DUCHENNE MUSCULAR DYSTROPHY IN SOUTH AFRICA: MOLECULAR ASPECTS

by

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September, 1992

GUILLAUME BENJAMIN AMAND DUCHENNE

1806 - 1875

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, except in a very slight degree, and on account of the conditions in which it occurs. It is a disease of early life and of early growth. Manifesting itself commonly at the transition from infancy to childhood, 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 R. Gowers

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ABSTRACT

September, 1992

Duchenne muscular dystrophy in South Africa: Molecular aspects.

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Duchenne muscular dystrophy (DMD) is a lethal X-linked neuromuscular disorder, characterised by progressive muscle wasting and weakness. DMD has its onset in early childhood, leading to physical handicap by the mid teens and usually death by the age of twenty years. Becker muscular dystrophy (BMD) is the allelic form of DMD and is differentiated by its age of onset and milder phenotype. DMD and BMD are incurable and the most effective way of managing affected families is by preventing the recurrence of the disorder. The use of intragenic and closely linked flanking markers, facilitates the identification of the defective X chromosome in female carriers and their affected male foetuses.

DMD is thought to be the most common of the heritable muscle disorders, having an incidence of 1 in 3 300. When extrapolated to the large South African population, it presents a significant socioeconomic problem. For this reason it was decided to develop a molecular genetic service for carrier identification and diagnostic predictions.

The first step in the South African study involved the collection of biological material from affected individuals. In so doing, minimum prevalences in the four major ethnic groups of Black, Caucasian, Indian and Mixed ancestry, were established. Although ascertainment was incomplete for a number of reasons, a markedly increased DMD frequency in the Indian population and a low frequency in the Black population was apparent. In the Caucasian group, an unexpectedly high BMD frequency, compared to DMD, was observed.

110 males affected with DMD and 18 with BMD were screened for deletions using genomic and cDNA probes and multiplex polymerase chain reaction (PCR) technology. Deletions were detected in the dystrophin gene of 47 DMD and 6 BMD patients, occurring predominantly in the 3' region of the gene (65%) and to a lesser extent in the 5' region of the gene (28%). The deletion frequency within individual ethnic groups,

revealed a markedly reduced frequency in Black patients (less than 30%) compared to deletions in 50% of each of the other groups.

The use of polymorphic DNA markers were investigated for carrier detection and for identifying the disease-associated alleles in patients in which a deletion was not detected. Allele frequencies of restriction fragment length polymorphisms (RFLPs) and tandem repeat sequences were established for the four South African ethnic groups.

Carrier status determination was undertaken in 98 families with one or more affected male relative(s). DNA analysis reduced the carrier risks of 100 females and increased the risks of 44 others. The DNA investigations were inconclusive for 109 females. Four male foetuses with DMD were diagnosed by deletion screening and RFLP analysis; one male foetus was shown to be unaffected.

This study illustrates the sensitivity and reliability of molecular genetic analysis in DMD and BMD. The findings presented in this thesis have an immediate and wide application in the effective management of DMD and BMD patients and their families.

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FIGURES AND ILLUSTRATIONS

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ABBREVIATIONS AND SYMBOLS

A	adenine
autorad	autoradiograph
bp	base pair
BMD	Becker muscular dystrophy
C	cytosine
$_{o}C$	degree Celsius
CaCl	calcium chloride
cDNA	complimentary deoxyribonucleic acid
cmo	centimorgan
CPK	serum creatine phosphokinase
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
e.g.	for example
et al.	and others
G	guanine
hr(s)	hour(s)
i.e.	that is
kb	kilobase(s)
kV	kilovolts
M	molar
Medunsa	Medical University of Southern Africa
ug	microgram(s)
min	minute(s)
ml	millilitre(s)
ul	microlitre(s)
mM	millimolar
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
PIC	polymorphic information content
RFLP(s)	restriction fragment length polymorphism(s)
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
T	Thymine
UCT	University of Cape Town
uv	ultra-violet

CHAPTER 1

INTRODUCTORY BACKGROUND

1.1 PLAN OF THE THESIS

This thesis is divided into six chapters. The first chapter deals with a general overview of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), outlining the historical, clinical, genetic and molecular genetic background. In the latter the progression of developments in molecular technology which led to the current understanding of the gene involved in this disorder, is briefly outlined.

Chapters 2-5 deal with the developments related to genetic management of DMD families and each section is preceded by an introduction of the specific issue. In the second chapter DMD and BMD are discussed in the South African context. The focus is the frequency of the disorders in the major ethnic groups.

In Chapters 3-5 data relating to the experimental work undertaken in this project, is presented. Since the detection of deletion mutations in the dystrophin gene is of major importance in the diagnosis of DMD and BMD, this issue is discussed first, followed by the alternative method of haplotype analysis. A combination of the two

approaches then follows in Chapter 5 in which family studies are used to illustrate the advantages and limitations of the available molecular technology. The sixth and final chapter is devoted to an assessment of the effectiveness of the available molecular technology, on the basis of the results of this project.

1.2 CLINICAL BACKGROUND OF DMD AND BMD

Duchenne muscular dystrophy (DMD) which follows an X-linked recessive mode of inheritance affecting only males, was first described in detail in 1868, by a physician, Guillame Benjamin Amand Duchenne. The condition is the most common of the heritable muscle disorders [Moser, 1984], and it is characterised by progressive wasting and weakness of skeletal muscle. The clinical onset is usually between ages 3 and 6 years, but histopathological investigations have revealed that muscle abnormalities are present in the foetus as early as the second trimester of pregnancy [Emery, 1987]. Affected boys are usually late walkers and tend to fall frequently and tire easily with mild physical exercise. A notable feature of the disorder is pseudohypertrophy of the calf muscles which results from these muscles being replaced, initially by fibrous tissue, and later by fat.

As the disease progresses, affected individuals become weaker, and by the end of the first decade, they are usually confined to a wheelchair. With increasing muscle weakness the upper limbs and the intercostal muscles become affected. A brace is eventually required to support the body and prevent thoracic deformities. Death due to respiratory and, in some cases, cardiac problems usually occurs before the age of twenty years [Appel and Roses, 1983]. A photograph of males in the progressive stages of DMD appears in Fig. I-1.

In comparison to DMD, Becker muscular dystrophy (BMD) follows a similar but milder clinical course, with phenotypic manifestations ranging from mild, bordering on normality, to severe physical handicap [Hart et al., 1987]. Affected males generally become symptomatic at the age of about 12 years and, if severely affected, might become incapacitated from the age of 14 years. In many instances the affected individuals reach adulthood before developing weakness of their lower limbs and the majority are capable of procreating [Beggs et al., 1991]. The clinical differentiation between DMD and BMD is conventionally based on the different ages of onset and wheelchair confinement [Malhotra et al., 1988; Beggs et al., 1991].

Clinical evidence of a muscular dystrophy can be supported by electromyography or biochemically by measuring the serum creatine phosphokinase (CPK) level which is markedly elevated in affected persons due to the enzyme leaching out from the dystrophic muscle [Emery, 1987]. Elevation of the CPK level is non-specific, however, since it is influenced by moderate exercise and many disease processes. For this reason firm diagnostic confirmation is conventionally achieved by histological examination of a muscle biopsy specimen.

