM, NTSi,** and **NTF.**

B: **MJ,** deletion of 7.8/1.0 kb (exon 53). Presence of 2.0 kb extra fragment in **AR, MO, MOB1, MOB2, MOB3, RB,** and **RBB.**


(kb)
M
MN
MNM
MNB
MNF
NT
NTM
NTSi
NTF
AC
ACM
C
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
0.564
1.2
A

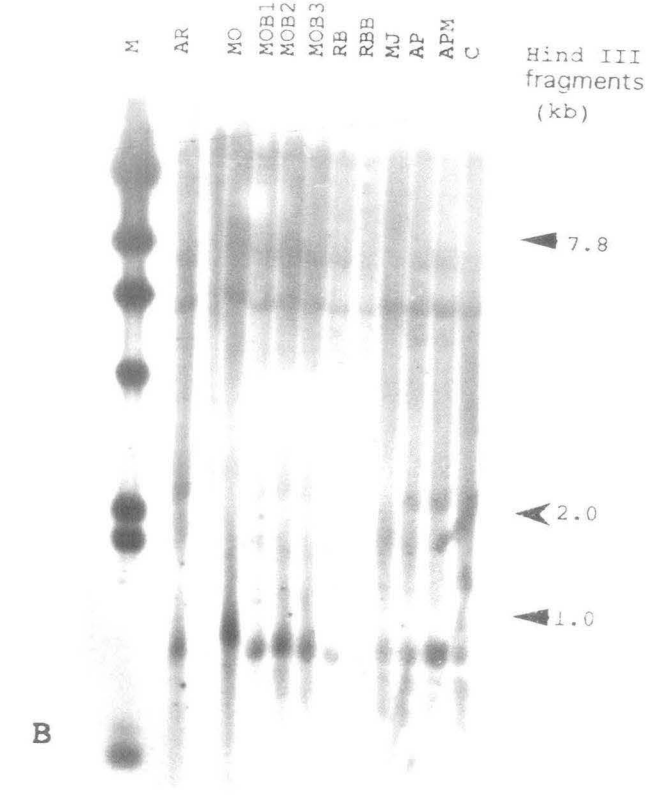
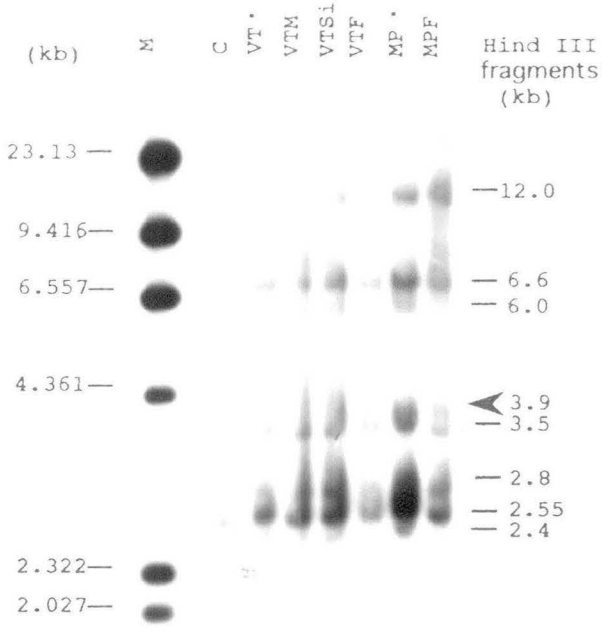

M
AR
MO
MOB1
MOB2
MOB3
RB
RBB
MJ
AP
APM
C
Hind III fragments (kb)
7.8
2.0
1.0
B

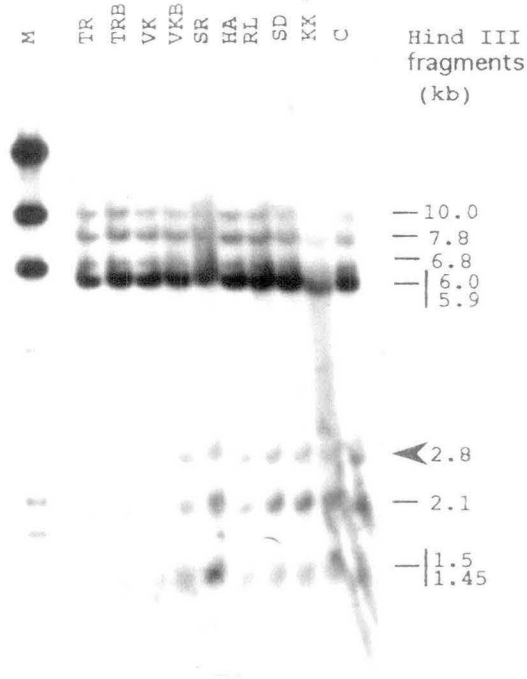
Fig. 11 A and **B**: Southern blot analysis with probe 10 and 11-14 respectively showing extra fragments (➤).

A: Presence of ~3.9 kb extra fragment in **VT**, **VTM**, **VTSi**, **VTF**, **MP** and **MPF**.

B: New RFLP showing acquisition of 2.8 kb fragment with loss of 1.9 and 1.8 kb fragments.


(kb)
M
C
VT·
VTM
VTSi
VTF
MP·
MPF
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
12.0
6.6
6.0
3.9
3.5
2.8
2.55
2.4

A


M
TR
TRB
VK
VKB
SR
HA
RL
SD
KX·
C
Hind III fragments (kb)
10.0
7.8
6.8
6.0
5.9
2.8
2.1
1.5
1.45

B

NOVEL HindIII RESTRICTION FRAGMENTS

During the course of a study of deletion analysis in muscular dystrophy patients and their family members by Southern hybridization, novel HindIII fragments were observed in 35/37 patients and their family members with 7 probes 1-2a, 2b-3, 4-5a, 5b-7, 8, 9 and 10. These were absent in the unrelated controls, with no family history of any muscle disease, in the respective blots. Fragments of ~5.2 kb and ~750 bp were most common in both, patients and their healthy family members. (Table 6; Figs. 6B, 7A-B, 8A-E, 9A-D, 10A-B, 11A). Extra fragments identified in patients with DMD/BMD/LGMD/SMA are summarized in Table 7.

The extra fragments were present in both patients with or, without deletions (Table 8). These were also identified in the mother (13/14), father (9/9), sisters (6/6), brothers (8/8), sons (2/2), daughter (1/1) and uncle (1/1) of the patients.

Probe 11-14 identified a novel polymorphism in the dystrophin gene with a loss of two bands (1.8 kb and 1.9 kb) and gain of ~2.8 kb band in both the patients and unrelated controls (Table 5; Fig. 11B).

To study the origin of these fragments, one patient (with extra fragment of ~750 bp detected by probe 8 was analyzed by 5 different restriction enzyme (TaqI, BamHI, BglII, EcoRI, and PstI). No novel fragment was identified with any of these enzyme. A junction fragment was identified with PstI in **VK** (Fig. 12).

A seperate study of DNA samples from 18 unrelated controls (9 males and 9 females) with no family history of any muscle disorder did not reveal the presence of these novel HindIII fragments with any of the five cDNA probes (1-2a, 2b-3, 4-5a, 5b-7 and 8).

Table 6 : Novel HindIII restriction fragments observed in the present study

S.No.	CODE	DELETION		EXTRA FRAGMENTS IDENTIFIED BY PROBE						
		PCR	S.H.	1-2a	2b-3	4-5a	5b-7	8	9	10
DMD PATIENTS AND THEIR FAMILIES										
1.	RS	None	None	-	-	-	-	750bp	-	-
	RSF			-	-	-	-	750bp	-	-
2.	AP	50-51	50-52	-	-	-	-	-	-	-
	APM			-	-	-	-	750bp	-	-
3.	NT	None	None	-	4.6kb	5.2kb	5kb	750bp	1.2	-
	NTM			-	4.6kb	5.2kb	5kb	750bp	1.2	-
	NTF			-	4.6kb	5.2kb	5kb	750bp	1.2	-
	NTSi			-	-	5.2kb	5kb	750bp	1.2	-
4.	RH	None	None	-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	RHM			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	RHSi			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
5.	MS	45-50	45-50	-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	MSB			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	MSM			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	MSF			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
6.	SO	45-50	45-52	-	-	-	-	750bp	-	-
	SOM			-	4.6kb	5.2kb	5kb	750bp,5kb	5kb	-
	SOF			-	-	-	-	750bp	-	-
	SOB			-	4.6kb	5.2kb	5kb	750bp,5kb	-	-
	SOSi			-	4.6kb	5.2kb	5kb	750bp,5kb	5kb	-
7.	SD	45	45	-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp,5kb	-	-
8.	HA	6	5-6	-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp,5kb	-	-

Continued on next page

S.No.	CODE	DELETION		EXTRA FRAGMENTS IDENTIFIED BY PROBE						
		PCR	S.H.	1-2a	2b-3	4-5a	5b-7	8	9	10
BMD PATIENTS AND THEIR FAMILIES										
1.	AR	45-58	45-48	-	-	5.2kb	-	750bp	2kb	-
	ARB	45-48	45-48	-	-	5.2kb	5kb		-	-
2.	SR	45-48	45-48	-	4.6kb	5.2kb	-	750bp,5kb	-	-
3.	RB	None	None	-	4.6kb	5.2kb	5kb		2kb	-
	RBB	None	None	-	4.6kb	5.2kb	5kb		2kb	-
4.	TR	45-47	45-47	-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp	-	-
	TRB			-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp	-	-
5.	JD	None	None	-	5kb	5.2kb	5kb	750bp	2kb	-
	JDB			-	5kb	5.2kb	5kb	750bp	2kb	-
	JDM			-	5kb	5.2kb	5kb	750bp	2kb	-
	JDF			-	5kb	5.2kb	5kb	750bp	2kb	-
6.	DH	45-48	45-48	-	5kb	5.2kb	5kb	750bp	2kb	-
	DHM			-	5kb	5.2kb	5kb	750bp	2kb	-
7.	MO	None	None	-	4.6kb	5.2kb	5kb	750bp	2kb	-
	MOB1	None	None	-	4.6kb	5.2kb	5kb	750bp	2kb	-
	MOB2	None	None	-	4.6kb	5.2kb	5kb	750bp	2kb	-
	MOB3	None	None	-	4.6kb	5.2kb	5kb	750bp	2kb	-
8.	RL	16-44	13-44	-	2.5kb,4.6kb	5.2kb	5kb	750bp,5kb	-	-
9.	MJ	51	51-52	-	-	5.2kb	-	750bp	-	-
	MJF							750bp		
10.	PN	None	None	-	5kb	5.2kb	5kb	750bp	2kb	-
	PNM	None	None	-	5kb	5.2kb	5kb	750bp	2kb	-
11.	VK	45-47	45-47	-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp,5kb	-	-
	VKB	None	None	-	2.5kb 4.6kb,5kb	5.2kb	5kb	750bp,5kb	-	-

Continued on next page

S.No.	CODE	DELETION		EXTRA FRAGMENTS IDENTIFIED BY PROBE						
		PCR	S.H.	1-2a	2b-3	4-5a	5b-7	8	9	10
LGMD PATIENTS AND THEIR FAMILIES										
1.	PG	None	None	-	4.6kb	5.2kb	5kb	750bp	-	-
2.	NC	None	None	-	4.6kb	5.2kb	5kb	750bp	-	-
3.	KK	None	None	-	5kb	-	5kb	5kb	-	-
4.	SA	None	None	-	5kb	5.2kb	5kb	750bp	2kb	-
	SAM			-	5kb	5.2kb	5kb	750bp	2kb	-
5.	RC	None	None	-	4.6kb	5.2kb	5kb	750bp	-	-
6.	MP	None	None	-	4.6kb	5.2kb	5kb	750bp	-	3.9kb
	MPF	None	None	-	4.6kb	5.2kb	5kb	750bp	-	3.9kb
7.	MN	None	None	-	-	5.2kb	-	750bp	-	-
	MNM			-	-	5.2kb	-	750bp	-	-
	MNB	None	None	-	-	-	-	750bp	-	-
	MNF	None	None	-	-	-	-	750bp	-	-
8.	HP (F)			-	4.6kb	5.2kb	5kb	750bp	-	-
	HPB	None	None	-	4.6kb	5.2kb	5kb	750bp	-	-
	HPM			-	4.6kb	5.2kb	5kb	750bp	-	-

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S.No.	CODE	DELETION		EXTRA FRAGMENTS IDENTIFIED BY PROBE						
		PCR	S.H.	1-2a	2b-3	4-5a	5b-7	8	9	10
9.	PO(F)			-	4.6kb	5.2kb	5kb	750bp	-	-
	POU			-	4.6kb	5.2kb	5kb	750bp	-	-
10.	NG(F)			4.8kb	4.6kb,5kb	5.2kb	5kb	750bp,5kb	2kb	-
	NGS	None	None	4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
	NGD			4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
11.	SC(F)			4.8kb	4.6kb,5kb	5.2kb	5kb	750bp,5kb	2kb	-
	SCM			4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
	SCSi1			4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
	SCSi2			4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
	SCS	None	None	4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
	SCB	None	None	4.8kb	4.6kb	5.2kb	5kb	750bp,5kb	2kb	-
SMA PATIENTS AND THEIR FAMILIES										
1.	AC	None	None	-	-	-	-	-	-	-
	ACM			-	-	-	-	-	-	-
2.	VT	None	None	-	4.6kb	5.2kb	5kb	750bp	2kb	-
	VTM			-	4.6kb	5.2kb	5kb	750bp	2kb	-
	VTF			-	4.6kb	5.2kb	5kb	750bp	2kb	-
	VTSi			-	4.6kb	5.2kb	5kb	750bp	2kb	-

Table 7: Summary of novel HindIII fragments

	NO. OF PATIENTS WITH NOVEL FRAGMENT BY PROBE						
	1-2a	2b-3	4-5a	5b-7	8	9	10
DMD	None	5	5	5	7	None	None
BMD*	None	11	14	11	13	6	None
LGMD	2	11	11	11	12	4	1
SMA	None	1	1	1	1	1	None
Total	2	28	31	28	33	11	1

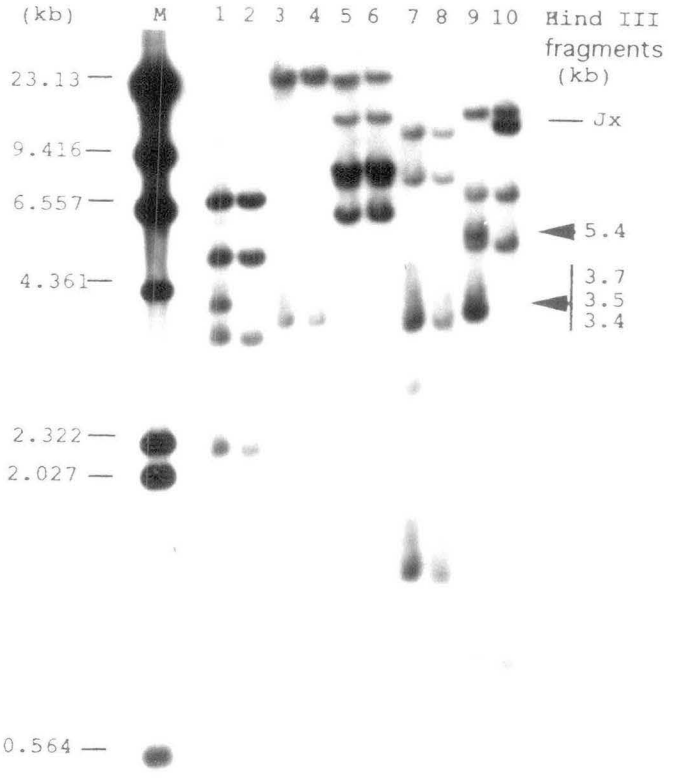
* = includes patient with QM

Table 8: Novel HindIII fragments in patients with or without deletions

	NO. OF PATIENTS WITH NOVEL FRAGMENTS		
	WITH DELETION	WITHOUT DELETION	TOTAL
DMD	4/5	3/3	7/8
BMD*	8/8	7/7	15/15
LGMD	None	12/12	12/12
SMA	None	1/2	1/2

* = includes patient with QM

Fig. 12: Southern blot analysis with probe 8 to check the presence of extra fragments. Lanes 1, 3, 5, 7, and 9 contain control DNA and lanes 2, 4, 6, 8, and 10 contain DNA from **VK** digested with five different enzymes.
Lanes 1 and 2 = TaqI, lanes 3 and 4 = BamHI, lanes 5 and 6 = EcoRI, lanes 7 and 8 = BglII, lanes 9 and 10 = PstI. Extra fragments were not observed in **VK** with any of the enzyme. Lane 2 shows deletion of 3.7 kb TaqI fragment (exon 47). Lane 8 shows deletion of 3.5 kb BglII fragment (exon 47). Lane 10 shows deletion of 5.4 kb PstI fragment (exon 47) and deletion of 3.4 kb PstI fragment (exon 51) along with a junction fragment of ~11 kb (Jx).