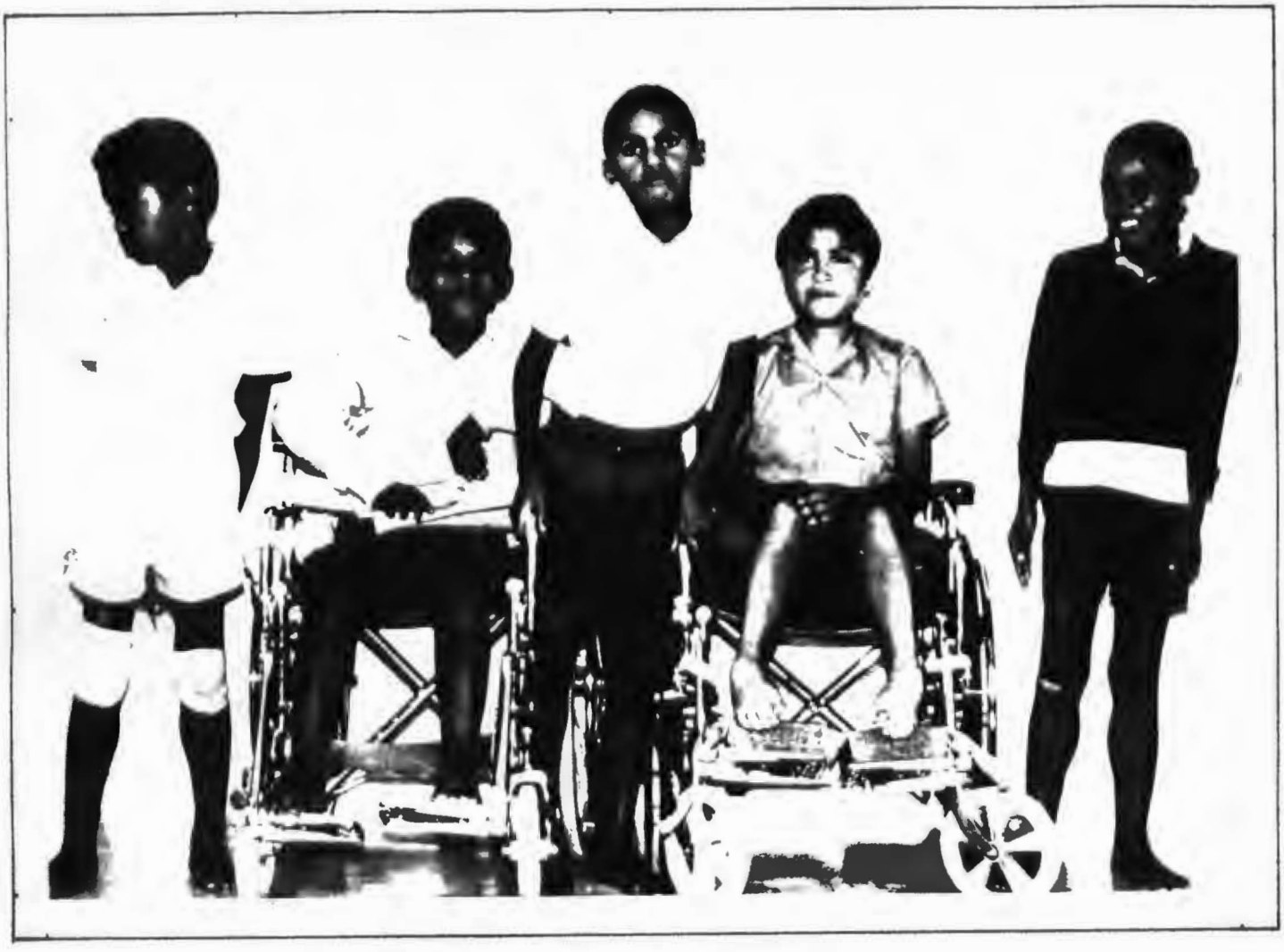

Figure I.1

Affected males in the progressive stages of Duchenne muscular dystrophy.

1.3 FREQUENCY OF DMD AND BMD

The frequency of DMD and BMD can either be expressed as incidence (i.e. the number of affected males born in the population in a specific period of time), or prevalence (i.e. the number of affected individuals present in the population, ascertained over a given period, or at a particular point in time) [Emery, 1987]. However, since the disorder is not clinically diagnosable in a newborn male, an affected neonate might go unnoticed unless his mother is known to carry the defective dystrophin gene. This factor may contribute towards the underestimation of frequency in a population.

Over a period of forty years (1939-1979) in countries worldwide, the incidence of DMD has been estimated to be about 1 in 3 300 male births [Emery, 1987]. BMD is estimated to have an incidence of 1 in 30 000 newborn males [Emery, 1987]. Frequency estimates in South Africa, which are a complex issue, are discussed in Chapter 2.

1.4 GENETICS OF DMD AND BMD

Mode of Inheritance

DMD follows an X-linked recessive mode of inheritance. This means that a female carrying the defective gene does not manifest the disorder because a normal gene on the homologous X chromosome masks the effect of the abnormality. Each of the carrier's offspring has a 50% chance of inheriting the DMD mutation from her. A male who inherits the defective gene will have the disorder since he has no normal gene to counteract the effects of the mutated one. A female offspring, however, will be a non-manifesting carrier like her mother. BMD, which is allelic to DMD, follows the same pattern of inheritance.

Mutations in the dystrophin gene which result in DMD, are regarded as being fully penetrant because all affected males have similar ages of onset and wheelchair confinement [Hoffman et al., 1988; Abbs et al., 1990; Love et al., 1990]. BMD is more heterogeneous in its clinical phenotype with considerable inter- and intra-familial variability being reported in members of the same family [Medori et al., 1989].

The mutation rate of the dystrophin gene has been estimated to be higher than that in any other gene resulting in an X-linked disorder; evidence suggests that about 30% of all DMD patients have the disorder as a result of a new mutation [Haldane, 1935]. However, several investigators have suggested that the proportion of DMD patients with a new mutation, might

be lower than the predicted 30% which is usually quoted [Roses et al., 1977; Bucher et al., 1980; Roncuzzi et al., 1986].

Manifesting females

Individuals affected with DMD and BMD are usually males since these disorders are X-linked recessive traits. However, manifesting female carriers are well documented. Possible explanations for females having a DMD-like phenotype are: translocations which disrupt the DMD gene resulting in DMD [Boyd and Buckle, 1986]; random inactivation (lyonisation) of a large proportion of the normal X-chromosomes in muscle tissue [Norman and Harper, 1989]; the absence of an X-chromosome, as in Turner syndrome [Emery, 1987]; the autosomal recessive form of muscular dystrophy, which mimics the DMD phenotype [Francke et al., 1989].

1.5 MOLECULAR GENETICS OF DMD AND BMD

Localisation of the dystrophin gene

The putative gene for DMD was mapped to the short arm of the X chromosome following cytogenetic evaluation of manifesting females with X-autosome translocations. In every instance the translocation breakpoint on the X chromosome occurred within the Xp21 region [Lindebaum et al., 1979; Verellin-Dumoulin 1984; Zatz et al., 1981]. On re-examination of the translocated Xp21 region, using high resolution banding techniques, Boyd and Buckle [1986] showed that 6 out of 9 translocations had a breakpoint in the Xp21.2 band and two others had breakpoints close to that region. This clustering of breakpoints within one area of the chromosome, and the apparent association of the translocation with the disorder in the affected females, indicated that the gene was nearby and had been disrupted by this rearrangement.

Confirmation regarding the localisation of DMD to Xp21, was provided by linkage studies using DNA probes encompassing this region. Davies et al. [1983] showed linkage of the DMD gene to two single copy probes, L-RC8 and L1.28, from the Xp21 region. RFLP analyses showed that the 2 markers were situated at a distance of about 15cmo on either side of the DMD locus with a 30% recombination rate between them.

In an attempt to increase the number of utilizable X-chromosome probes, panels of somatic cell-hybrids were used

to isolate and map new clones relative to the existing probes. The two closest extragenic markers, 754 and C7, were mapped within 3cmo and 10cmo of the gene, respectively. The marker 754, which mapped between L1.28 and DMD (Xp21-p11.3), detected high frequency RFLPs in the populations studied [Brown et al., 1985; Hofker et al., 1985]. The clone, C7, mapped to the 3' end of the DMD gene.

Isolation of intragenic probes

Subsequent research led to the isolation of probes from the 5' region of the gene which identified submicroscopic deletions in 7-10% of affected males [Kunkel et al., 1985; Ray et al., 1985]. These deletions did not occur in normal males and were presumed to represent the primary genetic defect in DMD and BMD [Hart et al., 1986; Kunkel et al., 1986; Thomas et al., 1986]. Linkage analysis using the probes from the 5' end of the gene, revealed a 5% recombination rate between the markers and the inheritance of DMD [Thompson et al., 1986], implying that the gene involved was large.

Additional information about the gene and its intragenic mutations was gleaned from techniques such as field inversion gel electrophoresis (FIGE) which resolves large DNA fragments from 50kb-2 000kb. Results from FIGE indicated that the gene was greater than 2 000kb in length and prone to large deletions in a region distal to the probes already isolated. Subsequent cloning distal to the 5' probes, led to the

isolation of a probe which detected deletions in about 16% of males affected with DMD [Burmeister and Lehrach, 1986; van Ommen et al., 1986; den Dunnen et al., 1987; Kenwrick et al., 1987].

The identification of the mRNA transcript

A major advance in the understanding of the molecular genetics of DMD, following the precise localisation of the gene, was the isolation and identification of its 16kb mRNA transcript in fetal muscle [Monaco et al., 1986]. The entire complementary DNA (cDNA) derived from the mRNA was subsequently cloned and used to screen the DNA of males affected with DMD [Koenig et al., 1987]. The cDNA subclones revealed deletions in more than 60% of affected boys and identified two regions of the gene which were predisposed to mutational events [Koenig et al., 1987; Darras et al., 1988]. Partial duplications of exonic fragments were detected in about 16% of males affected with either DMD or BMD [Hu et al., 1990].

The isolation of the protein product

A recent development in the progress of DMD genetics was the identification and characterisation of the gene's protein product, dystrophin. Sequence analysis of the complete cDNA of the DMD gene predicted the amino acid sequence and the structure of the protein product [Koenig et al., 1988;

Davison et al., 1988]. These predictions gave direction to immunological assays which localised the protein in the plasma membrane of muscle cells [Hoffman et al., 1987]. Subsequent characterisation of dystrophin in muscle biopsy specimens from healthy males and individuals with DMD and BMD revealed a low abundance of the protein in DMD patients (less than 3% of normal) and normal quantities of an abnormally structured protein in BMD patients [Hoffman et al., 1988; Patel et al., 1988]. The correlation of the severity of the disorder with the abundance of dystrophin, supported the findings at the molecular level which correlated the severity of the disorder with the effect of a deletion on the translational reading frame of the mRNA trascript [Monaco et al., 1988].

An abnormality within the dystrophin molecule is evidence for an unequivocal diagnosis of either DMD or BMD. Analysis of the protein is therefore invaluable in view of the existence of an autosomal recessive (AR) form of DMD which mimics the classical X-linked form of DMD and has been misdiagnosed as such, in sporadic patients [Francke et al., 1989; Vainzof et al., 1991]. Since the AR form of DMD does not show dystrophin abnormalities, analysis of the protein in muscle biopsy specimens from sporadic DMD patients provides the correct differentiation between the two forms of DMD [Hoffman et al., 1988; Norman et al., 1989].

Prospects for genetic management

The application of the above developments in the management of DMD and BMD kindreds has solved problems which previously seemed insurmountable. The accurate diagnosis of the disorders in male foetuses in more than 60% of "at risk" pregnancies is possible with the molecular advances. For the remaining families, alternative DNA-based approaches for identifying female carriers and tracking the defective gene, are available.

CHAPTER 2

DUCHENNE MUSCULAR DYSTROPHY IN SOUTH AFRICA

2.1 MOTIVATION FOR THIS STUDY

The reported high incidence of DMD in previously studied populations [Emery, 1987; Lindlof et al., 1988; Sugino et al., 1990] poses a major socioeconomic problem when extrapolated to the large South African population. Since no curative treatment is available, the most effective way to assist affected kindreds is by identifying the female carriers of the defective DMD gene and providing genetic counselling to prevent the birth of affected children.

In the past, female carriers were identified by pedigree data and increased serum CPK levels [Mahoney et al., 1977; Griggs et al., 1983]. However, levels of this enzyme are raised only in about 70% of the mothers of affected males [Caskey et al., 1980]. As a result, the CPK tests have no value for women with a high prior risk for carrying the defective gene and who have normal enzyme levels. Furthermore, the available tests had no capacity for distinguishing between normal and affected male foetuses. For these reasons the only option for high risk pregnancies was termination based on foetal sexing.

In the last decade, molecular research, using recombinant DNA technology, has made revolutionary progress in defining the basic defects which underlie DMD and BMD [Kunkel et al., 1985;

Ray et al., 1985; Wapenaar et al., 1988]. The detection of specific mutations within more than 60% of affected males has radically altered the overall approach to genetic counselling and prediction. Furthermore, groups of alleles of intragenic and flanking markers, which segregate together (i.e. as a unit or haplotype) and which are associated with either the defective or the normal X-chromosome, can be established. The segregation of these haplotypes within families of affected males, facilitates the identification of female relatives who are carrying the defective DMD gene [Bakker et al., 1985; Kingston, 1985; Darras et al., 1987; Prior et al., 1989a; Ward et al., 1989].

The Department of Human Genetics, U.C.T. Medical School (hereafter referred to as the study centre), is actively engaged in molecular genetic research of DMD and BMD, and close contact has been established with affected families who can benefit from the molecular advances. This study was based upon the development of a molecular predictive testing facility for the diagnosis of DMD and BMD. In this regard, this thesis describes the molecular approaches and assessment of methods for screening affected individuals and their "at risk" relatives.

2.2 ASCERTAINMENT OF AFFECTED INDIVIDUALS

2.2.1 Collection of patient data and biological material

A DMD/BMD survey was initiated in the South African population in 1987 prior to molecular investigations for the implementation of a formal service. Clinicians and nursing staff from the study centre screened institutions and special schools for the physically disabled, for males with a DMD or BMD phenotype. In addition, on a national level, staff of the State genetic services, Department of National Health and Population Development, screened rural populations during clinics held in peripheral centres. Clinicians and nursing staff at universities and hospitals throughout South Africa, with an interest in muscle disorders, were informed about the proposed genetic service. In this regard, they were offered the opportunity to refer DMD and BMD patients and their families to the study centre.

BMD and DMD were differentiated on the basis of the age of onset and wheelchair confinement, as described by Emery [1987]. In all instances the clinical diagnosis was established by a specialist paediatrician or physician, and confirmed by appropriate ancilliary investigations including elevated CPK levels. In some instances, the diagnosis of the muscle dystrophy was confirmed by histological studies and electromyography.

Since ethnic variation has been reported for other genetic disorders in South Africa [Greenberg et al., 1991], it was necessary to examine ethnic differences for DMD and BMD. For this reason, in this study, patients were subdivided into the four major South African ethnic groups.

Group 1 (Black): These are persons from the indigenous Black population.

Group 2 (Indian): These persons had their ancestral origins on the Indian subcontinent.

Group 3 (Mixed Ancestry): These are persons of mixed ancestry (with admixtures of Javanese, Western European, West African, Madagascan, San and Khoi-Khoi).

Group 4 (Caucasian): This group incorporated persons of European descent, mainly British, Dutch, German and French.

Patients were also grouped as "familial" (more than one affected individual per family) and "sporadic" (referring to a sporadic phenotype i.e. a single, isolated occurrence of the disorder within the family).

Establishment of a DNA Bank for DMD and BMD in South Africa

With informed consent, 30 ml of peripheral blood was collected by venepuncture into EDTA tubes for DNA isolation. The blood

was separated into 10 ml aliquots and either processed immediately or frozen at -70°C. Samples sent from peripheral clinics were allowed a maximum transport time of 3 days at 4°C. DNA was extracted as described in Appendix A and stored at -70°C.