(kb)
M
1 2 3 4 5 6 7 8 9 10
Hind III fragments (kb)
23.13
9.416
6.557
4.361
2.322
2.027
0.564
Jx
5.4
3.7
3.5
3.4

DISTRIBUTION OF DELETION BREAKPOINTS

Having precisely localized the deletion boundaries, the distribution of breakpoints was evaluated. The absence of a HindIII fragment was presumed to indicate that the exons therein were completely deleted in the patient's DNA. Breakpoints were identified as either 5′ or 3′ relative to the direction of transcription.

Patients with Duchenne muscular dystrophy

In **MS, SO** and **SD**, the 5′ breakpoint was in intron 44. **AP** had 5′ breakpoint in intron 49, and **HA** in 3. The 3′ breakpoint was in intron 52 in **AP** and **SO**, 50 in **MS**, 45 in **SD** and 6 in **HA**.

Patients with Becker muscular dystrophy

Five patients, **AR, ARB, SR, DH, TR** had their 5′ breakpoint in intron 44 while **RL** had in intron 12 and **MJ** in intron 50. The 3′ breakpoint in **AR, ARB, SR** and **DH** was in intron 48 while in **TR** it was in intron 47. **RL** had a 3′ breakpoint in intron 44 and **MJ** in 53.

Patient with quadriceps myopathy

Patient **VK** had the 5′ breakpoint in intron 44 and 3′ breakpoint in intron 47.

SPECIFIC DELETION TRENDS ASSOCIATED WITH DMD OR BMD

The deletions in both DMD and BMD patients were concentrated in the region of probes 5b-7 and 8. The deletions in DMD patients were heterogeneous with respect to the extent of the deletions (Fig. 13). On the other hand, the deletions in BMD patients were homogeneous, with deletion of exons 45-48 being the most common.

DELETION FREQUENCY

The deletions were identified in 12 out of 19 (63%) unrelated DMD/BMD families by Southern blot analysis with eight cDNA probes. The frequency of deletions in DMD patients (62.5%) was similar to that of BMD patients (63.6%) (Table 9).

The number of deletions detected with different cDNA probes is summarized in Table 10. A total of 77 deletions were identified in 12 DMD/BMD patients and one male sib. In DMD patients, majority of the deletions were detected by probe 8 (13/23, 56.5%) and a significant proportion of these (6/13, 46%) extended into the distal segment of probe 5b-7. On the other hand, probe 5b-7 identified maximum number of deletions in BMD patients (28/54, 52%). The 10 kb HindIII fragment (exon 47) was common to both the probes. A small number of deletions were localized in the 5′ region of the cDNA corresponding to probe 1-2a (3/23, 13.4%) in DMD and 2b-3 (7/54, 13%) in BMD patients.

Of the 5 families with familial disease (where there were at least two affected males), deletions were found in 3 (60%). In one family, the deletion was identical in both the affected brothers (**AR** and **ARB**). In one non-deleted family, three affected brothers of the proband (**MO**) were studied. All the four brothers had no deletion. Out of the 14 families with sporadic disease (where only one affected male was known) deletions were detected in 9 (64.28%) (Table 11).

DELETION ANALYSIS BY mPCR AND SOUTHERN HYBRIDIZATION

DNA samples from 23 DMD/BMD patients (19 probands and 4 sibs) were assayed for deletions both by mPCR and Southern analysis. Fig. 13 shows the extent

Table 9: Percentage cDNA deletions in patients with DMD and BMD

CLINICAL DIAGNOSIS	NO. OF FAMILIES	NO.(%) OF cDNA DELETIONS
DMD	8	5(62.5%)
BMD*	11	7(63.6%)
TOTAL	19	12(63.15%)

* = includes one patient with QM

Table 10: Number of deletions detected with cDNA probes

CLINICAL DIAGNOSIS	NUMBER OF DELETIONS DETECTED BY PROBE						
	1-2a	2b-3	4-5a	5b-7	8	9	Total
DMD (n=6)	3	0	0	7	13	0	23
BMD* (n=8)	0	7	6	28	12	1	54
Total	3	7[a]	6[a]	35[b]	25[b]	1	77

* = includes one patient with QM
a = exon 20 shared by each probe in 1 patient
b = exon 47 shared by each probe in 8 patients

Table 11: Frequency of deletions detected in familial and sporadic cases (DMD/BMD)

	NO. OF FAMILIES* WITH DELETION	NO.OF FAMILIES* WITHOUT DELETION
Sporadic (n=14)	9 (64.28)	5(35.7)
Familial (n=5)	3 (60)	2(40)

n = No. of families studied

* = Figures in parenthesis indicate percentage

Table 12: Detection of deletions by multiplex PCR and the Southern blot analysis using cDNA probes

METHOD	NO. OF FAMILIES EXAMINED	NO. OF FAMILIES WITH DELETIONS	DETECTION RATE (%)
mPCR	19(8DMD,11BMD)	12(5DMD,7BMD)	63%
Southern Blotting	19(8DMD,11BMD)	12(5DMD,7BMD)	63%

Fig. 13 : Extent of 13 deletions related to the dystrophin exon map.

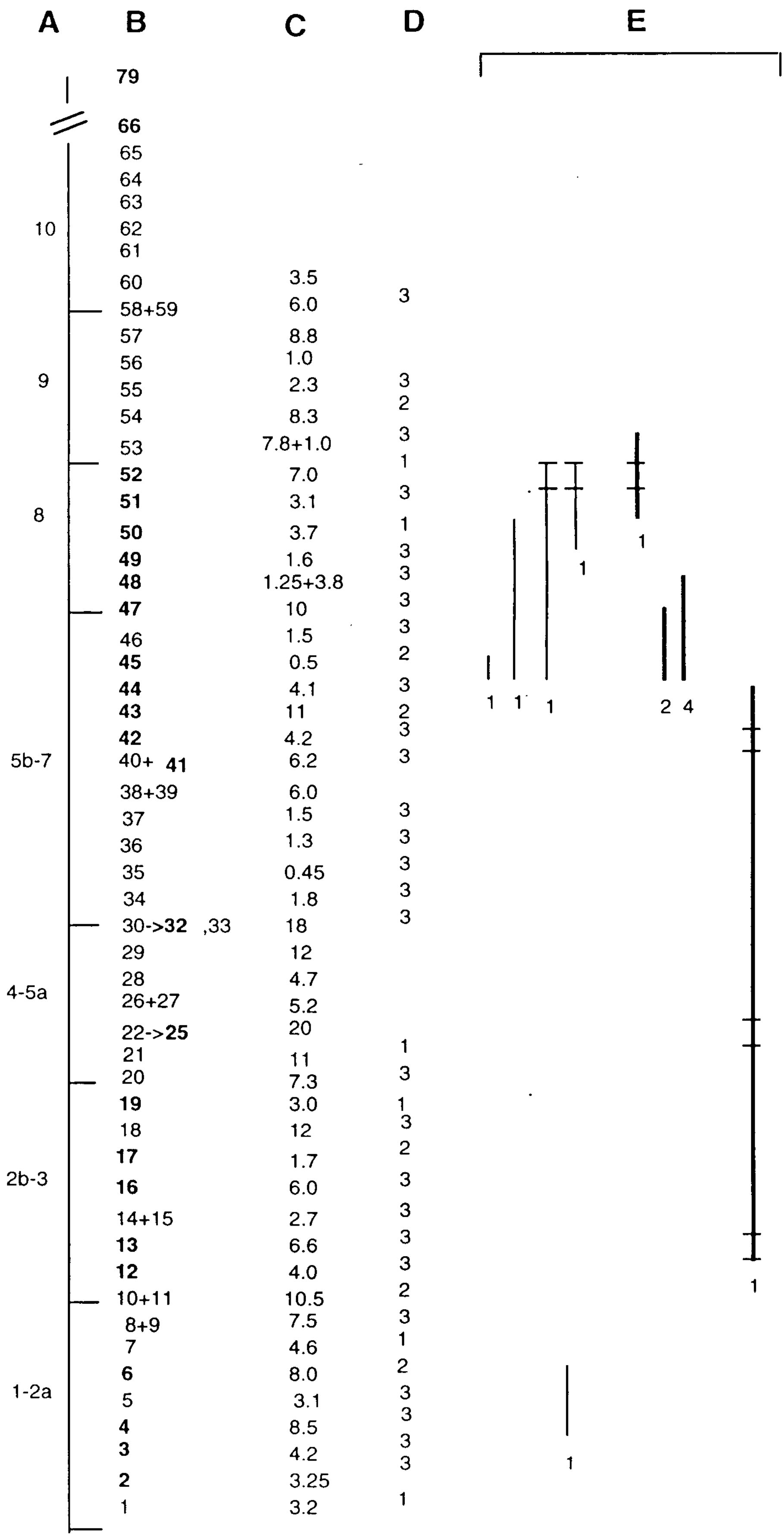
A : dystrophin cDNA probes used for the detection and delineation of deletions. The most distal probes (11-14) are not shown, since no deletions were detected with them.

B : exon numbers, numbers in bold indicate exons amplified by PCR.

C : exon containing HindIII genomic fragments (sizes in kb).

D : exon border type as per Koenig et al., 1989.

E : Extent of deletions found in 13 DMD/BMD patients. Thin vertical bars denote DMD patients; thick bars denote BMD patients; horizontal bars on vertical bars denote exons not deleted by PCR. Number below the bars indicate the number of patients sharing deletions of the same exons.



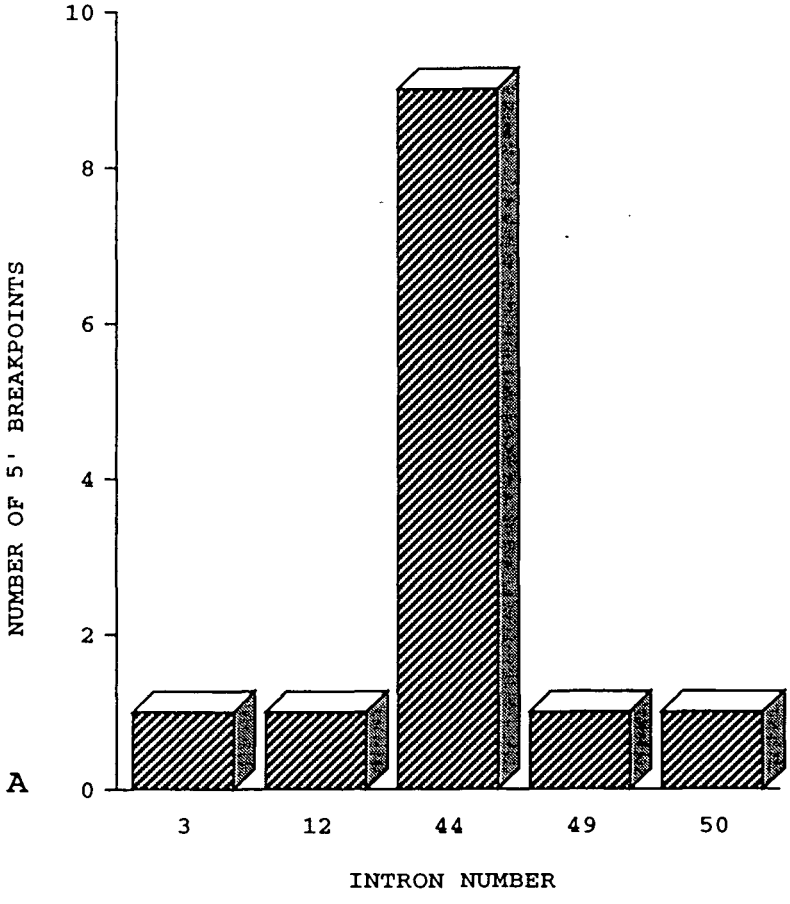
A
B
C
D
E
79
66
65
64
63
10
62
61
60 3.5
58+59 6.0 3
57 8.8
56 1.0
9
55 2.3 3
54 8.3 2
53 7.8+1.0 3
1
52 7.0
51 3.1 3
8
50 3.7 1
49 1.6 3
48 1.25+3.8 3
47 10 3
46 1.5 3
45 0.5 2
44 4.1 3
43 11 2
42 4.2 3
5b-7
40+ 41 6.2 3
38+39 6.0
37 1.5 3
36 1.3 3
35 0.45 3
34 1.8 3
30->32 ,33 18 3
29 12
28 4.7
4-5a
26+27 5.2
22->25 20
21 11 1
20 7.3 3
19 3.0 1
18 12 3
17 1.7 2
2b-3
16 6.0 3
14+15 2.7 3
13 6.6 3
12 4.0 3
10+11 10.5 2
8+9 7.5 3
7 4.6 1
6 8.0 2
1-2a
5 3.1 3
4 8.5 3
3 4.2 3
2 3.25 3
1 3.2 1
1
1
1
1
1
1
2
4
1
1