By June 1992, DNA had been banked from 130 DMD and 23 BMD patients in whom the clinical diagnosis was confirmed. Clinical details and pedigree data were obtained from the referring clinicians in most instances.

In addition, blood was collected from 401 unaffected family members comprising of (i) 107 mothers of affected children, (ii) 150 female relatives (i.e. sisters and maternal aunts of the patient) at risk of carrying the defective gene, and, (iii) 136 unaffected male relatives from whom DNA was required in order to determine carrier status in the females.

The numbers of affected DMD and BMD males and their relatives, from whom DNA is currently stored for molecular investigation, is listed in terms of ethnic origin in Table II-1.

Table II-1. Numbers of affected DMD males and their family members from whom DNA has been stored for molecular investigations.

Key for Table II-1

N= the number of families involved in each ethnic group.

ETHNIC GROUP	AFFECTED MALES DMD (N) BMD (N)	MOTHERS OF PATIENTS		POTENTIAL FEMALE CARRIERS		UNAFFECTED MALES	
		DMD	BMD	DMD	BMD	DMD	BMD
BLACK	40 (45) 2 (2)	24	1	18	0	15	1
INDIAN ORIGIN	25 (27) 3 (3)	17	2	26	1	28	1
MIXED ANCESTRY	33 (27) 4 (4)	23	1	44	0	25	0
CAUCASIAN	32 (41) 14 (9)	30	12	47	14	48	18
TOTAL	**130(140)** **23 (18)**	**94**	**16**	**135**	**15**	**116**	**20**

2.2.2 Estimation of frequency of DMD and BMD in South Africa

As stated in section 1.3, frequency of a disorder can be expressed as incidence or prevalence. Because DMD is not recognisable at birth it is not possible to ascertain the affected newborn population. This factor together with the rural distribution of a large proportion of the South African population contribute to incomplete ascertainment of frequency in this study. For this reason, in this thesis DMD and BMD frequencies are expressed as minimum prevalence.

Minimum prevalences of DMD and BMD in the different populations of South Africa was estimated on a basis of the number of living affected males ascertained during the period 1987-1992 and are summarised in Tables II-2 and II-3. Ninety-five per cent of the affected boys of Indian origin were referred from regions in Natal, whereas the majority of patients of Mixed Ancestry (97%) were ascertained at institutions in the Cape province. About 40% of the Black patients were seen in Cape Town and the remaining patients were referred by clinicians in the other provinces. The Caucasian referrals were from all parts of the country. The expected minimum prevalence for DMD based on figures ascertained in Cape Town (for Blacks, Mixed Ancestry and Caucasian) where ascertainment should have been the highest due to it being the focal centre of study, and Natal for the Indian population, is compared to the observed DMD prevalence in Table II-4. The expected prevalence for BMD is calculated as 1/10th of the

in Table II-4. The expected prevalence for BMD is calculated as 1/10th of the observed DMD prevalence. A map of South Africa showing the area covered by this study, is depicted in Fig. II-1.

Table II-2. Minimum prevalences calculated for DMD in South Africans of different ethnic origin.

Key to Table II-2

(F) = familial patients (S) = sporadic patients

ETHNIC GROUP	MALE POPULATION x 10^3	AFFECTED DMD (F)	(S)	MINIMUM PREVALENCE $\times 10^3$
BLACK	CP 965	18		
	TVL 3 218	12		
	N 5 571	9		
	OFS 951	4		
	10 700	43		1:248,8
		(20)	(23)	
INDIAN ORIGIN	CP 18	0		
	TVL 72	0		
	N 373	33		
	OFS 0	0		
	465	33		1: 14,1
		(19)	(14)	
MIXED ANCESTRY	CP 1 332	32		
	TVL 150	0		
	N 53	3		
	OFS 35	1		
	1 570	35		1: 44,8
		(15)	(20)	
CAUCASIAN	CP 692	8		
	TVL 1 408	13		
	N 320	6		
	OFS 190	5		
	2 610	32		1: 81,5
		(12)	(20)	
TOTAL	15 092	143		1:105,5
		(66)	(77)	

Table II-3

Minimum prevalences calculated for BMD in South Africans of different ethnic origins.

Key to Table II-3

Esimates include all living affected individuals during the period 1986-1990.

(F) = familial cases; (S) = sporadic cases

ETHNIC GROUP	MALE POPULATION X 10^3	AFFECTED BMD (F) (S)	MINIMUM PREVALENCE X 10^3
BLACK	10 700	2	1:5 350
INDIAN ORIGIN	465	2	1: 232,5
MIXED ANCESTRY	1 570	2	1: 785
CAUCASIAN	2 610	14	1: 186,4
TOTAL	15 092	20 (12) (8)	1: 754,6

Table II-4. A comparison of expected and observed minimum prevalences for DMD and BMD in the South African population.

ETHNIC GROUP	EXPECTED MINIMUM PREVALENCE DMD $x10^3$ BMD $x10^3$	EXPECTED NUMBER OF PATIENTS DMD BMD	OBSERVED NUMBER OF PATIENTS DMD BMD	PATIENTS NOT DIAGNOSED DMD BMD
BLACK	1: 53,6	200	43	157
	1:2 488	5	2	3
INDIAN ORIGIN	1: 11,3	41	33	8
	1: 141	3	2	1
MIXED ANCESTRY	1: 41,6	38	35	3
	1: 448	3	2	1
CAUCASIAN	1: 86,5	30	32	0
	1: 815	3	14	0
TOTAL	1: 48,8	309	143	166
	1:1 078,0	14	20	5

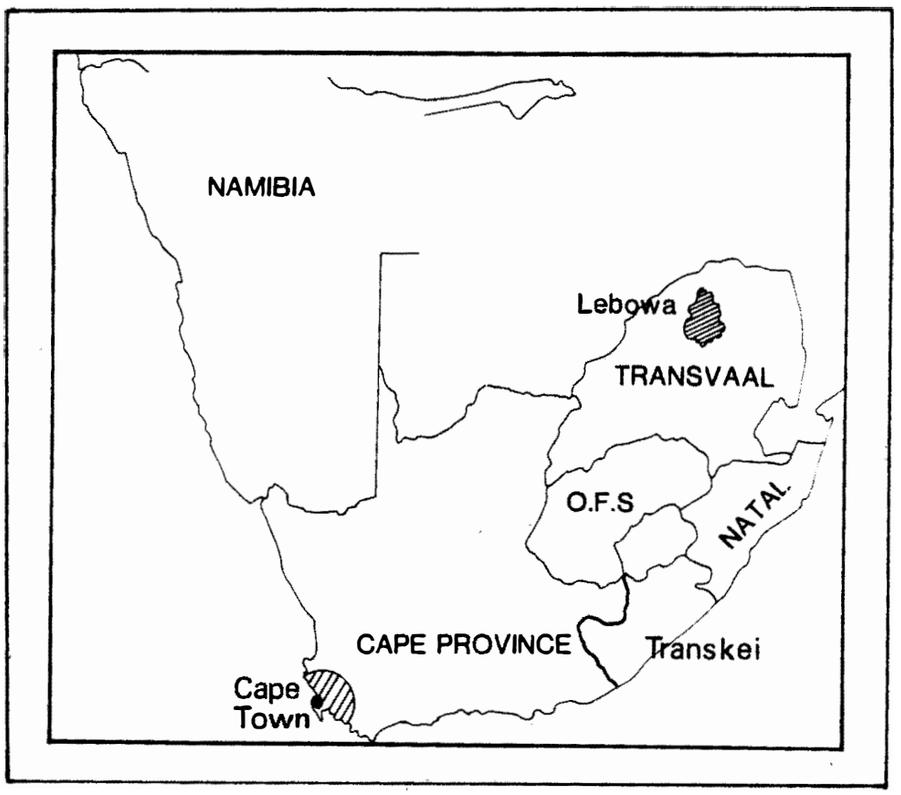

Figure II-1

A map of South Africa showing the areas from which biological material was obtained in this study.

2.2.3 Discussion of observed DMD and BMD frequencies in South Africa

The determination of DMD and BMD prevalences in South Africa is incomplete, for the following reasons; (i) since a significant proportion of the South African population is rural it is unlikely, for logistical reasons, that all of the male individuals affected with either DMD or BMD, have been identified; (ii) it is probable that population estimations based upon population census figures are an underrepresentation of the true situation in South Africa, and (iii) some families had no knowledge of their family histories. In light of these factors, complete ascertainment could not be achieved.

When estimating the disease frequency, cognisance must be taken of the fact that some sporadic DMD patients may be misdiagnosed instances of the phenotypically similar AR form of muscular dystrophy [Salih et al., 1983; Francke et al.,1989; Vainzof et al., 1991]. The inclusion of these misdiagnosed patients in DMD frequency estimates will result in falsely elevated figures. Precise frequency estimates can be made on the basis of biochemical evidence of dystrophin abnormalities in sporadic patients [Hoffman et al., 1988; Norman et al., 1989] (see section 1.5).

DMD Prevalence

The numbers obtained in this study are specific for the South African context and cannot be directly compared with reported prevalences from other parts of the world. Minimum prevalence estimates in the South African population reveal a higher DMD frequency in members of the Indian group ($1:14 \times 10^3$) when compared to the other ethnic populations ($1:44,8 \times 10^3$ - $1:248,8 \times 10^3$). A possible explanation for this situation, might be an increased occurrence of the AR form of DMD in the Indian population. If this were so, however, it would be expected that a significant proportion of affected females would also be ascertained in the population. The fact that no Indian females manifesting a DMD-like disorder have been encountered in the present study, argues against the increased frequency of the AR form of DMD in this population. Interestingly, similar findings of a high frequency of X-linked DMD in the Indian population of the West Midlands region of the United Kingdom, were recently reported [Roddie and Bundey, 1992]. It is therefore possible that there is a genuine increase in the occurrence of classical X-linked DMD in this ethnic group.

The frequency of DMD within South Africans of Mixed Ancestry is intermediate between that calculated for the Indian and Caucasian groups. This situation possibly reflects the admixture of genes from several ethnic groups.

Since the male Black population in South Africa is much larger than in any of the other ethnic groups ($1,07x10^7$ compared to $4,65x10^6$ for a combination of the others), the observed number of affected Black males were expected to be higher than in the other groups. In the present study, however, the Black population showed the lowest frequency of DMD.

In light of the sociopolitical situation in South Africa, which leads to the underestimation of Black births and the low assessment of clinical disorders in this group (especially in the rural populations) this low observed DMD frequency is not unexpected. Patient data was collected at hospitals which serve large Black townships in Cape Town (Red Cross Childrens' Hospital), Natal (King Edward VIIIth Hospital) and Transvaal (Medunsa). Clinicians at these hospitals have verified the real paucity of DMD patients within this population. Peculiarly, socioeconomic circumstances have not prevented Black mothers from having their children examined for other muscular disorders (Spinal muscular atrophy is 15 times more prevalent than DMD in the South African Black population [personal communication. A.Moosa]). For this reason it may be assumed that a Black mother would seek professional help for a child with a progressive physical handicap e.g. DMD, at some stage during the patient's life. The observed decreased frequency of DMD may therefore reflect a genuine low occurrence of the disorder in the indigenous Black population of South Africa.

BMD Prevalence

The overall prevalence observed for BMD in the local population is slightly higher (14%) than 10% of the DMD prevalence (as expected from previously reported incidences of DMD and BMD [Emery, 1987]). However, within the Black, Indian and Mixed Ancestry groups the BMD frequency is similar to the expected value. The frequency of BMD in the Caucasian population is much higher (46%) than the 10% of the DMD frequency expected from reported values. This observation could be partially explained by an increased awareness of the disorder or an increase in the severity of the phenotype in this ethnic group. These patients are therefore more likely to come to the attention of the clinicians interacting with this study centre.

CHAPTER 3

THE ANALYSIS OF DYSTROPHIN GENE DELETIONS IN SOUTH AFRICAN DMD AND BMD PATIENTS

3.1 INTRODUCTORY BACKGROUND

3.1.1 Deletion detection by Southern analysis

The detection of deletions in the dystrophin gene of males affected with DMD or BMD is the unequivocal method of diagnosis of the disorder. A number of genomic probes as well as the cDNA probes spanning the entire mRNA transcript, are available for deletion screening using the conventional methods of Southern blot hybridisations [Vandenplas et al., 1984]. A historical background relating to the isolation of the probes used in the present study and their application in the diagnosis of the disorders is outlined below.

Probes from the 5' region of the gene

In 1985, Ray et al. reported the cloning of the region of DNA spanning an (X;21) translocation breakpoint in a female with a BMD-like phenotype. Since the autosomal breakpoint in this case lay within a cluster of ribosomal-RNA genes, existing rRNA probes were used to clone the translocation breakpoint. The two translocation-derived chromosomes were separated in somatic cell hybrids and rRNA sequences were used to clone DNA fragments spanning the breakpoint. One of the clones generated from the X-chromosome region, XJ1.1, identified restriction

fragment length polymorphisms (RFLPs) in the normal population and showed close linkage to the disorder in affected families. Analysis of RFLPs in additional families revealed a 5% recombination rate between this clone and the DMD gene. In an early study, a deletion of XJ1.1 was detected in one of fifty affected males. The probe was later used in bidirectional chromosome "walk" techniques to expand this genomic region, resulting in the isolation of more than 60kb of the X-chromosome. The clones derived from these "walks" were designated XJ and assigned to the DXS206 locus.

While the XJ probes were being cloned, Kunkel's group, working independently in Boston, isolated the pERT87 clones [Kunkel et al., 1985]. They used a subtractive hybridisation technique to clone DNA fragments which were absent in a male affected simultaneously with three X-linked disorders viz. DMD, chronic granulomatous disease and retinitis pigmentosa. An excess of the patient's DNA was sheared and combined with partially digested DNA isolated from a multiple-X human lymphoid cell line. Following phenol-enhanced reassociation, the appropriately reassociated molecules were cloned and localised. Homologues mapping to the Xp21 region were designated pERT84 and pERT87 and assigned to the DXS142 locus and DXS164 loci, respectively.

The DXS164 region was expanded by chromosome walking to cover a stretch of DNA of 137kb. Three subclones, pERT87-1, pERT87-8 and pERT87-15, spanning over 50kb, were used to screen the DNA

from affected males. Deletions were detected in about 7% of these patients [Monaco et al., 1985; Kunkel et al., 1986]. All of the subclones revealed RFLPs which could be tracked in affected families. A combination of the subclones were informative for linkage analyses in about 89% of unrelated females [Monaco et al., 1985]. Although these findings suggested that the dystrophin gene had been found, it was apparent that there was a 5% recombination rate between the DXS164 locus and the remaining region of the gene.

Genomic probes in the 3' region of the gene

Screening DMD patients with DNA probes which had been isolated from the proximal (5') region of the DMD gene led to the detection of deletions in 7-10% of affected males. Subsequent genetic studies, using cDNA probes and the technique of FIGE located several DMD mutations distal to the 5' region [Monaco et al., 1987; Van Ommen et al., 1986]. Further research resulted in the isolation of probes from the distal region of the DMD gene.

The P20 probe was isolated using a hybrid cell-mediated cloning and selection procedure [Wapenaar et al., 1988]. This probe mapped to a position near the middle half of the DMD gene and consists of two DNA segments, normally 25-40kb apart. Further studies with cDNA probes mapped P20 more precisely to a large intron of 180kb within which a significant proportion of detectable deletions have a breakpoint. In spite of its

high propensity for deletions in DMD and BMD, this intron is highly conserved in the human species.