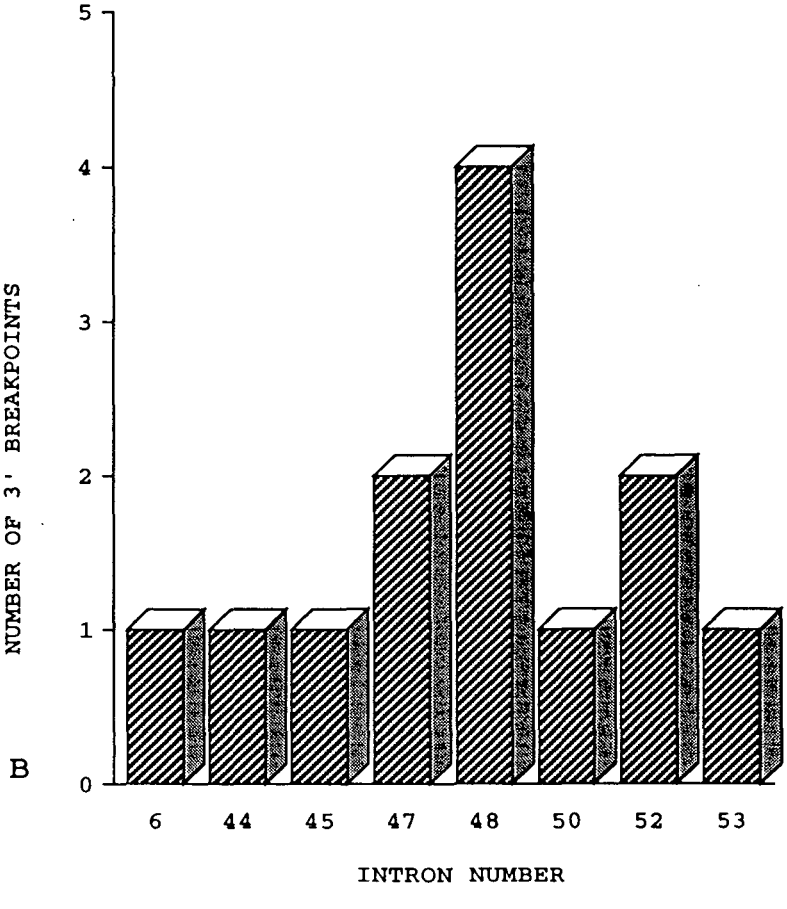
and location of deletions detected by eight cDNA probes in relation to 27 exons amplified in the PCR. Deletions detected in 12 DMD/BMD families by PCR were also identified by Southern blot analysis (Table 12). The only difference observed by these two techniques was in the deletion pattern. One patient **RL** with a deletion of exons 13-44 by Southern analysis showed amplification of exons 13, 25 and 42 by PCR. Another observation was the presence of 5.2 kb HindIII fragment (exons 26, 27) by probe 4-5a. Since an extra fragment of the same size is also observed in other patients, it was not clear whether the 5.2 kb fragment was the original fragment or the novel fragment. The deletion of exon 26 and 27 could not be confirmed since the primer pairs for these two exons were not included in the analysis by three multiplex sets. The deletion (by mPCR) in three patients (**AP, SO, MJ**) was extended by Southern blot analysis. In **AP**, the deletion identified by PCR (50-51) was extended to 50-52, in **SO** from 45-51 to 45-52 and in **MJ** from 51 to 51-53.

ANALYSIS OF DELETION BREAKPOINTS

The deletion analysis by mPCR and cDNA analysis revealed that intragenically the deletions were preferentially localized to two regions of the gene, one in the 5′ end of the cDNA detected by probe 1-2a (1/13, 7.7%), and a second larger one associated with exons around the middle part of the cDNA detected by probes 5b-7 and 8 (12/13, 92.3%). Of the total deletions, 92.3% have one or both of their breakpoints within the major "hot spot". Fig. 14 shows the distribution of 5′ and 3′ deletion breakpoints. The intron 44, which spans about 180 kb of the dystrophin gene, was the most frequently involved in deletion breakpoints. A total of 10 breakpoints were present in intron 44 (nine 5′ and one 3′), and 3′ breakpoint of 4 deletions were present in intron 48.

Fig. 14: Histogram showing distribution of 5'(**A**) and 3' (**B**) breakpoints for dystrophin gene deletions. The X-axis indicates the intron number while the Y-axis indicates the number of breakpoints localized within any given intron. Data include a total of 5 DMD and 8 BMD deletions.


A
NUMBER OF 5' BREAKPOINTS
10
8
6
4
2
0
3
12
44
49
50
INTRON NUMBER


B
NUMBER OF 3' BREAKPOINTS
5
4
3
2
1
0
6
44
45
47
48
50
52
53
INTRON NUMBER

PATTERN OF DELETIONS AND INTELLIGENCE

Information regarding the school performance was available for 16/23 DMD/ BMD patients. Of these, 6 (3 DMD, 3 BMD) were mentally subnormal and 10 (3 DMD, 7 BMD) had normal intelligence. The proportion of deletions was greater in families with mentally subnormal probands (83%) than in the families with normal intelligence (50%) (Table 13). The deletions in the central "deletion prone" region were identified in 11 patients (4 DMD, 7 BMD) with cDNA probes 5b-7 and 8 (Table 14). Out of these, 3 DMD and 2 BMD patients had mental subnormality. The deletions were different in each of these cases (deletion of exons 45, 45-48, 45-50, 50-52, 51-53). The common deletions were of exon 45 in 3, and exons 50 and 52 in 2 patients each. Most deletions apparently did not show any association with mental subnormality. For example, in BMD, one mentally subnormal and 3 patients of normal intelligence had the same deletions involving exons 45-48. Hence, there is no obvious correlation between the deletion pattern and mental subnormality in this group of patients.

EFFECT OF DELETION ON THE TRANSLATIONAL READING FRAME

The consequence of the deletion on the reading frame was determined by examining the type of intron-exon borders of the remaining two exons that flanked the deletion. The exon borders were classified as one of the three types (1, 2 or 3) depending on their position in the coding triplets (Koenig et al., 1989). The reading frame is maintained if the deletion juxtaposes exons with exon/intron borders that would restore the normal triplet codons in the DNA sequence. The effect that the deletion would exert on the reading frame is given in Table 15.

Table 13: Proportion of cDNA deletions in mentally normal and subnormal patients

MENTAL SUBNORMALITY	NO. OF PATIENTS	NO.(%) OF PATIENTS WITH CDNA DELETIONS
NO	10	5 (50)
YES	6	5 (83)
TOTAL	16	10 (62.5%)

Table 14: Deletion pattern and mental subnormality (MR)

S.NO.	PATIENT	DELETION	PROBE USED	MR
DMD Patients				
1.	AP	50-52	8	YES
2.	MS	45-50	5b-7, 8	YES
3.	SO	45-52	5b-7, 8	NA
4.	SD	45	5b-7	YES
BMD Patients				
5.	AR	45-48	5b-7, 8	NO
6.	ARB	45-48	5b-7, 8	NO
7.	SR	45-48	5b-7, 8	NO
8.	DH	45-48	5b-7, 8	YES
9.	TR	45-47	5b-7, 8	NA
10.	VK	45-47	5b-7, 8	NO
11.	MJ	51-53	8	YES

NA = NOT AVAILABLE

Table 15: Effect of deletions on the open reading frame

S.NO.	CODE	DELETIONS OF EXONS		BORDER TYPE*	READING FRAME
		PCR	SH		
1.	AP	50-51	50-52	3/1	SHIFTED
2.	MS	45-50	45-50	3/1	SHIFTED
3.	SO	45-51	45-52	3/1	SHIFTED
4.	SD	45	45	3/2	SHIFTED
5.	HA	4-6	4-6	3/2	SHIFTED
6.	AR	45-48	45-48	3/3	INFRAME
7.	ARB	45-48	45-48	3/3	INFRAME
8.	SR	45-48	45-48	3/3	INFRAME
9.	DH	45-48	45-48	3/3	INFRAME
10.	TR	45-47	45-47	3/3	INFRAME
11.	VK	45-47	45-47	3/3	INFRAME
12.	RL	16-44	13-44	3/3	INFRAME
13.	MJ	51	51-53	1/3	SHIFTED

* based on Southern analysis

There were 13 deletions observed in the present study. The consequence of the deletion on reading frame could be determined for all. In 5 DMD patients with deletions, the reading frame was disrupted because of the deletion (out-of-frame or frame-shift deletions). Seven out of eight BMD patients had deletions that maintained the reading frame (in-frame deletions). Thus, the correlation between deletion and phenotype fit the reading frame model in 12 (92.3%) out of 13 cases. One patient with BMD was in contradiction with the model. He had a frame-shift deletion of exons 51-53.

GENOTYPE - PHENOTYPE CORRELATIONS (Table 16)

Having analyzed the effect of deletion on the translational reading frame, an attempt was made to study the impact of exonic deletion on the phenotype of the DMD/BMD patients. Five of 8 patients with DMD showed deletions by Southern blot analysis that cause a shift in the open reading frame. The initial symptoms in these patients started between 2 to 5.6 years of age. Four probands (**MS, SD, HA** and **SO**) are now in the age range of 6-9 years and ambulatory. One patient (**AP**) became wheelchair bound at the age of 10.6 years. In this patient, the deletion identified initially by PCR was in-frame (50-51). It was found to include exon 52 by cDNA analysis, thus making it an out-of-frame deletion (Table 15). The absence of exon 52, but the presence of exon 53 indicated that the splicing of exon 49 (border type 3) to exon 53 (border type 1) may be responsible for the appearance of severe phenotype. Similarly, in **SO**, the in frame deletion by PCR for exons 45-51 was extended to 45-52 by cDNA analysis. The absence of exon 52 and presence of 53, both in **AP** and **SO**, indicated that the splicing of exon 44 (border type 3) to exon

53 (border type 1) may be responsible for the severe phenotype. When last seen at age 6, he had a waddling gait and experienced great difficulty in getting up from squatting position. The clinical investigations revealed a severe phenotype.

Three DMD patients did not show any deletions with either mPCR or Southern blot analysis. One of these (**RS**) was wheelchair bound at age 7 years, and had an elder brother suffering from the same disease who became non-ambulatory at age 6 years. Another patient **RH** had an early onset at 3 years of age with high CK. He had calf hypertrophy, and Gowers' sign was positive. When last seen at 9 years, he had great difficulty in walking. **NT** presented to the clinic at the age of 12 years as suffering from DMD. He died soon after.

Deletion analysis revealed an in frame deletion comprising exons 45-47 of the dystrophin gene in an isolated case of clinically diagnosed quadriceps myopathy. This was a 43-year-old patient (**VK**) with mild calf hypertrophy. He had slight difficulty in walking and in climbing stairs. The first symptoms of muscle weakness appeared very late at the age of 33 years. His disease was characterized as Becker muscular dystrophy by mPCR and Southern blot analysis. Another BMD patient (**TR**) had the same deletion. Onset in this patient was at age 14 years and he was ambulatory at 22 years of age.

The analysis of intron-exon border type of deletions comprising exons 45-48 was in accordance with the reading frame hypothesis in four BMD patients (**AR, ARB, SR** and **DH**) as expected. Two (**AR, ARB**)were brothers who had identical deletion and more or less similar progression of the disease. Both were ambulatory at age 20 and 43 years respectively. The symptoms of muscle disease appeared late

in **SR** (at age 17 years) and **DH** (at age 10 years). The progression of the disease was slow and both were ambulatory at age 19 and 14 years respectively.

One BMD patient **RL**, had a large deletion of exons 13-44 (in frame) with mild progression of the disease. Symptoms first appeared at 18 years and the patient was ambulatory at 22 years.

One BMD patient (**MJ**) was an exception to the reading frame theory. He had an apparent frame shift deletion of exons 51-53. He was ambulatory at 10 years with a mild phenotype. This exception to the reading frame rule could be due to the fact that the clinical progression of some patients less than 13 years old may be different from other patients. His condition is being monitored.

No deletions were observed in four BMD patients (**JD, RB, PN, MO**) and three symptomatic sibs of patient **MO** (**MOB1, MOB2, MOB3**). Two patients (**PN, MO**) had a positive family history. The progression of the muscle weakness was mild in both and they were ambulatory at the age of 12 and 16 years respectively. Two brothers **MOB1** and **MOB2** were wheelchair bound at 32 and 34 years and the third (**MOB3**) showed the first symptoms at 14 years (Table 1B). The age of onset was 6 years in **RB** and 11 years in **JD**. The progression of the disease was mild and both were ambulatory.

The dystrophin gene did not reveal any deletions in the patients with LGMD and SMA as would be expected in them since these disorders are not caused by mutations in the dystrophin gene.

Table 16 : Genotype-phenotype correlation

PATIENT	CLINICAL DIAG.	AGE (yrs.) D	ONSET	WC	MR	FH	DELETION BY SH	MOLECULAR DIAG.
AP	DMD	10	4	10.6	+	-	50-52	DMD
SO	DMD	6	2	A	NA	-	45-52	DMD
MS	DMD	6	2	A	+	-	45-50	DMD
SD	DMD	9	2	A	+	-	45	DMD
HA	DMD	8	5.6	A	-	+	4-6	DMD
AR	BMD	20	13	A	-	+	45-48	BMD
ARB	BMD	43	14	A	-	+	45-48	BMD
SR	BMD	19	17	A	-	-	45-48	BMD
DH	BMD	14	10	A	+	+	45-48	BMD
TR	BMD	20	14	A	NA	-	45-47	BMD
RL	BMD	22	18	A	NA	-	13-44	BMD
MJ	BMD	10	2	A	NA	-	51-53	DMD/BMD
VK	QM	43	33	A	-	-	45-47	BMD

MR = MENTAL SUBNORMALITY
FH = FAMILY HISTORY
SH = SOUTHERN HYBRIDIZATION
WC = WHEEL CHAIR BOUND
NA = NOT AVAILABLE

D = DIAGNOSIS
A = AMBULATORY
- = ABSENT
+ = POSITIVE

QUANTITATIVE mPCR (QM-PCR) ANALYSIS

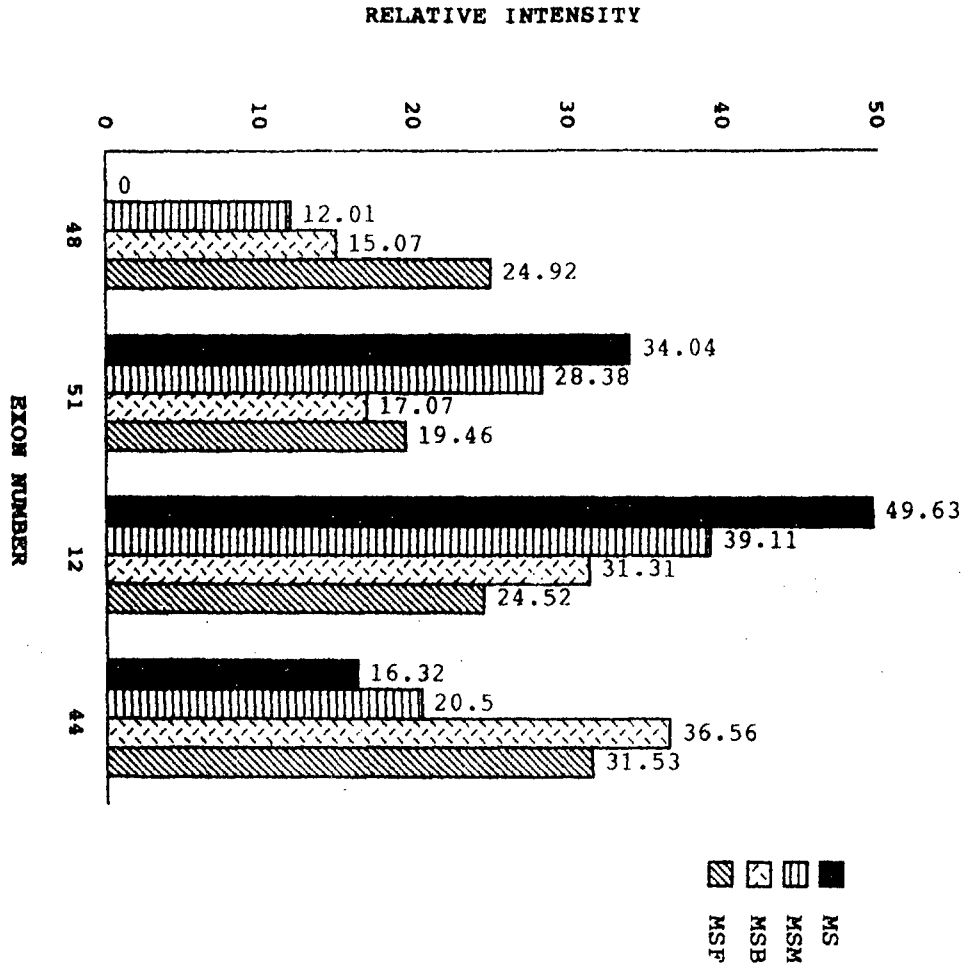
Quantitative PCR analysis was done to ascertain the carrier status of the female relatives of the patients with deletions in the dystrophin gene. A combination of primers for deleted and non-deleted exons was used as described in Methods. A total of 5 females and 6 normal males from the families with identifiable deletions were examined. Of the 4 mothers in these families, 3 were carriers of the mutation found in their respective DMD/BMD sons (one obligatory carrier), and one mother did not show the expected deletion in the DNA from her peripheral blood (Figs. 15-18).