Blonden et al. [1989] set out to characterise the P20 intron using whole cosmids as probes in competitive DNA hybridisations. The cosmids derived from 90kb of the P20 region detected a number of breakpoints within a proximal sequence of the intron. More recently a collaborative effort, in which 242 breakpoints in this intron were mapped, showed that the breakpoints are widely scattered and that there is no correlation between the size of the deletion and the position of the breakpoint [Blonden et al., 1991].

cDNA probes

In the subsequent search for a candidate mRNA-derived cDNA, the genomic regions from which the closely linked markers were derived, were screened for sequences which were conserved across species. In this regard, Burghes et al. [1987] expanded the DXS206 locus in the direction of DXS164 to yield 350kb of contiguous DNA. cDNA libraries were constructed and subclones which were common to a number of different species, were used to isolate a 16kb mRNA transcript in muscle. The exons recognised by the cDNA were localised to the DXS164 and DXS206 loci, and to the centromeric region flanking this domain, spanning an area of more than 200kb.

At the same time, in a search for sections of the DXS164 locus which might be transcribed, Monaco et al. [1986] expanded this region to yield a 220kb stretch of DNA. Fifty different subclones were used as hybridisation probes against HindIII digests of DNA from animals of different species. One of two subclones which were conserved across species, recognised a 16kb mRNA fragment from human fetal tissue. A 1kb cDNA clone, isolated from a cDNA library, recognised potential exons over a region of 110-130kb spanning the DXS164 locus and representing about 10% of the 16kb transcript. Koenig et al. [1987] predicted the direction of transcription of the DMD transcript from the 1kb cDNA fragment and generated a 14kb cDNA molecule. The complete cDNA detected 65 different HindIII fragments on Southern blots, each containing one or more exons [Koenig et al., 1987].

The HindIII hybridisation pattern of cDNA probes, consists of a number of non-polymorphic fragments, each representing an entire exon or part of one [Koenig et al., 1987]. The absence of one or more hybridising bands is indicative of a deletion of one or more exons [Koenig et al., 1987]. The contiguous genomic arrangement of the exons in the gene has been determined by examining the deletion patterns observed in DMD and BMD patients [Koenig et al.,1987; Darras et al.,1988] .

3.1.2 Deletion detection by the polymerase chain reaction (PCR)

Deletion screening by Southern analysis can be tedious and slow, especially when a result is urgently required. Because each deletion-prone region in the dystrophin gene is spanned by at least two cDNA probes, a time period of up to four weeks is necessary for attempts at deletion detection. For this reason, the sophisticated polymerase chain reaction (PCR) technology, which is rapid and less costly than Southern analysis, has been incorporated into the molecular diagnosis of DMD and BMD patients. The attributes of this technology are outlined below.

Introduction

The PCR is a procedure which results in the amplification of a defined sequence within a DNA sample [Saiki et al., 1989]. The method utilises a heat-stable DNA polymerase (isolated from the thermophilic unicellular alga, *Thermus aquaticus*) and synthetic oligonucleotide primers which are homologous to the sequences flanking the target region.

The standard PCR protocol involves repetitive cycles of incubation at three different temperatures. In the first step, double-stranded template DNA is denatured at 90-95°C. In the second step the DNA is allowed to reanneal to the oligonucleotide primers, at 50°C to 60°C. The third step

involves the extension of the primers by incorporating free nucleotides to generate a strand complementary to the template DNA. In this way the template is effectively doubled and the new extension product can be used as a template for the subsequent cycles. Thus, at the end of 25 to 30 cycles, the sequence of interest is amplified more than a million fold and can be visualised by electrophoresis and ethidium-bromide staining on an agarose gel.

The PCR reaction, where applicable, is therefore a major improvement on Southern analysis. One of the major advantages of the PCR is that it obviates the need for radioisotopes. Further advantages of PCR are that it requires minimal quantities of biological material as template and that it has the potential to provide results within 1-2 days. These attributes of PCR are especially useful when the quantity of sample material is limited or when an urgent test is required.

Since each hotspot within the DMD gene contains a number of different exons which could be deleted in an affected male, deletion screening of these domains may require independent PCR amplifications of more than 30 single exons. To simplify this process, Chamberlain et al. [1988] and Koenig et al. [1989] combined 14-22 different oligonucleotides in a single reaction mixture, so achieving the simultaneous amplification of 7-11 different exons. In this way, multiprimer reactions have detected 80-98% of all deletions [Koenig et al., 1989; Beggs et al., 1990].

3.1.3 Deletion distribution within the dystrophin gene

DNA deletions have been detected in the dystrophin gene of 40-60% of males affected with DMD and BMD [Koenig et al., 1987; Darras et al., 1988; Read et al., 1988; Sugino et al., 1989]. Deletions within the DMD gene are extremely heterogeneous regarding size and location. Studies worldwide [Bartlett et al., 1988; Darras et al.,1988; Gilgenkrantz et al., 1989; Herrman et al.,1989; Lindlof et al.,1989; Sugino et al., 1989] have shown that these mutational events are non-randomly distributed in the large gene, occurring preferentially in two regions; near the 5' end (exons 3-19), about 500kb from the promoter, and near the middle region of the gene (exons 42-53), about 1 200kb from the promoter [Covone et al., 1991]. These observations suggest that there might be a mechanism which predisposes these deletion-prone regions (hotspots) to mutation. In contrast to the two hotspots, only two deletions have been reported to date in the distal 3' region spanned by probes 11-14 [Darras and Francke, 1988b;Passos-Bueno et al., 1990].

Within each hotspot, deletion breakpoints appear to cluster in a large intron (i.e. intron 7 in the 5' end, and intron P20 in the 3' end) each spanning about 150kb. The deletions reported thus far occur predominantly in the P20 domain, and to a lesser extent in the 5' region [Koenig et al., 1987; Darras et al.,1988; Gilgenkrantz et al., 1989; Asano et al.,1991]. Clusters of deletion endpoints which have been

identified within the 3' hotspot occur either 5' to the P20 intron, within the intron, or 3' to P20 [Gilgenkrantz et al.,1989].

3.1.4 Genotype/Phenotype Correlation

The size and location of deletions in the dystrophin gene apparently has no correlation with the severity and progression of the resultant disorder [Hart et al., 1987; Darras et al., 1988; Davies et al., 1988; Medori et al., 1989b]. In order to explain the clinical differences observed between DMD and BMD, Monaco et al. [1988] proposed the reading frame rule. These authors suggested that DMD is likely to be the result of a deletion which removes the frame of the protein coding message, resulting in a nonsense code from the mutation point. The resultant protein product would be truncated and degraded within the cell. This concept has been supported by research which has revealed the low abundance or complete lack of the dystrophin molecule in muscle cells of males affected with DMD [Hoffman and Kunkel, 1989].

It has been shown that in BMD the deletion causing the disorder removes part of the coding message but leaves the reading frame intact [Monaco et al. 1988]. The partially deleted message codes for a protein which is smaller than the normal protein but retains partial activity. In muscle biopsy specimens from BMD patients the dystrophin protein appears to be present in normal quantities but has an abnormal molecular weight [Hoffman et al., 1988].

The description of exon/intron boundaries encompassing the first 60 exons [Koenig et al., 1988; Malhotra et al., 1988; Baumbach et al., 1989; Koenig et al., 1989], facilitated the correlation of phenotype with deletion size and location in affected males. Molecular and protein studies subsequently showed that the majority of deletions conform with the reading frame rule. A number of deletions which do not have the predicted effect on the translational reading frame have, however, been reported [Baumbach et al., 1989; Love et al., 1990; Malhotra et al., 1988]. Explanations proposed for in-frame deletions which result in a severe phenotype [Gillard et al., 1989], include :

i) the generation of a novel splice site in the flanking intronic sequences, through altered splicing;
ii) the disruption of the reading frame by the inclusion of intronic sequences during splicing;
iii) the deletion of an exon, crucial for protein synthesis.

DMD and Mental retardation

The expression of the DMD gene in the brain suggests that there might be a causal relationship between a mutation in the dystrophin gene and mental retardation which is present in 18-63% of males affected with DMD [Emery, 1987; Hoffman et al., 1988; Rapaport et al., 1991].

The need to understand the association between MR and DMD has resulted in considerable research at the molecular level. In this regard, Rapaport et al. [1991] conducted a study in Brazilian patients to determine whether the pattern of DMD deletions correlated with mental retardation. Their results showed that a significant proportion of patients with a deletion in the region of exon 52, had some form of developmental delay. In view of their findings, it was deemed necessary to comment on the mental capacity of the South African patients with a similar deletion.

3.2 MATERIALS AND METHODS FOR DELETION SCREENING IN SOUTH AFRICAN DMD AND BMD PATIENTS

3.2.1 Strategy for deletion screening

In the present study, before the availability of the probe, P20, from the 3' mutation hotspot, and the cDNA subclones which encompassed the entire coding region of the dystrophin gene, DNA from affected persons was screened by Southern blot hybridisation analysis with the genomic XJ and pERT87 probes from the 5' region of the gene. Later, when the P20 probe was obtained, the DNA samples in which the probes from the 5' hotspot had not detected deletions, were probed with P20.

With the availability of the cDNA probes, the extent of the deletions detected with the genomic probes could be more accurately determined. Patient samples were reprobed with the cDNA subclones which span the two hotspots viz. cDMD8, cDMD7 from the 3' hotspot, which detect most deletions, and cDMD1-2a and cDMD2b-3, which span the lesser deleted 5' hotspot. In instances where no deletions were detected with these probes, the subclones cDMD9, cDMD4-5a, cDMD5b-6, cDMD10 and cDMD11-12a, were used to screen the regions of the gene which are known to have a lower deletion frequency. Based on the results of other investigators, the probe cDMD12b-14 was not used in this study since it corresponds to the 3' untranslated region of the gene and is least likely to detect deletions [Liechti-Gallati et al., 1989; Lindlof et al., 1989].

In keeping with the chronological advances of available technology, PCR was more recently applied in DMD/BMD screening. All of the detected cDNA deletions which involved an exon for which the primers were available, were confirmed by multiplex PCR. DNA from patients who did not have a deletion detectable with cDNA probes was amplified with the oligonucleotide primers specific for exon 45. This approach was necessary because the HindIII fragment containing exon 45 is small and difficult to analyse on Southern blots.

3.2.2 Probes and oligonucleotide primers

Genomic probes

In order to screen the 5' region of the dystrophin gene in affected DMD/BMD males for deletions, the following intragenic genomic probes were used: pERT84-10, XJ1.1 and XJ2.3 from the DXS206 locus and pERT 87-8, pERT 87-1 and pERT 87-15 from the DXS164 locus. The P20 probe (DXS269) was used to screen the 3' mutation hotspot. The references for the probes and the relevant restriction enzymes used for these investigations are listed in Table III-1.

cDNA probes

The cDNA subclones, cDMD 1-2a, 2b-3, 4-5a, 5b-7, 8 and 9-14 used for deletion screening, were obtained from The National Institutes of Health (NIH), USA. The probes were prepared as

described in Appendix A. The subclones cDMD 5b-7 and 9-14 were further subdivided using double digests with the restriction enzymes, HincII/BamHI and HincII/EcoRI into probes cDMD 5b-6 and 7 and 9, 10, 11-12a respectively as described by Darras et al. [1988]. These cloned sub-fragments yielded fewer bands in the hybridisation patterns on autoradiographs, thereby making them easier to resolve. For deletion screening with the cDNA probes, DNA from affected persons was digested with the restriction enzyme, HindIII (Table III-2).

Primers for PCR

The PCR primers were synthesized in the Department of Biochemistry, U.C.T. The primers used in this study and the exons amplified are listed in Table III-3.

Origin of PCR primers

Chamberlain et al. [1988] used cDNA subclones to screen human genomic libraries and isolated the exons contained in the 4,1kb and 0,5kb HindIII fragments identified by cDMD7, and the exon contained in the 1,2kb and 3,8kb fragments recognised by cDMD8. The exon/intron sequences for exons 19 and 8 were derived by Monaco et al. [1986] and Heilig et al. [1987], respectively. The sequence for exon 60 was derived from cDNA analysis by Koenig [1988]. All of the other primers, including those amplifying the entire promoter region, were designed by Beggs et al. [1990].

Table III-1. Genomic probes and restriction enzymes used for deletion screening in the South African study.

PROBE	ENZYME FOR INSERT	ENZYME FOR RFLP	FRAGMENT SIZE (KB)	REFERENCE
pERT 84-10	HindIII	TaqI	4,5/ 2,8	Kunkel et al., 1985
XJ 1.1	XbaI	TaqI	3,1/ 3.8	Ray et al., 1985
XJ 2.3	BamHI/ EcoRI	TaqI	6,6/ 5,8	Thompson et al., 1986
pERT 87-1	KpnI/SalI	MspI	4,05/1,8	Kunkel et al., 1985
pERT 87-8	XbaI	TaqI	1,1/ 2,7/ 3,8	Kunkel et al., 1985
pERT 87-15	HindIII	TaqI	3,3/ 3,1	Kunkel et al., 1985
P 20	PstI	PstI	2,5/ 8,0	Wapenaar et al., 1988

Table III-2. cDNA probes and enzymes used for deletion screening in the South African study.

CDNA CLONE	ENZYME FOR INSERT	ENZYME FOR DETECTION	REFERENCE
cDMD 1-2a	EcoRI/ HindIII	HindIII	Liechti - Gallati et al., 1989
cDMD 2b-3	EcoRI	HindIII	Liechti - Gallati et al., 1989
cDMD 4-5a	EcoRI	HindIII	Liechti - Gallati et al., 1989
cDMD 5b-6 cDMD 7	EcoRI/ HincIII	HindIII	Liechti - Gallati et al., 1989
cDMD 8	EcoRI/ HincIII	HindIII	Liechti - Gallati et al., 1989
cDMD 9 cDMD 10 cDMD 11-12a	EcoRI/ BamHI	HindIII	Liechti - Gallati et al., 1989

Table III-3. A summary of multiplex primer sets used for deletion screening in this project.
F= forward primer; R= reverse primer

Exon/ HindIII Fragment	5' Primer Sequence 3'	PCR Product
44 / 0.5kb	F-AAACATGGAACATCCTTGTGGGGAC R-CATTCCTATTAGATCTGTCGCCCTAC	547 bp
45 / 4.1kb	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCTTCAGAACCTGATCT	268 bp
42	F-CACACTGTCCGTGAAGAAACGATGATG R-TTAGCACAGAGGTCAGGAGCATTGAG	155 bp
48	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506 bp
51	F-GAAATTGGCTCTTTAGCTTGTGTTTC R-GGAGAGTAAAGTGATTGGTGGAAAATC	388 bp
60	F-AGGAGAAATTGCGCCTCTGAAAGAGAACG R-CTGCAGAAGCTTCCATCTGGTGTTCAGG	139 bp
Promoter	F-GAAGATCTAGACAGTGGATACATAACAAATGCATG R-TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC	535 bp
4	F-TTGTCGGTCTCCTGCTGGTCAGTG R-CAAAGCCCTCACTCAAACATGAAGC	196 bp
8	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360 bp
19	F-TTCTACCACATCCCATTTTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp

3.2.3 Methodology used for deletion screening

Southern blot analysis

Southern analysis with genomic and cDNA probes were standard methods, as described in Appendix A.

DNA amplification by the PCR

Amplifications for deletion screening were performed in 50ul volumes containing 250ng genomic DNA; 0,5uM of each primer; 1 X buffer (Appendix A); 200uM (dCTP, dGTP, dATP, dTTP); 5units Taq DNA polymerase. Each reaction mixture was overlaid with a drop of sterile liquid paraffin to prevent evaporation.