Fig. 15A illustrates the autoradiographic results in one DMD patient (**MS**) and his family, and Fig. 15B shows the corresponding densitometric scans represented by histogram. The affected patient was found to carry deletions of exons 45-50. Exons 12, 44, 48 and 51 were analyzed by QM-PCR. Examination of the densitometric scans of the father and mother indicated that the mother had reduced relative band intensities for exon 48 which was deleted in the patient. Clearly, the relative intensity for deleted exon in the mother was about 50% of the level found in the father, showing that the mother in this family was a carrier for the deletion of the missing exon in her son.

Fig. 16A illustrates the autoradiographic results in another DMD family (**SO**) and Fig. 16B shows the corresponding densitometric scans. The affected patient was found to carry deletions of exons 45-52. Exons 12, 44, 48 and 51 were analyzed by QM-PCR. Examination of the densitometric scans of the father and mother indicated the mother was not a carrier for the deletion in exons 48 and 51. The sister

Fig. 15 A: Quantitative mPCR analysis of family **MS**, as described in Methods, using the primers for exons 44, 48, 51 and 12. The fragments are numbered according to the exons to which they correspond (left). This patient has a deletion of exon 48.

B: Graphical representation of densitometric analysis for family of patient **MS** using the same set of primers as in **A**. The patient in this family lacks exon 48 and the mother is a carrier for this deletion.


RELATIVE INTENSITY
0
10
20
30
40
50
EXON NUMBER
48
51
12
44
0
12.01
15.07
24.92
34.04
28.38
17.07
19.46
49.63
39.11
31.31
24.52
16.32
20.5
36.56
31.53
MSF
MSB
MSM
MS

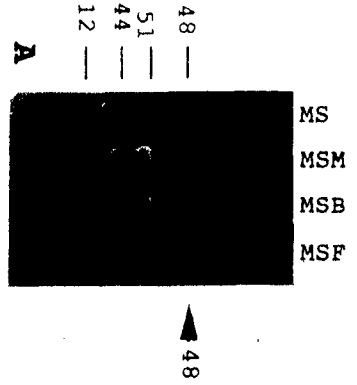
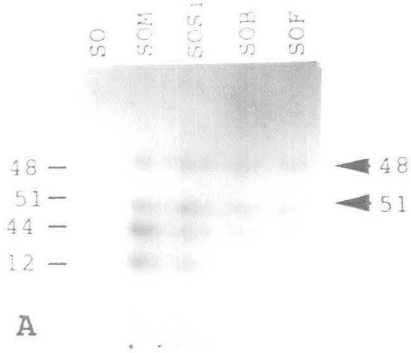
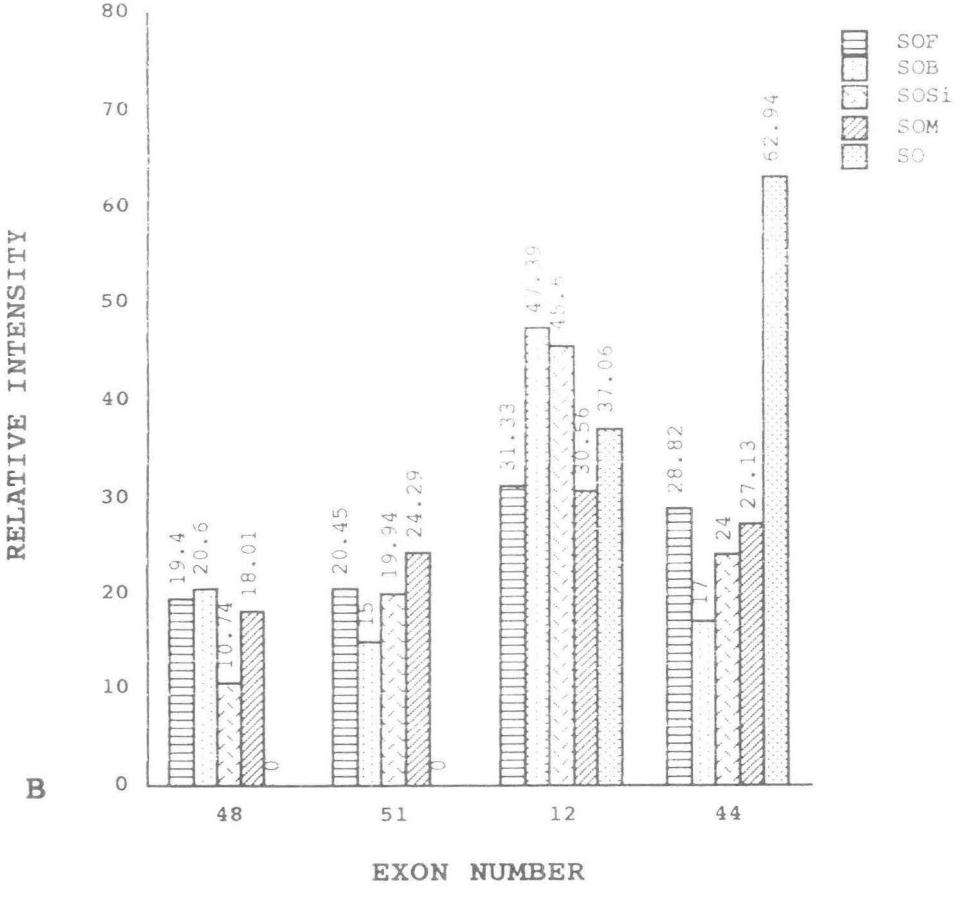

A
48
51
44
12
MS
MSM
MSB
MSF
48

Fig. 16 A: Quantitative mPCR analysis of family **SO**, as described in Methods, using the primers for exons 44, 48, 51 and 12. The fragments are numbered according to the exons to which they correspond (left). This patient has a deletion of exons 48 and 51.

B: Graphical representation of densitometric analysis for family of patient **SO** using the same set of primers as in **A**. The patient in this family lacks exons 48 and 51, but the mother is clearly not a carrier for this deletion.


SO
SOM
SOSi
SOB
SOF
48
51
44
12
48
51

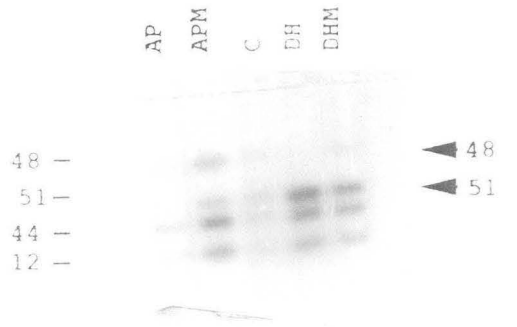
A


RELATIVE INTENSITY
80
70
60
50
40
30
20
10
0
19.4
20.6
10.74
18.01
0
20.45
15
19.94
24.29
0
31.33
47.39
45.5
30.56
37.06
28.82
17
24
27.13
62.94
48
51
12
44
EXON NUMBER
SOF
SOB
SOSi
SOM
SO

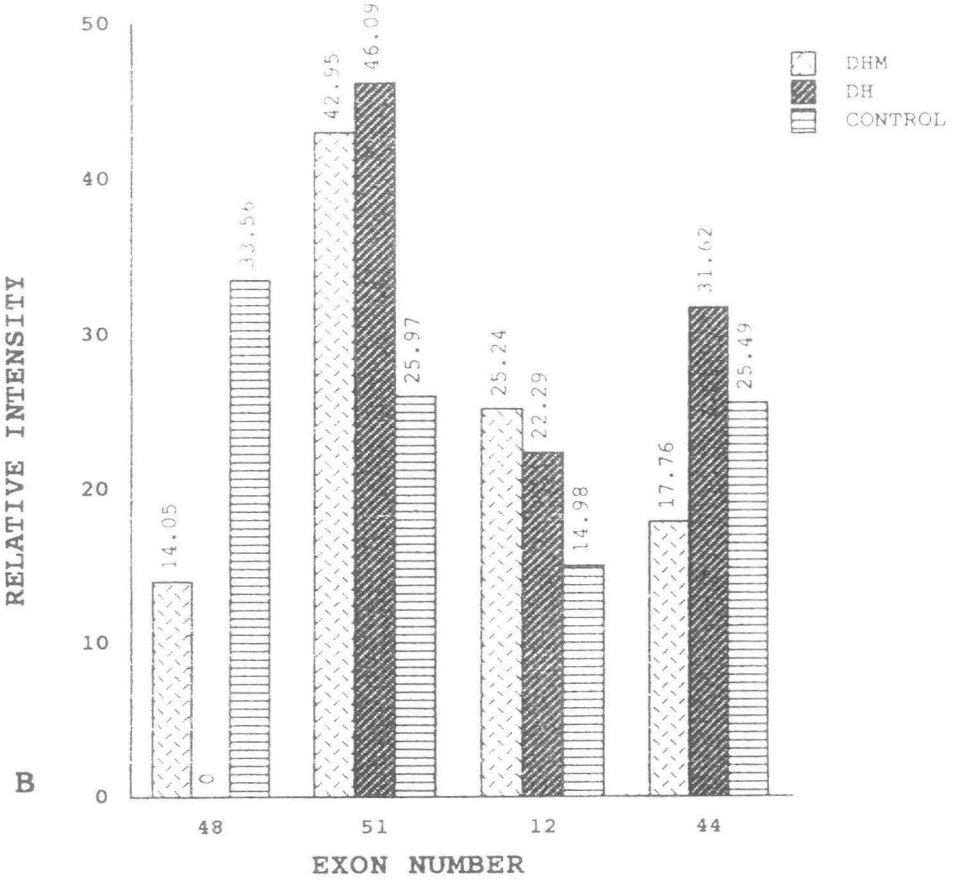
B

Fig. 17: **A**: Quantitative mPCR analysis of family **DH**, as described in Methods, using the primers for exons 44, 48, 51 and 12. The fragments are numbered according to the exons to which they correspond (left). This patient has a deletion of exon 48.

B: Graphical representation of densitometric analysis for family of patient **DH** using the same set of primers as in **A**. The patient in this family lacks exon 48 and the mother is a carrier for this deletion.

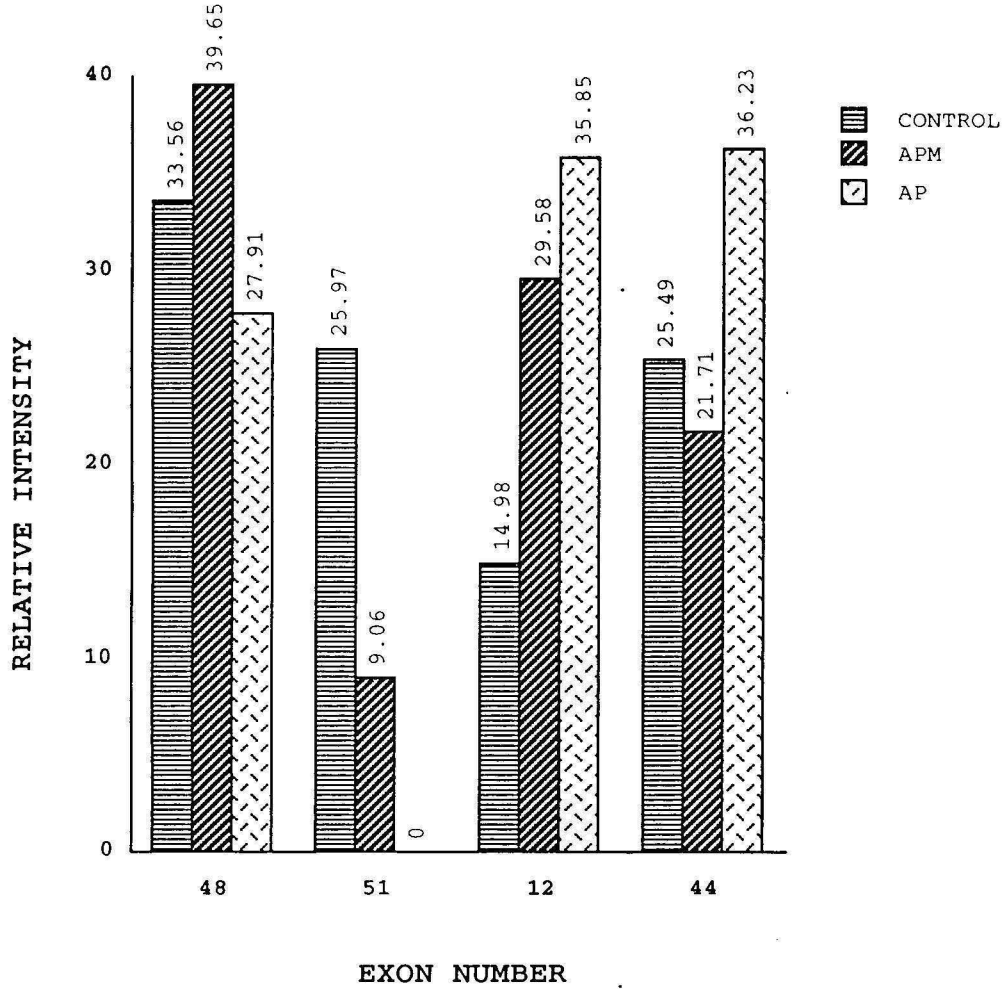

AP
APM
C
DH
DHM
48
51
44
12
48
51

A


RELATIVE INTENSITY
50
40
30
20
10
0
14.05
0
33.56
42.95
46.09
25.97
25.24
22.29
14.98
17.76
31.62
25.49
48
51
12
44
EXON NUMBER
DHM
DH
CONTROL

B

Fig.18: Graphical representation of the densitometric analysis for family of patient **AP** using the same set of primers as in Fig. 17**A**. The patient in this family lacks exon 51 and the mother is a carrier for this deletion.


40
30
20
10
0
RELATIVE INTENSITY
33.56
39.65
27.91
25.97
9.06
0
14.98
29.58
35.85
25.49
21.71
36.23
48
51
12
44
EXON NUMBER
CONTROL
APM
AP

appeared to be carrier for exon 48 but not for 51. This observation could either arise from an experimental error or may be an event which requires in depth study for an explanation.

Similarly, Figs. 17 and 18 show that the mother of **DH** and **AP** were both carriers for the deletion of exons in their sons.

The Southern blots have also been prepared for the above females but but the densitometric analysis could not be done due to time constraint.

PCR PRODUCT ANALYSIS FOR EXON 52

To understand the amplification of exon 52 by PCR and its absence by Southern blot analysis, first the DNA samples of **AP**, **SO** and **MJ**, were subjected to mPCR analysis by set II. The amplified products were transferred to nylon membrane and the blot was probed with cDNA 8. Exon 52 was identified on the autoradiogram as 113 bp amplified product. In another experiment, the amplified product for exon 52 was used as a probe to analyze HindIII-digested DNA from these patients. Due to random binding of the probe with the DNA, the result was inconclusive.

DISCUSSION

DELETION ANALYSIS BY mPCR AND SOUTHERN HYBRIDIZATION

A total of 23 male DMD/BMD patients from 19 unrelated families were analyzed for deletions in the dystrophin gene by mPCR (27 exons) and Southern hybridization (8 cDNA probes). An overall 63% of deletions were identified. This is in conformity with majority of the earlier reports (Koenig et al., 1987; Darras et al., 1988a; Forrest et al., 1988; den Dunnen et al., 1989; Gillard et al., 1989; Liechti-Gallati et al., 1989; Cooke et al., 1990; Claustres et al., 1991; Battaloglu et al., 1992; Multicenter study group, 1992; Simard et al., 1992; Vitiello et al., 1992; Katayama et al., 1993; Florentin et al., 1995). In other studies, a low frequency of deletions (32-52%) have been reported among these patients (Lindlöf et al., 1989; Sugino et al., 1989; Upadhyaya et al., 1990; Soong et al., 1991; Lau et al., 1992; Gökgöz et al., 1993; Shomrat et al., 1994) (Table 17). The percentage of deletions is much higher (78-86%) whenever the initial clinical diagnosis has been confirmed by dystrophin protein abnormalities (Beggs et al., 1991; Specht et al., 1992; Nicholson et al., 1993a).

The percentage of deletions detected by mPCR depends on the number of primer pairs used in an amplification reaction when compared to Southern blot analysis. Chamberlain et al. (1988) designed PCR primers to amplify 6 exons commonly deleted in DMD/BMD patients and with the addition of three more primers (Chamberlain et al., 1990), it was predicted to detect about 80% of deletions that have been identified by cDNA probes. According to Beggs et al. (1990) and

TABLE 17: Frequencies of deletions in North-America, Europe and Asia in DMD/BMD patients

AUTHORS	SUBJECTS	DELETION BY cDNA PROBES
NORTH AMERICA AND EUROPE		
Baumbach et al., 1989	DMD	90/160 (66%)
den Dunnen et al., 1989	DMD	98/160 (61%)
Hodgson et al., 1989	DMD/BMD	163/287 (57%)
Liechti-Gallati et al., 1989	DMD	53/80 (66%)
Lindlöf et al., 1989	DMD/BMD	45/90 (50%)
Cooke et al., 1990	DMD/BMD	89/123 (67%)
Upadhyaya et al., 1990	DMD	82/164 (50%)
Clausters et al., 1991	DMD/BMD	26/38 (67%)
Battaloglu et al., 1992	DMD/BMD	51/81 (63%)
Simard et al., 1992	DMD	38/59 (64%)
Vitiello et al., 1992	DMD	37/72 (51%)
Gökgöz et al., 1993	DMD	38/76 (50%)
Nicholson et al., 1993a	DMD/BMD	72/92 (78%)
Florentin et al., 1995	DMD/BMD	57/90 (63%)
Morandi et al., 1995a	BMD	47/53 (89%)
ASIA		
Sugino et al., 1989	DMD	18/42 (43%)
Asano et al., 1991	DMD	9/28 (32%)
Soong et al., 1991	DMD	6/13 (46%)
Lau et al., 1992	DMD/BMD	9/24 (38%)
Hiyama et al., 1993	DMD/BMD	26/48 (54%)
Immoto et al., 1993	DMD	54/88 (61%)
Katayama et al., 1993	DMD	14/22 (64%)
Shomrat et al., 1994	DMD/BMD	23/62 (37%)
Present study	DMD/BMD	13/19 (63%)

Katayama et al. (1993), 98% and 93% of the deletions (detected by cDNA) could be detected by mPCR respectively, when a total of 19 different exons were analyzed. Fujishita et al. (1991) have reported that 92% of the deletions observed by Southern analysis were detected by PCR using only 7 primer pairs. In a comparative study by Claustres et al. (1991), 92% and in another study by the Multicenter study group (1992) 82% of the deletions detected by cDNA probes could be identified by amplification of 9 selected exons. In the present study, the deletion detection rate observed by cDNA was same as that observed by mPCR when 27 exons were amplified. It is observed that irrespective of the number of primer pairs used to amplify the exons, the percentage rate of deletion detection remains about the same. However, the use of 27 primer pairs has helped in the determination of exact endpoints of the deletions.

In this study, the proportion of deletions observed in familial cases (3/5, 60%) was almost similar to that in the sporadic (9/14, 64.28%). A similar observation has been reported earlier by Lindlöf et al. (1989) who observed deletions in 16/27 (59%) families with familial disease, and 29/63 (46%) families with sporadic disease. According to them, this could be interpreted as evidence against any major negative effect on either gamete production or gamete reproduction in women with these deletions. Similarly, Nicholson et al. (1993a) observed deletion/duplications in 21/25 (84%) families with more than one affected member and 54/67 (80.6%) in isolated cases. On the other hand, Passos-Bueno et al. (1990), found deletions in 16/22 (73%) of sporadic cases compared to only 7/23 (28%) of familial cases. Nicholson et al. (1993a) based this difference on large proportion of isolated cases (n=67) in their study, compared to Passos-Bueno (1990) (n=22).

It has been suggested that the region of the DMD locus detected by cDNAs 7 and 8 is the most prone to deletions (Forrest et al., 1987a, 1988; Koenig et al., 1987; Darras et al., 1988a; Upadhyaya et al., 1990; Gökgöz et al., 1993). Forrest et al. (1987a, 1988) found that 59 of 107 (55%) DMD patients had deletions detected by these two probes: 27 (46%) were identified by Cf23a (equivalent to cDNA 7) only while 32 (54%) were identified with Cf56 (equivalent to cDNA 8) alone. In another study of 104 DMD patients, Koenig et al. (1987) detected a total of 53 deletions: 27 (51%) with probe 8, and 19 (36%) among these started in the region detected with probe 7. Darras et al. (1988a) observed that 12 among 32 DMD/BMD patients had deletions in the region detected by probe 8. Among these, only three (25%) were confined to this region and six (50%) extended to the region seen by probe 5b-7. Passos-Bueno et al. (1990) analyzed 38 DMD patients, 17 (50%) had deletions detected by Cf56a: 5 (29%) of these extended to the region of probe Cf23a and only 2 (12%) were confined to Cf23a. Upadhyaya et al. (1990) observed 62/82 (76%) deletions with Cf56a and Cf56b (cDNA 6-7). Out of these 35 (42%) started in the region of Cf56b. In this study, it was observed that among 5 deleted DMD patients, 3 (60%) had deletions detected by probe 8, 2 (67%) of which extended to the region 5b-7 and in one (16.7%), it was confined to the region of probe 5b-7. This suggests that DMD deletions are confined mainly to probe 8, in accordance with Forrest et al. (1987a) who pointed out that the screening of DMD patients should begin with cDNA 8. Deletions in the 5′ end of the gene have been reported earlier in DMD patients (Darras et al., 1988a; Baumbach et al., 1989; Koenig et al., 1989; Cooke et al., 1990; Simard et al., 1992; Gökgöz et al., 1993; Hiyama et al., 1993; Katayama et al., 1993). In this study, one DMD patient showed deletions with probe 1-2a.

In a study of BMD patients, Forrest et al. (1988) reported that 17/36 (47%) had deletions detected with probe Cf23a and that 16 of these were also detected with Cf56a. Darras et al. (1988a) observed deletions in three BMD cases with probe 8, which extended towards the 3′ end. In a sample of 8 patients with BMD (present study), 7 (87.5%) had deletions detected with probe 5b-7, and 6 of these extended to the region of cDNA 8. The deletions observed in these families were similar to those reported by Forrest et al. (1987a, 1988), Passos-Bueno et al. (1990), Cooke et al. (1990), Matsumura et al. (1993) and Nicholson et al. (1993a) suggesting that BMD mutations are particularly concentrated in the 5b-7 region.

An interesting finding was one BMD patient in whom the deletion detected by probe 8 extended into the region of probe 9. In earlier studies, Koenig et al. (1989) and Lindlöf et al. (1989) have reported deletions in the region of probe 8 alone in BMD patients. Lindlöf et al. (1989), Rapaport et al. (1991) and Nicholson et al.(1993a) have reported deletions in DMD patients in the region of probe 8 and extending to probe 9. Cooke et al. (1990) identified one BMD deletion in the region of probe 7 extending to probe 9. Deletions starting in the region of probe 5b-7 and extending to probe 9 have also been reported in DMD patients (Lindlöf et al., 1989; Rapaport et al., 1991; Simard et al., 1992; Nicholson et al., 1993a).

In this study, no deletions were identified with probe 10 in both DMD and BMD patients. Nicholson et al. (1993a) reported one DMD and one BMD patient each with deletion in the region of probe 8 extending to probe 10. A few studies have reported deletions in the DMD patients with probe 9 extending to the region 10 (Liechti-Gallati et al., 1989; Vitiello et al., 1992; Hiyama et al., 1993).

Probe 11-14 did not reveal any deletion in both DMD and BMD patients in this study. Deletion studies in DMD/BMD patients with probe 11-14 have been carried out earlier (Hodgson et al., 1989; Koenig et al., 1989; Lindlöf et al., 1989; Cooke et al., 1990; Norman et al., 1990; Beggs et al., 1991; Lau et al., 1992; Simard et al., 1992; Gökgöz et al., 1993; Nicholson et al., 1993a) but none of these observed deletions in any DMD/BMD patient. According to Darras et al. (1988a), deletions near the 3' end of the gene may exist but not be found in DMD/BMD patients because they cause a milder, non-progressive myopathy. This possibility has been suggested by some patients with the glycerol kinase deficiency/adrenal hypoplasia/ myopathy microdeletion syndrome (Francke et al., 1987), who were found to have deletions of the 3' end of the dystrophin gene (Darras and Francke, 1988). However, Passos-Bueno et al. (1990) detected a deletion with the 3' cDNA (Cf115) in a boy with DMD. This patient had a clinical evaluation typical of severe DMD and had two affected maternal uncles who died at 13 and 15 years of age. He had no glycerol kinase deficiency or any other associated X-linked disorder. In 1993, Hiyama et al. also reported a single DMD case deleted in the region of cDNA 11.

In the present study, 11/13 (84.6%) deletions were detectable when only two cDNA probes (1-2a and 8) were used. The deletions were distributed non-randomly in the major (11/13, 84.6%) and minor (1/13, 7.7%) "hot spot" regions of the dystrophin gene in DMD/BMD patients. Liechti-Gallati et al. (1989) observed 54% and 22% deletions in major and minor "hot spot" regions of the gene respectively. Claustres et al. (1990) have observed 68% and 24% whereas, 75% and 16-20% were observed by Gillard et al. (1989), Koenig et al. (1989) and Nicholson et al.(1993a) in the major and minor deletion prone regions respectively. Lindlöf et al. (1989), Klamut

et al. (1990), Prior et al. (1990a) and Simard et al. (1992) also reported that distal and proximal "hot spots" accounted for 50-60% and 30% of deletions respectively. In a recent study among Filipino DMD/BMD patients by Cutiongco et al. (1995), more deletions were observed in the 5' region than in the central region of dystrophin gene. Although, their patient sample is too small, they observed 2 deletions in the minor "hot spot" and 1 in the major.

Thus, the frequency and distribution of these deletions in the Indian population were not unique but characteristic of general population (e.g. Darras et al., 1988a; Forrest et al., 1988; den Dunnen et al., 1989; Gillard et al., 1989; Koenig et al., 1989; Lindlöf et al., 1989).

In this study, one patient **RL**, showed a large deletion of exons 13-44 by cDNA analysis, but exons 13, 25 and 42 were amplified in the PCR. This difference may have been due to the partial conservation of a PCR primer annealing site on the template DNA (Katayama et al., 1993). In addition, the 5.2 kb HindIII fragment corresponding to exons 26-27 was detected on the Southern blot with probe 4-5a (Fig. 7B). This may be a novel fragment which was observed in 35/37 patients. Two distinct mutations in a single dystrophin gene have been described previously (Bartlett et al., 1989; Laing et al., 1992; Wilton et al., 1993; Morandi et al., 1995b) hence, the possibility of two distinct mutations in this patient cannot be ruled out. Further hybridizations on different restriction enzyme digests and PCR amplification with a different pair of primers specific for the same region may help to resolve this discrepancy.

In **MJ**, the deletion was between exons 51-53 by Southern blot analysis but exon 52 was amplified by PCR. Similarly, in both **AP** and **SO**, the deletion identified

by PCR (50-51 and 45-51 respectively) was extended to include exon 52 by Southern blot analysis. In a comparative study of 148 patients (Abbs et al., 1991), discrepancies between the Southern cDNA data and mPCR results were observed in a total of 8 patients. Detailed investigation revealed that this was due to low resolution of Southern analysis, and sequence variation within priming site which prevents annealing of primer. Claustres et al. (1991) identified the deletion of 3.1 kb HindIII fragment containing exon 51 by cDNA analysis but PCR revealed exon 51 and its flanking introns to be intact. They suggested that the breakpoint could be in the intronic 3.1 kb fragment. Katayama et al. (1993) observed variation in the results of PCR and Southern blot analysis in two patients. Gökgöz et al. (1993) also reported the presence of exon 52 by PCR, but its absence by Southern blot analysis in two cases. The use of another set of primers for exon 52, and Southern analysis with another restriction enzyme may resolve this discrepancy.

NOVEL HindIII RESTRICTION FRAGMENTS

Novel HindIII restriction fragments identified in 35/37 patients with or without exonic deletions may not be the junction fragments. Although, the extra fragments appeared in some of the deleted patients, these were also present in non-deleted patients and built-in controls (healthy family members of the patients). However, the extra fragments were not detected in 20 unrelated controls (with no family history of any muscle disease).

DNA from one patient (**VK**) was digested with TaqI, BglII, BamHI, EcoRI, and PstI and probed with cDNA 8 (Fig. 12). The restriction fragment pattern obtained was same as observed in the control male with all the enzymes except PstI (lane 10

and 11). A junction fragment of ~11 kb was revealed along with an additional deletion of 3.4 kb PstI fragment containing exon 51 which was not deleted by PCR and HindIII. To suggest any relation between this junction fragment and the novel HindIII fragments identified by probe 8, other samples need to be investigated by different restriction enzymes.

A new restriction fragment pattern was identified with probe 11-14 in both controls and patients (Table 5; Fig. 11B). There was a gain of one ~2.8 kb band with the loss of two bands of 1.8 and 1.9 kb each when compared with the pattern described by Koenig et al. (1987). This pattern is also different from the one reported by Bies et al. (1992). They had observed the gain of two bands of 2.4 and 3.4 kb each with the loss of one 6.0 kb band as compared to the pattern described by Koenig et al. (1987).

DISTRIBUTION OF DELETION BREAKPOINTS

In the present study, the pattern of deletions found in DMD patients is heterogeneous with respect to their size and location, but there appears to be a cluster of deletion endpoints between intron 44 and 52 (8/10). The deletions observed in BMD patients were homogeneous to the extent that the 5′ endpoint in 6/8 patients were located in the same intron 44. Intron 44, detected by probe 5b-7, has been reported to be 160-180 kb long and is the most frequent site for rearrangements in the dystrophin gene (den Dunnen et al., 1989). Upto 77% of the deletions both in DMD and BMD patients had a breakpoint in this region. Ten out of eleven deletions with breakpoint in intron 44 extended towards the 3′ end, as also observed by Kitoh et al. (1992) who suggested that the breakpoint in this intron is orientation specific.

But in this study, an in-frame deletion was observed which proceeds towards the 5′ end from intron 44 in one BMD patient (**RL**, Fig. 13). Koenig et al. (1989) studied 273 deletions in DMD/BMD patients and did not come across any BMD deletion that started in the large intron 44 and extended towards the 5′ end of the gene beyond exon 44. Subsequently, in a case study, Love et al. (1991) reported a BMD patient with deletion of exons 14-44. Beggs et al. (1991) described two inframe deletions with a breakpoint in intron 44 which extended to the 5′ region. One patient (del 10-44) was too young to be classified and the other (del 35-44) had high CK as the only presenting symptom. Matsumura et al. (1993) observed two BMD patients with inframe deletions starting in intron 44 and extending in the 5′ direction (13-44 and 35-44). Recently, in a study on BMD patients, Morandi et al. (1995a) reported two patients with deletions beginning in intron 44 and extending in the 5′ region of the gene (13-44 and 32-44).

The data in this study provides further evidence that the distribution of deletion breakpoints within the major "hot spot" region is not entirely random. Of a total of 23 breakpoints, 10 (43.47%) were in intron 44 and 4 (17.3%) were in intron 48, (Fig. 14). This is in conformity with an earlier report on BMD patients by Beggs et al. (1991) who observed 43.2% of breakpoints in intron 44 and 14.8% in intron 48 out of a total of 81 breakpoints in the major "hot spot" region. On the other hand, Simard et al. (1992) in a study on DMD/BMD patients observed equal number of breakpoints in intron 44 and 50 which could be due to the large number of DMD patients in their study. Florentin et al. (1995) have reported less breakpoints (17%) in intron 44 and more (29%) in intron 50.

PATTERN OF DELETIONS AND INTELLIGENCE

Dystrophin is found in brain as well as in muscle and expression of the dystrophin gene is controlled by different 5′ promoters in various cell types or tissues, the full length brain isoform having an alternative first exon (Chelly et al., 1989; Nudel et al., 1989; Barnea et al., 1990). It is possible that defects in the dystrophin gene might affect brain function just as they affect muscle function, although removal of the brain promoter is compatible with normal intellect (Boyce et al., 1991; den Dunnen et al., 1991; Rapaport et al., 1991).

The 38% of DMD patients with mental subnormality (MR) observed in the present study is within the range 18 to 63% reported earlier (Allen and Rodgin, 1960; Dubowitz, 1965; Murphy et al., 1965; Kozicka et al., 1971; Rabbi-Bartolini and Zatz, 1986; Emery, 1987; Hodgson et al., 1989; Rapaport et al., 1991). Lindlöf et al.(1989) reported a lower frequency (16%) of mentally subnormal (not able to attend school) patients. This could be explained partially by the inclusion of 20 families in their sample in which the patients were too young to be classified (Rapaport et al., 1991).

In the present study, mental subnormality was observed in 6/16 DMD/BMD patients. In five of the six mentally subnormal patients, deletions were identified which were of variable length (deletion of exons 50-52, 45, 45-50, 45-48, 51-53). However, it was observed that exon 45 was deleted in 3 and exons 50 and 52 were deleted in 2 patients each. This data might suggest that DNA of importance for normal mental development in males may occur in the region of cDNA 8 as also observed by Lindlöf et al. (1989). Although, probe 8 was the most frequently deleted

(comprising 84.6% of patients), four patients with deletions by this probe were normal.

Most deletions apparently did not show any association with MR. For example, in BMD, one MR and three patients with normal intelligence had the same deletions involving exons 45-48. Rapaport et al. (1991) reported two mentally retarded and three boys of normal intelligence with DMD who had the same deletion involving exons 45-49. They also observed that 4 DMD boys were deleted for exon 45, but only one had mental retardation. In this study, one DMD boy was deleted for exon 45 alone and he was mentally subnormal. None of the three DMD patients with deletion of exon 45 alone were mentally retarded in a study by Nicholson et al. (1993a, 1993b).

In this study, there were two MR patients (one DMD and one BMD) with deletions of DNA region encompassing exon 52. Rapaport et al. (1991) observed six unrelated mentally retarded DMD patients with an identical pattern of deletion of exons 48-52. They suggested that exon 52 of the dystrophin gene might be implicated in mental retardation. This conclusion was based on the greater number of DMD patients in their study with mental retardation who had deletion involving this exon. Hiyama et al. (1993) found two patients with mental retardation who had deletion encompassing exon 52. There were two patients with deletion of exon 52 alone, and only one of them was mentally retarded. In addition, mental retardation was not observed in a case having duplication of identical exon 52. Nicholson et al. (1993a, 1993b) found that 5/11 patients missing exon 52 were retarded. An involvement of the same region of dystrophin gene (probe 8) was also

found in some of the MR patients reported by Lindlöf et al. (1989), who suggested that DNA of importance for normal development in males may occur in this region. According to den Dunnen et al. (1989), the observation of a greater frequency of mental retardation among patients with a deletion encompassing the region covered by exon 52 could be due to the presence of essential sequences (which could be other genes) whose expression may even be independent from DMD gene expression, or it could be related directly to an important domain of brain dystrophin transcript in this region.

According to Rapaport et al. (1991) mental retardation is rare in BMD and none of their BMD patients had deletion involving the region covered by exon 52, suggesting a direct effect on an important domain of brain dystrophin which would be impaired in DMD but not in BMD. However, one BMD patient with deletion of exons 51-53, who is mentally subnormal, was observed in this study.

No clear relationship was found between mental subnormality and the occurrence, extent, or position of the gene deletions as also reported by Gillard et al. (1989), Hodgson et al. (1989), Hiyama et al. (1993) and Nicholson et al. (1993a, 1993b). None of the patients with MR had deletions in the proximal half of the gene which is in conformity with Hodgson et al. (1992) and Nicholson et al. (1993b).

GENOTYPE - PHENOTYPE CORRELATIONS

Two collaborative studies (Gillard et al., 1989; Koenig et al., 1989) have demonstrated that the effect of deletions on the translational reading frame of dystrophin mRNA may account for the phenotypic differences between DMD and

BMD, rather than the size and location of deletions, as predicted by Monaco et al. (1988). Results obtained in this study, with the exception of one patient, agree with the published series (Baumbach et al., 1989; Gilgenkrantz et al., 1989; Claustres et al., 1991).

In patients with deletions, the clinical severity of the disorder was examined for the frame-shift hypothesis (Monaco et al., 1988). Deletions that created a frame-shift mutation in the protein coding region caused DMD, whereas an in-frame deletion caused BMD, except in one case where a BMD phenotype was the result of an apparent frame-shift deletion. Hence clinical/molecular correlations based on the alteration of the reading frame were valid in 92% of the cases.

Five patients with classical DMD had deletions that cause a shift in the open reading frame. **HA** was deleted in exons 4-6. This frame-shift deletion has not been reported earlier. When last seen, he was 8 years old and still ambulatory. **SD** had a deletion of exon 45. This deletion has been previously reported (Cooke et al., 1990; Arahata et al., 1991; Claustres et al., 1991; Battaloglu et al., 1992; Kitoh et al., 1992; Nicholson et al., 1992; Simard et al., 1992; Gökgöz et al., 1993; Katayama et al., 1993; Nicholson et al., 1993a; Uchino et al., 1994). In this patient, initial symptoms appeared at the age of 2 years with difficulty in walking by 10 years of age. The deletion of exons 45-50 was documented in **MS** which has been associated with DMD phenotype (Lindlöf et al., 1989; Cooke et al., 1990; Claustres et al., 1991; Battaloglu et al., 1992; Simard et al., 1992; Gökgöz et al., 1993; Hiyama et al., 1993). Exons 45-52 were deleted in **SO**. This deletion pattern has been observed in DMD patients by Koenig et al., 1989; Cooke et al., 1990; Claustres et al., 1991; Battaloglu et al.,

1992, Lau et al., 1992; Gökgöz et al., 1993 and Nicholson et al., 1993a. A frame-shift deletion of exons 50-52 was observed in **AP**, who became wheelchair bound at the age of 10.6 years. This deletion pattern has been reported earlier in DMD patients (Claustres et al., 1991; Lau et al., 1992).

All the BMD patients had a mild clinical profile as indicated by their proximal muscle weakness. The majority of the mutations associated with this clinical profile were clustered in a very small region of the gene. Deletion of exons 45-47 and 45-48 accounted for 75% of the deletions detected in BMD patients.

There were two patients (**TR** and **VK**) carrying deletion of exons 45-47. **TR** was a typical BMD patient with onset at 14 years. But **VK** was clinically diagnosed as suffering from quadriceps myopathy. The symptoms of the muscle disease appeared quite late at the age of 33 years. The evidence that patients with QM have deletion of dystrophin locus, has been previously reported by Sunohara et al. (1990), Beggs et al. (1991) and Vonmitzlaff et al. (1993) in their studies on dystrophin protein and gene from QM patients. Both **TR** and **VK** were ambulatory when last seen (Table 16). This common BMD deletion of exons 45-47 has been widely reported (Witkowski, 1988; Norman et al., 1989, 1990; Koenig et al., 1989; Cooke et al., 1990; Arahata et al., 1991; Battaloglu et al., 1992; Simard et al., 1992; Palmucci et al., 1992; Gökgöz et al., 1993; Hiyama et al., 1993; Matsumura et al., 1993; Nicholson et al., 1993a; Uchino et al., 1994; Morandi et al., 1995a). A comparison of this deletion pattern with the exon-intron boundaries sequences by Koenig et al. (1989) confirms that this deletion should not disrupt the translational reading frame of the protein. This may explain the mild clinical expression of the disease in skeletal muscle, in line with the hypothesis of Monaco et al. (1988).

An in-frame deletion of exons 45-48 was observed in four patients (**AR, ARB, SR, DH**). Two brothers **AR** and **ARB** had a similar progression of the disease. Both were ambulatory at age 20 and 43 years respectively. In **SR**, the symptoms of muscle disease appeared at 17 years and he was ambulatory at age 19 years. **DH** also had a late onset at age 10 years and was ambulatory when last seen at age 14 years. This pattern has been previously reported in BMD patients (Koenig et al., 1989; Arahata et al., 1991; Claustres et al., 1991; Battaloglu et al., 1992; Simard et al., 1992; Driguzzi et al., 1993, Matsumura et al., 1993; Nicholson et al., 1993a; Morandi et al., 1995a).

Few BMD patients have been described with large intragenic deletions (Hodgson et al., 1989; Koenig et al., 1989; England et al., 1990; Love et al., 1991; Matsumura et al., 1993; Morandi et al., 1995a). One patient 324 (England et al., 1990) had a deletion of exons 17-48 and was able to walk with the aid of a stick at 61 years of age. Patient number 1 (Matsumura et al., 1993) who had a deletion of exons 13-44 was very young (6.5 years), and the status of the dystrophin and dystrophin associated proteins was near normal. This large deletion of exons 13-44 was observed in one BMD patient (**RL**) in this study. His initial symptoms appeared at 18 years of age and he had a mild phenotype as did the case reported by Morandi et al. (1995a). This deletion has been suggested for the development of potential DMD therapies using dystrophin minigenes (Love et al., 1991).

There was one case, which is a rare instance, where the frame-shift theory could not predict the course of the disease. One patient (**MJ**) clinically diagnosed as BMD, had a deletion of exons 51-53 with a border type 1/3 so that when exon 50 gets spliced with 54, a frame-shift results. Although, this case should be diagnosed

as DMD according to the frame-shift theory, the clinical course of the disease was more mild. Recent studies (Chelly et al., 1990; Matsuo et al., 1991; Narita et al., 1993; Hagiwara et al., 1994) have shown that the genomic mutations, even when accurately defined, cannot always predict the sequence of the post transcriptionally modified dystrophin mRNA. In these studies, transcript sequence analysis has shown that alternate splicing events could produce an unpredictable coding sequence. Such data illustrate the importance of analyzing gene mutations at the mRNA level to avoid erroneous assignment of the mutation boundaries based solely on genomic DNA. In **MJ**, alternative splicing can be a likely explanation for the observed deviation from the frame-shift theory, as splicing out exon 50, would maintain the reading frame. However, this hypothesis remains to be verified experimentally. The other explanation is that this exception to the reading frame rule may be due to the fact that the clinical progression of some patients less than 13 year old may be different from what was predicted at initial diagnosis (Koenig et al., 1989). In **MJ**, the initial symptoms appeared at the age of 2 years but, the clinical progression of the disease was mild and he was ambulatory at age 10 years. The progression of the disease in this patient is being monitored. Asano et al. (1990) have also reported BMD cases having out-of-frame deletions among Japanese muscular dystrophy patients. Chelly et al. (1990) reported BMD cases that showed truncated proteins despite having out-of-frame deletions due to the use of new splice sites that excluded the out-of-frame deletion sequences.

There appears to be no correlation between deletion size and disease severity. At the extremes of the spectrum are a DMD patient with a 52-bp deletion within exon 19 (Matsuo et al., 1990) and a mildly affected BMD patient in whom a large

deletion removes 46% of the dystrophin gene (England et al., 1990). Deletion of a single exon 45 has caused DMD in **SD** while, a large deletion of exons 13-44 resulted in a milder BMD phenotype in **RL**. Similar results have been reported by Monaco et al. (1988), Malhotra et al. (1988), Gillard et al. (1989), Koenig et al. (1989), Lindlöf et al. (1989), Cooke et al. (1990), Beggs et al. (1991) and Nicholson et al. (1993a, 1993b).

Earlier studies have reported no detectable deletion or duplication in about 40% of patients with DMD/BMD. It is presumed that these patients have point mutations of the dystrophin gene affecting either transcription, mRNA processing, translation or protein stability. Point mutations in DMD/BMD patients have been reported (Bulman et al., 1991a; Roberts et al., 1992b, Prior et al., 1993a, 1993b; Lenk et al., 1993b; Knepper et al., 1995; Lasa et al., 1995; Tuffery et al., 1995 and Yu et al., 1995). There were 3 DMD and 4 BMD families in whom no exonic deletion were observed. The disease in these patients may be due to the presence of point mutations. Patients with LGMD and SMA did not reveal any deletion in the dystrophin gene. Dystrophin protein studies need to be done before ruling out the possibility of Xp21 myopathy in them.

CARRIER DIAGNOSIS

The deletion of specific exons in the dystrophin gene can be easily detected in DMD/BMD patients by PCR. The presence of only one copy of the gene results in a complete loss of the corresponding PCR fragments. Since the females have two copies of the gene, it becomes difficult to detect deletions in the carrier females by mPCR. The deletions of some exons on one X chromosome are masked by the PCR fragments produced from the normal X chromosome.

The mPCR products were analyzed during the exponential phase of the PCR process, a stage at which the genomic proportions of the different exons are expected to be maintained. It has been possible to detect the deletion of specific exons in probable carriers by a reduction in the relative intensities of the corresponding PCR fragments to about 50% of the level in normal individuals. Of the 19 families, with 23 DMD/BMD patients, deletions were detected in 12 families. QM-PCR was carried out in 4 families.

In three families (2 sporadic and 1 familial), the mother was found to be a carrier for the deleted exon in her son. In one family, the mother was a non-carrier. Ioannou et al. (1992) performed QM-PCR on 14 DMD/BMD families where deletions were identified by standard PCR. They were able to assign carrier status in 9 of 14 (64%) mothers with familial disease, and in 6 of 22 (27%) of possible carriers, while 36% of families where deletions were detected in DMD patients the mothers were found to be non-carriers.

QM-PCR is not useful for carrier determination in DMD/BMD families where no deletions are detectable in any of the 27 exons examined. In such families, carrier determination has to be based on haplotype analysis alone. Since exon deletions are not detectable in about 30% of the families, and germline mosaicism or new mutations are encountered in about 30% of DMD/BMD families, misdiagnosis may occur in 9% of these families (Ioannou et al., 1992). Sufficient attention has to be paid to this aspect for genetic counseling of such families.

CONCLUSIONS

Duchenne and Becker muscular dystrophies (DMD/BMD) are progressive muscle disorders caused by mutations in the dystrophin gene. Dystrophin gene analysis revealed deletion of one or more exons in 63% of the DMD/BMD patients; in the remaining 37% no deletions were observed.

The deletions were non-randomly distributed in the major (92.3%) and minor (7.7%) "hot spot" regions of the dystrophin gene, and majority of these had their proximal breakpoint in intron 44, which agrees well with the earlier reports. Thus, the frequency and distribution of these deletions in the Indian population were not unique but characteristic of general population.

The DMD and BMD patients who had shown no deletions at the genomic DNA level are presumed to have point mutations in the dystrophin gene affecting either transcription, mRNA processing, translation or protein stability.

Examination of mutations in the dystrophin gene is very important for the diagnosis and prognosis of patients with atypical presentation of muscle syndromes.

In four DMD/BMD patients, genetic analysis has shown variations between the result of PCR and Southern hybridization. Such cases need further hybridizations on different restriction enzyme digests and PCR amplification with a different pair of primers specific for the same region.

The proportion of deletions in familial (3/5) versus sporadic cases (9/14) was about the same.

The deletion pattern was observed to be homogeneous in BMD and heterogeneous in DMD, which agrees well with earlier studies.

There was no relationship observed between MR and the occurrence, extent or position of the gene deletions in DMD/BMD patients although, the frequency of deletions was greater in families with mental subnormality compared with normal intelligence.

The present data has been useful in establishing the diagnosis for individual patients. The clinical and molecular correlation based on the alteration of the reading frame was valid in 92% of the cases.

No intrafamilial variability in the severity and clinical progression of the disease was observed in families with more than one affected sib.

Although, QM-PCR is very successful in identifying the carrier status of the female members of the deleted DMD/BMD families, their identification for the non-deleted could be made possible by linkage studies, RFLP markers, polymorphic dinucleotide sequences or other sequence polymorphism within or flanking the dystrophin gene. The identification of possible carriers in these families is important for genetic counseling.

Analysis of the DNA fragments generated by HindIII has identified a novel polymorphism with probe 11-14 in muscular dystrophy patients, their family

members and unrelated controls. To come to a concrete conclusion a large population of healthy controls need to be screened.

To understand the origin and relevance of novel HindIII fragments observed with 7 cDNA probes in 35/37 muscular dystrophy patients and their healthy relatives besides their absence in unrelated controls, a detailed investigation based on cloning and sequencing of these fragments is necessary.

SUMMARY

Duchenne and Becker muscular dystrophies (DMD/BMD) are allelic, recessive, neuromuscular disorders caused by the mutations in the dystrophin gene at Xp21. This gene is the largest known gene (2.4 Mb) with 79 exons. The dystrophin gene codes for a 14 kb mRNA which is translated into a 427 kDa protein. The dystrophin protein is absent in DMD and a partially functional/truncated protein is present in BMD. Although, the dystrophin protein is reported to be normal in patients with LGMD, QM and SMA, abnormalities of the dystrophin have been observed in some of them (Samaha and Quinlan, 1996). Majority of the mutations in the dystrophin gene are intragenic deletions, but duplications and point mutations have also been identified.

To study the frequency and distribution of deletions and to evaluate the concordance between the clinical phenotype and the reading frame hypothesis propounded by Monaco et al. (1988), the molecular analysis of the dystrophin gene was undertaken in 37 patients with muscular dystrophy. Of these patients, 8 were diagnosed as suffering from DMD, 14 BMD, 12 LGMD (8 males and 4 females), 2 SMA and one with QM. Genomic DNA was isolated from a total of 78 peripheral blood samples of these patients and their family members.

The dystrophin gene was analyzed by mPCR for 27 exons and Southern hybridization with 8 cDNA probes. Frame-shift deletions were identified in 5/8 DMD and in-frame in 7/14 BMD and 1 QM patients. Due to the presence of an

in-frame deletion, the clinically diagnosed patient with quadriceps myopathy (**VK**) is considered to have Becker muscular dystrophy. Patients with LGMD/SMA did not reveal any deletion as was expected, since these disorders are not caused by mutations in the dystrophin gene.

The overall deletion frequency (63%) in Indian DMD/BMD patients is in conformity with the earlier reports on American-European population (55-65%). However, it is higher than that reported for some Asian populations (32-52%). The deletions were distributed non-randomly in the major (92.3%) and minor (7.7%) "hot spot" regions, and majority of them had their proximal breakpoint in intron 44, as also reported in earlier studies. Thus, the frequency and distribution of these deletions in the Indian population were not unique but characteristic of general population.

Deletion pattern in BMD patients was homogeneous, with in-frame deletions of exons 45-48 being the most common. On the other hand, the deletions in the DMD patients were heterogeneous with respect to their size and location. This agrees well with earlier studies.

In four patients (DMD/BMD), variations were found between the deletion pattern observed by PCR and Southern analysis. In **AP**, **SO** and **MJ**, exon 52, deleted by Southern hybridization, was amplified by PCR. In **RL**, a large deletion of exons 13-44 was observed by Southern analysis but intermittent exons 13, 25 and 42 were amplified by PCR. These patients need further hybridizations on different restriction enzyme digests and PCR amplification with a different pair of primers specific for the same region. Similar variations between the results of PCR and Southern blot analysis have been observed by other workers.

The proportion of deletions observed in familial cases (3/5) was about the same as in sporadic cases (9/14).

Mental subnormality was observed in 38% of DMD patients which is within the range 18-63% observed in earlier reports. There was no obvious difference between the size and distribution of deletions in patients with MR and those with normal intelligence. However, the frequency of deletions was greater in families with MR than in the families with normal intelligence.

The present data has been useful in establishing the diagnosis for individual patients. The clinical and molecular correlation based on the alteration of the reading frame was valid in 92% of the cases. The deletions that caused a shift in the reading frame resulted in DMD whereas those that maintained it caused BMD, except in one case (**MJ**), where BMD was due to an apparent frame-shift deletion. He is now 10 years old and needs follow up.

Intrafamilial variability in the severity of the disease in terms of its clinical progression was not observed in families with more than one affected sib.

In three DMD patients no exonic deletions were observed. One of these (**RS**) became wheelchair bound at the age of 7 years, and had an elder brother suffering from DMD who became non-ambulatory at 6 years. Another patient (**RH**) had symptoms typical of DMD, and when last seen at 9 years, he experienced great difficulty in walking. The third DMD patient (**NT**) died soon after presentation to the clinic at the age of 12 years.

Similarly, no deletions were observed in four BMD patients (**JD, RB, PN, MO**) and three symptomatic sibs of **MO**. Two patients had positive family history (**PN** and **MO**). The progression of the muscle weakness was mild in both and they were ambulatory at 12 and 16 years respectively. Two elder brothers of **MO** became

wheelchair bound at the age of 32 and 34 years and the third showed the first symptoms at 14 years. The progression of the disease was mild in both **RB** and **JD** and both are still ambulatory.

The disease in the DMD/BMD patients with no deletions may be due to the presence of point mutations which may affect the mRNA splicing, translation or stability of dystrophin protein.

To ascertain the carrier status of the mothers in the DMD/BMD families with identifiable deletions, QM-PCR was done for 4 families. Carrier mothers were identified in 3 families (2 DMD and 1 BMD) out of which one was an obligate carrier. One of the 4 mothers did not show the expected deletion in the DNA from her peripheral blood.

A novel HindIII polymorphism was observed by probe 11-14 in 8 DMD, 15 BMD, 11 LGMD, 2 SMA patients, and their family members. In addition, this polymorphism was also seen in three unrelated healthy controls who had no family history of any muscle disease. There was a gain of an ~2.8 kb fragment and loss of two fragments of 1.8 and 1.9 kb.

Novel HindIII fragments with 7 cDNA probes (1-2a, 2b-3, 4-5a, 5b-7, 8, 9 and 10) were observed in 35/37 muscular dystrophy patients. The extra fragments were identified in both patients with deletions (12/13) and without deletions (23/24). In addition, the extra fragments were identified in the family members of the patients; mother (13/14), father (9/9), sisters (6/6), brothers (8/8), sons (2/2), daughter (1/1) and uncle. These fragments were not observed in 20 unrelated control DNA samples.

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APPENDIX

STOCK SOLUTIONS

All the chemicals used were of AR grade. Sterile double distilled water was used to make the final volume of the respective solution. Sterilization was done by autoclaving at 15 lbs/in for 15 minutes if not specified otherwise.

1. *1 M Tris.Cl pH 8.0/7.5*

   121.1 g Trizma (Sigma, USA) was dissolved in 800 ml water. The pH was adjusted to 8.0/7.5 with concentrated HCl and the volume was made up to 1000 ml. The solution was sterilized and stored at 4°C.

2. *2 M Tris.Cl pH 7.5/8.0*

   121.1 g Tris 7-9 buffer (Sigma, USA) was dissolved in 400 ml water. The pH was adjusted to 7.5/8.0 with concentrated HCl. The volume was made up to 500 ml. The solution was sterilized and stored at room temperature.

3. *0.5 M EDTA (pH 8.0)*

   186.1g disodium ethylenetetracetate (Sigma, USA) was dissolved in 800 ml water. The solution was stirred vigorously on a magnetic stirrer. Sodium hydroxide pellets were added simultaneously till the pH was 8.0. The volume was made up to 1000 ml. The solution was sterilized and stored at 4°C.

4. *3 M Sodium acetate pH 5.2*

   40.81 g Sodium acetate was dissolved in 80 ml water. The pH was adjusted to 5.2 with glacial acetic acid and the volume made up to 100 ml. The solution was sterilized and stored at room temperature.

5. *5 M NaCl*

   292.2 g Sodium chloride was dissolved in 1000 ml water. The solution was sterilized and stored at room temperature.

6. *10% SDS*

   10 g Sodium dodecyl sulfate (Sigma, USA) was dissolved in 100 ml water. The solution was stored at room temperature.

7. *1 M $MgCl_2$*

   20.33 g Magnesium chloride was dissolved in 100 ml water. The solution was sterilized and stored at 4°C.

8. *1 M Sucrose*

   54.76 g Sucrose was dissolved in 160 ml water. The solution was sterilized and stored at 4°C.

9. *0.1 M Tris.Cl (pH 8.0) + β-Mercaptoethanol*

β-Mercaptoethanol (E Merck)	40.00 µl
2 M Tris.Cl (pH 8.0)	2.00 ml
Water	17.96 ml

   The solution was stored at 4°C.

10. *O.5 M Glucose*

    9 g Glucose was dissolved in 100 ml water. The solution was filter sterilized and stored at 4°C.

11. *10 N NaOH*

    200 g Sodium hydroxide was dissolved in 500 ml water. The solution was stored at room temperature.

12. *5 M Potassium acetate*

    49 g Potassium acetate was dissolved in 100 ml water. The solution was sterilized and stored at 4°C.

13. *50% Dextran sulfate*

    10 g Dextran sulfate (Sigma, USA) was dissolved in 18 ml water. The solution was kept at 42°C overnight to accelerate dissolution and stored at -20°C.

14. *100% TCA*

    500 g Trichloro acetate was dissolved in 227.0 ml water. The solution was stored at 4°C.

15. *50X TAE Buffer*

Tris 7-9 buffer (Sigma, USA)	242.00 g
Glacial acetic acid	57.10 ml
0.5M EDTA (pH 8.0)	100.00 ml

    The volume was made up to 1000 ml. The solution was sterilized and stored at room temperature.

16. *10X TBE Buffer*

Tris 7-9 Buffer (Sigma, USA)	108.00 g
Boric acid	55.00 g
0.5M EDTA (pH 8.0)	40.00 ml

The volume was made up to 1000 ml. The solution was sterilized and stored at room temperature.

17. *20X SSC*

Sodium chloride	175.30 g
Trisodium citrate	88.20 g
Water	800.00 ml

The pH was adjusted to 7.0 and the volume was made up to 1000 ml. The solution was sterilized and stored at room temperature.

18. *Developer (Kodak)*

16.0 g of powder from smaller bag was dissolved in 500 ml water by stirring constantly. 97.5 g of the powder from larger bag was added slowly till the contents were slowly dissolved. The volume was made up to 1000 ml. The developer was stored in a dark bottle at room temperature.

19. *Fixer (Kodak)*

253 g of the powder was dissolved in 1000 ml water and stored in dark bottle at room temperature.

SOLUTIONS FOR GENOMIC DNA ISOLATION

1. *Lysis Buffer*

1 M $MgCl_2$	2.50 ml
1 M Tris.Cl (pH 8.0)	5.00 ml
Triton X-100	5.00 ml
1 M Sucrose	160.00 ml

The volume was made up to 500 ml. The solution was filter sterilized and stored at 4°C.

2. *Nucleus Buffer*

5 M NaCl	7.50 ml
0.5 M EDTA (pH 8.0)	24.00 ml

The volume was made up to 500 ml and the solution was stored at 4°C.

3. *TE Buffer (pH 8.0)*

1 M Tris.Cl (pH 8.0)	1.00 ml
0.5 M EDTA (pH 8.0)	0.20 ml

The volume was made up to 100 ml and the solution was stored at 4°C.

4. *Proteinase K*

20 mg Proteinase K Type XVIII (Sigma, USA) was dissolved in 1 ml water. The solution was stored at -20°C and stored in small aliquotes at -20°C.

5. *Equilibrated Phenol*

Distilled phenol was equilibrated with equal volume of 1 M Tris.Cl (pH 8.0) overnight. The aqueous layer was removed and 0.1 volume of 0.1 M Tris.Cl (pH 8.0) containing 0.2%β-Mercaptoethanol was added. It was stored in a dark bottle at 4°C.

6. *Chloroform:Isoamyl alcohol (24:1) (Freshly prepared)*

Chloroform	96.00 ml
Isoamyl alcohol	4.00 ml

SOLUTIONS FOR PLASMID ISOLATION

1. *Luria Broth (LB)*

Bactopeptone (Difco, USA)	10.00 g
Yeast extract (Hi Media, India)	5.00 g
Sodium chloride	10.00 g
Water	950.00 ml

The pH was adjusted to 7.0 with 5 N NaOH. The volume was made up to 1000 ml. The solution was sterilized and stored at 4°C.

2. *LB Plates*

15 g bacto agar (Difco, USA) was added to 1000 ml of LB just before autoclaving. The solution was swirled gently to distribute the agar evenly throughout. The medium was cooled to 50°C before adding 1 ml of ampicillin (100 mg/ml), and poured into petri plates.

3. *STE Buffer*

5 M NaCl	10.00 ml
1 M Tris.Cl (pH 8.0)	5.00 ml
0.5 M EDTA (pH 8.0)	1.00 ml

The volume was made up to 500 ml. The solution was stored at 4°C.

4. *Solution I*

0.5 M Glucose	50.00 ml
1 M Tris.Cl (pH 8.0)	12.50 ml
0.5 M EDTA (pH 8.0)	10.00 ml

The volume was made up to 500 ml. The solution was stored at 4°C.

5. *Solution II (Freshly prepared)*

10 N NaOH	0.40 ml
10% SDS	2.00 ml

The volume was made up to 20 ml.

6. *Solution III*

5 M Potassium acetate	60.00 ml
Glacial acetic acid	11.50 ml

The volume was made up to 100 ml. It was stored at 4°C.

7. *Lysozyme (10 mg/ml) (Freshly prepared)*

Lysozyme (Sigma, USA)	20.00 mg
1 M Tris.Cl (pH 8.0)	0.02 ml

The volume was made up to 2 ml.

8. *1000X Ampicillin*

100 mg ampicillin was dissolved in 1 ml water. It was stored at -20°C in small aliquotes.

SOLUTIONS FOR POLYMERASE CHAIN REACTION

1. *10 mM dNTP stock*

100 mM dATP (Promega, USA)	10.00 µl
100 mM dCTP (Promega, USA)	10.00 µl
100 mM dGTP (Promega, USA)	10.00 µl
100 mM dTTP (Promega, USA)	10.00 µl
Milli Q water	60.00 µl

Stored at -20°C.

2. *Taq DNA Polymerase 10X Buffer (Promega, USA)*

Supplied with the Taq DNA polymerase.

3. *25 mM $MgCl_2$ (Promega, USA)*

Supplied with the Taq DNA polymerase.

4. *Multiplex primer Set I (IMMCO ,USA)* (Table I A)

Supplied as a set of 9 primer pairs at a concentration of 0.5 nano moles each in 400 µl. Stored at -20°C.

5. *Multiplex primer Set II (IMMCO, USA)* (TableI B)

Supplied as a set of 9 primer pairs at a concentration of 0.5 nano moles each in 400 µl. Stored at -20°C.

6. *Multiplex primer Set III (IMMCO, USA)* (TableI C)

20 µl of each primer (5 nmoles) was added to 40 µl of milli Q water to prepare 400 µl of set III. Stored at -20°C.

SOLUTIONS FOR SOUTHERN HYBRIDIZATION

1. *Depurination solution*

5 ml concentrated HCl in 250 ml water.

2. *Denaturation solution*

5 M NaCl	150.00 ml
10 N NaOH	25.00 ml

The volume was made up to 500 ml.

3. *Neutralization solution*

2 M Tris.Cl (pH 7.5)	250.00 ml
5 M NaCl	150.00 ml

The volume was made up to 500 ml.

4. *Prehybridization solution*

0.29 g NaCl was dissolved in 1 ml of water and the following were added:

50% Dextran sulfate	1.00 ml
10% SDS	0.50 ml
Formamide (Sigma, USA)	2.50 ml

The solution was kept at 42^{o}C for 15-30 minutes.

5. *Hybridization solution*

To the prehybridization solution, denatured probe containing 50 µl of 10 mg/ml Herring sperm DNA (Promega, USA) and 5 µl of labeled λ-HindIII digested marker was added.

6. *Wash solution I (Freshly prepared)*

Tetrasodium pyrophosphate	0.18 g
100% TCA	1.00 ml

The volume was made up to 20 ml.

7. *Wash solution II*

20X SSC	5.00 ml
10% SDS	1.00 ml

The volume was made up to 100 ml.

8. *Wash solution III*

20X SSC	2.50 ml
10% SDS	5.00 ml

The volume was made up to 500 ml.

9. *Deprobing solution*

2 M Tris. Cl (pH 7.5)	2.00 ml
0.5 M EDTA (pH 8.0)	0.80 ml
10% SDS	40.00 ml

The volume was made up to 500 ml.

10. *Sephadex G-50*

Approximately 1 g sephadex G-50 (Sigma, USA) was soaked in 100 ml water for 2 hours. It was washed with sterile water twice. 100 ml of TE buffer (pH 7.5) was added and left overnight. It was sterilized and stored at room temperature.

TABLE I : Sequence of polymerase chain reaction (PCR) primers for 27 exons of dystrophin used in the present study

(A) : SET I by CHAMBERLAIN et al. (1990)

PRI[a]	SIZE (bp)	PRIMER LOCATION		SEQUENCE
4	196	I/I	F	TTG TCG GTC TCC TGC TGG TCA GTG
			R	CAA AGC CCT CAC TCA AAC ATG AAG C
8	360	I/I	F	GTC CTT TAC ACA ATT TAC ATG TTG AG
			R	GGC CTC ATT CTC ATG TTC TAA TTA G
12	331	I/I	F	GAT AGT GGG CTT TAV TTA CAT CCT TC
			R	GAA AGC AGG CAA CAT AAG ATA CAC CT
17	416	I/I	F	GAC TTT CGA TGT TGA GAT TAC TTT CCC
			R	AAG CTT GAG ATG CTC TCA CCT TTT CC
19	459	I/I	F	TTC TAC CAC ATC CCA TTT TCT TCC A
			R	GAT GGC AAA AGT GTT GAG AAA AAG TC
44	268	I/I	F	CTT GAT CCA TAT GCT TTT ACC TGC A
			R	TCC ATC ACC CTT CAG AAC CTG ATC T
45	547	I/I	F	AAA CAT GGA ACA TCC TTG TGG GGA C
			R	CAT TCC TAT TAG ATC TGT CGC CCT AC
48	506	I/I	F	TTG AAT ACA TTG GTT AAA TCC CAA CAT G
			R	CCT GAA TAA AGT CTT CCT TAC CAC AC
51	388	I/I	F	GAA ATT GGC TCT TTA GCT TGT GTT TC
			R	GGA GAG TAA AGT GAT TGG TGG AAA ATC

a = Primers are named for the exon they amplify using numbering of Koenig et al.(1989)

I = Primer sequences inside or overlapping the neighboring intron and splice signals, E = Primers that bind entirely within the exon

F = Forward primers (5'-3'), R = Reverse primers (5'-3'), relative to the coding sequence

(B) : SET II by Beggs et al. (1990)

PRI[a]	SIZE (bp)	PRIMER LOCATION		SEQUENCE
Pm	535	I/E	F	GAA GAT CTA GAC AGT GGA TAC ATA ACA AAT GCA TG
			R	TTC TCC GAA GGT AAT TGC CTC CCA GAT CTG AGT CC
3	410	I/I	F	TCA TCC ATC ATC TTC GGC AGA TTA A
			R	CAG GCG GTA GAG TAT GCC AAA TGA AAA TCA
6	202	I/I	F	CCA CAT GTA GGT CAA AAA TGT AAT GAA
			R	GTC TCA GTA ATC TAC TTA CCT ATG ACT ATG G
13	238	I/I	F	AAT AGG AGT ACC TGA GAT GTA GCA GAA AT
			R	CTG ACC TTA AGT TGT TCT TCC AAA GCA G
43	357	I/I	F	GAA CAT GTC AAA GTC ACT GGA CTT CAT GC
			R	ATA TAT GTG TTA CCT ACC CTT GTC GGT CC
47	181	I/I	F	CGT TGT TGC ATT TGT CTG TTT CAG TTA C
			R	GTC TAA CCT TTA TCC ACT GGA GAT TTG
50	271	I/I	F	CAC CAA ATG GAT TAA GAT GTT CAT GAA T
			R	TCT CTC TCA CCC AGT CAT CAC TTC ATA G
52	113	E/E	F	AAT GCA GGA TTT GGA ACA GAG GCG TCC
			R	TTC GAT CCG TAA TGA TTG TTC TAG CCT C
60	139	E/E	F	AAG AGA AAT TGC GCC TCT GAA AGA GAA CG
			R	CTG CAG AAG CTT CCA TCT GGT GTT CAG G

a = Primers are named for the exon they amplify using numbering of Koenig et al.(1989)

I = Primer sequences inside or overlapping the neighboring intron and splice signals, E = Primers that bind entirely within the exon

F = Forward primers (5'-3'), R = Reverse primers (5'-3'), relative to the coding sequence

(C): SET III designed in our laboratory (based on the published sequences)

PRI[a]	SIZE (bp)	PRIMER LOCATION		SEQUENCE
49	439	I/I	F	GTG CCC TTA TGT ACC AGG CAG AAA TTG
			R	GCA ATG ACT CGT TAA TAG CCT TAA GAT C
16	290	I/I	F	TCT ATG CAA ATG AGC AAA TAC ACG C
			R	GGT ATC ACT AAC CTG TGC TGT ACT C
41	270	I/I	F	GTT AGC TAA CTG CCC TGG GCC CTG TAT TG
			R	TAG AGT AGT AGT TGC AAA CAC ATA CGT GG
32	253	I/I	F	GAC CAG TTA TTG TTT GAA AGG CAA A
			R	TTG CCA CCA GAA ATA CAT ACC ACA CAA TG
42	195	E/E	F	CAC ACT GTC CGT GAA GAA ACG ATG ATG G
			R	CTT CAG AGA CTC CTC TTG CTT AAA GAG AT
2	174	E/I	F	AGA T GA AAG AGA AGA TGT TCA AAA G
			R	AAT GAC ACT ATG AGA GAA ATA AAA CGG
79	137	E/E	F	GAA AGA TTG TAA ACT AAA GTG TGC
			R	GGA TGC AAA ACA ATG CGC TGC CTC
25	113	E/E	F	CAA TTC AGC CCA GTC TAA AC
			R	CTG AGT GTT AAG TTC TTT GAG
66	68	E/E	F	CAG GGA GGA TCC GTG TCC TG
			R	GTC TTC CAA ATG TGC TTT AC

a = Primers are named for the exon they amplify using numbering of Koenig et al.(1989)

I = Primer sequences inside or overlapping the neighboring intron and splice signals, E = Primers that bind entirely within the exon

F = Forward primers (5'-3'), R = Reverse primers (5'-3'), relative to the coding sequence

TABLE II: Human dystrophin cDNA clones

*cDNA CLONE	VECTOR	EXCISION BY	TOTAL SIZE (kb)	INSERT SIZE (kb)
57666	pBS	EcoRI & HindIII	4.80	1.538
57668	pBS	EcoRI	4.30	1.150
57670	pBS	EcoRI	5.20	1.850
57672	pBS KS	EcoRI	5.50	2.500
57674	pBS KS	EcoRI	3.80	0.900
57676	pBS KS	EcoRI	9.10	6.100

* = INDEXED BY ATCC® (American Type Cell Culture) NUMBER

ABBREVIATIONS

bp	Base pair
BMD	Becker muscular dystrophy
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CK	Creatine kinase
cM	Centi morgan
cpm	Counts per minute
C-terminal	Carboxy-terminal
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleoside triphosphate
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDTA	Ethylene diamine tetra-acetic acid
EMG	Electromyogram
FIGE	Field inversion gel electrophoresis
g	Gram
HCl	Hydrochloric acid
IU/L	International units per liter
kb	Kilobase
kDa	Kilodalton
LB	Luria broth
LGMD	Limb girdle muscular dystrophy
M	Molar
Mb	Mega base
ml	Milli liter
mg	Milli gram
mM	Milli molar
$MgCl_2$	Magnesium chloride
mPCR	Multiplex polymerase chain reaction

MR	Mental subnormality
mRNA	Messenger ribonucleic acid
N	Normal
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ng	Nano gram
ORF	Open reading frame
PCR	Polymerase chain reaction
pERT	Phenol enhanced reassociation technique
PFGE	Pulsed field gel electrophoresis
Pm	Muscle specific promoter
QM	Quadriceps myopathy
QM-PCR	Quantitative multiplex polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SMA	Spinal muscular atrophy
SSCP	Single strand conformation polymorphism
TAE	Tris:Acetic acid:EDTA solution
TBE	Tris:Boric acid:EDTA solution
TCA	Trichloro-acetic acid
TE	Tris:EDTA solution
Tris	Tris (hydroxymethylaminomethane)
UV	Ultra violet
U	Units
V	Volts
α	Alpha
β	Beta
μg	Microgram
μl	Microliter
^{o}C	Degree celsius
λ	Lambda
~	Approximately