Cycling conditions were as follows:
1 cycle of denaturation at 94°C/3mins; 30 cycles comprising denaturation at 94°C/48secs, annealing at 60°C/48secs, and extension at 72°C/2mins; and a final cycle of extension at 72°C/15mins. For deletion screening, 15ul of the amplification products were electrophoresed on 3% agarose gels and visualised by ethidium bromide staining.

3.3 RESULTS OF SCREENING FOR DYSTROPHIN GENE DELETIONS IN SOUTH AFRICAN DMD AND BMD PATIENTS

3.3.1 Technical observations: potential pitfalls

Southern Analysis

Since the technical quality of Southern blots might hinder the ability to detect deletions of some of the smaller exons of the dystrophin gene, conditions have to be optimised in each laboratory. It was therefore deemed necessary to present data of Southern blot hybridisations used in this study.

(i) Genomic Probes

The hybridisation patterns of the genomic probes on Southern blots were generally easy to interpret. In most cases, the RFLP system involved either two or three hybridising bands representing the different alleles. In the case of a male, only one of the alleles would hybridise. A deletion removed the allelic band completely, leaving an empty lane. Deletions were therefore easily identified in the patients' hybridisation patterns (Fig.III-1). However, partial digests or low amounts of DNA sometimes yielded faint signals which could be misinterpreted as a deletion. To provide evidence for complete digestion of the DNA, photographs were taken of the agarose gels prior to Southern blotting and filed for comparison with the autoradiograph results.

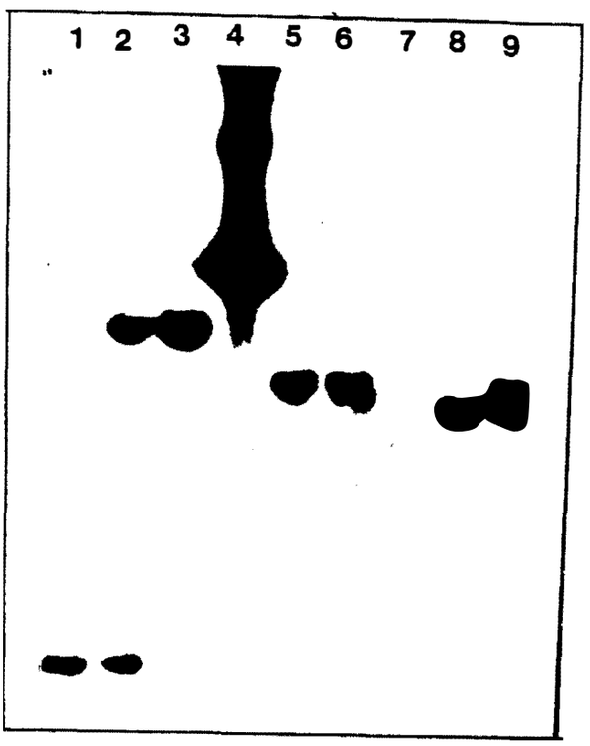

Figure III-1.

An autoradiograph showing the clarity of Southern blots for deletion detection using genomic probes.

Key to Figure III-1

DNA specimens in lanes 1 and 3 (pERT87-1/MspI) and lanes 5, 6 and 8 (pERT87-15/TaqI) illustrate homozygosity (in females) or hemizygosity (in males). Heterozygosity of the normal alleles for the two polymorphisms are shown in lanes 2 and 9. The sample in lane 7 is deleted at the pERT87-15 locus. Lane 4 is a HindIII digest of phage lambda DNA used as a size marker.

Deletions were not easily detected in carrier females because of the presence of the corresponding undeleted allele. Theoretically, in these individuals the deletions should result in hybridising bands of reduced intensities. However, because DNA concentrations and blotting procedures vary from process to process, conditions could not be optimised to give reproducible results. Differences in intensities in the banding pattern were therefore not relied upon for the identification of deletion carriers. This difficulty with dosage analysis has also been observed by other investigators [Kimber et al., 1989; Read et al., 1988].

In some instances, the polymorphic bands of the probes are closely spaced and require longer electrophoretic runs for resolution. Examples of these are the alleles of the 754/PstI, C7/EcoRV and P20/EcoRV polymorphisms. Gels for analysis of these RFLPs were routinely electrophoresed for 24 hours or longer (i.e. until the 2,0kb fragment of a phage lambda DNA /HindIII digest had reached the end of the gel).

(ii) cDNA probes

The HindIII fragments in the cDNA banding patterns were easily resolved on 0,6% agarose gels. Problems were usually experienced with the following probes: cDMD1-2a, which identifies closely spaced fragments of 7,5kb, 8,0kb and 8,5kb; cDMD2b-3 which hybridises to large fragments of 10kb and 12kb; cDMD4-5a which recognises fragments of 11kb, 12kb and

20kb. For optimal resolution these probes required gels to be run for periods of up to 24 hours or more. In order to retain the smaller fragments during these long runs, the gel length was increased from 18cm to 24cm. The banding patterns of some of the cDNA probes which were difficult to analyse because of rare HindIII-RFLPs, or faint bands, are shown in Figs. III-2, III-3 and III-4. The contiguous arrangement of the HindIII fragments within the gene are tabulated within each figure.

The autoradiograph in Fig. III-2 shows the normal banding pattern of nine fragments (lane 1) expected with cDMD1-2a. However, a number of extra bands have been observed on autoradiographs when using this probe. The first of these is a band of about 8,2kb which is allelic with the 7,5kb band. As a result, when the 8,2kb fragment is present in a male, the 7,5kb band is absent. If the missing 7,5kb fragment is an isolated occurrence on an autoradiograph, it could be misinterpreted as a deletion of exons 8 and 9 (lane 4). It was not possible to separate the bands of 8,5kb, 8,3kb and 8,0kb which migrated together as a very dark fragment in that region (lanes 2, 3 and 4). The 7,5/8,2 kb RFLP follows a Mendelian pattern of inheritance [Prior et al., 1989].

The second unusual band seen with cDMD1-2a, is 4,8kb in size, and occurs in association with the 7,5kb band in some families (Fig.III-2: lanes 2 and 3). This aberrant band is not associated with the DMD disorder as it is detected in unaffected males. Because it is rarely detected, the mode of

inheritance of the 4,8kb fragment has not been determined in the South African population.

The third unusual band seen with cDMD1-2a, is about 12kb in size, and co-segregates with the 8,2kb fragment (Fig. III-2: lanes 6 and 7), and has only been seen in one family, by the author. This kindred was too small to determine the inheritance pattern of the polymorphism.

The HindIII hybridisation pattern of cDMD5b-6 is shown in Fig.III-3. This autoradiograph shows the faintness of the smaller (less than 2kb) and larger (greater than 10kb) fragments which, in some instances, makes resolution of these fragments difficult.

In the hybridisation patterns of some patients, a single fragment was absent, due to the presence of an exonic deletion, or a rare HindIII-RFLP. In order to resolve this problem, a BglII digest of the sample was probed with the cDNA subclone. A deletion would alter the banding pattern of the BglII digest whereas an RFLP would have no effect [Darras et al., 1988]. An autoradiograph of HindIII- digested DNA, probed with cDMD9, is shown in Fig. III-4 (a). The sample in lane 1, demonstrated a deletion of the 8,3kb fragment. However, when a BglII digest of the DNA sample was probed with cDMD9 (Fig.III-4 (b)), no alteration in the migration of the hybridising bands was observed. This indicated that the missing 8,3kb HindIII fragment was due to an RFLP.

cDMD1-2a

EXON	1	2	3	4	5	6	7	8+9	10
BAND (kb)	3,2	3,25	4,2	8,5	3,1	8,0	4,6	7,5	10,5

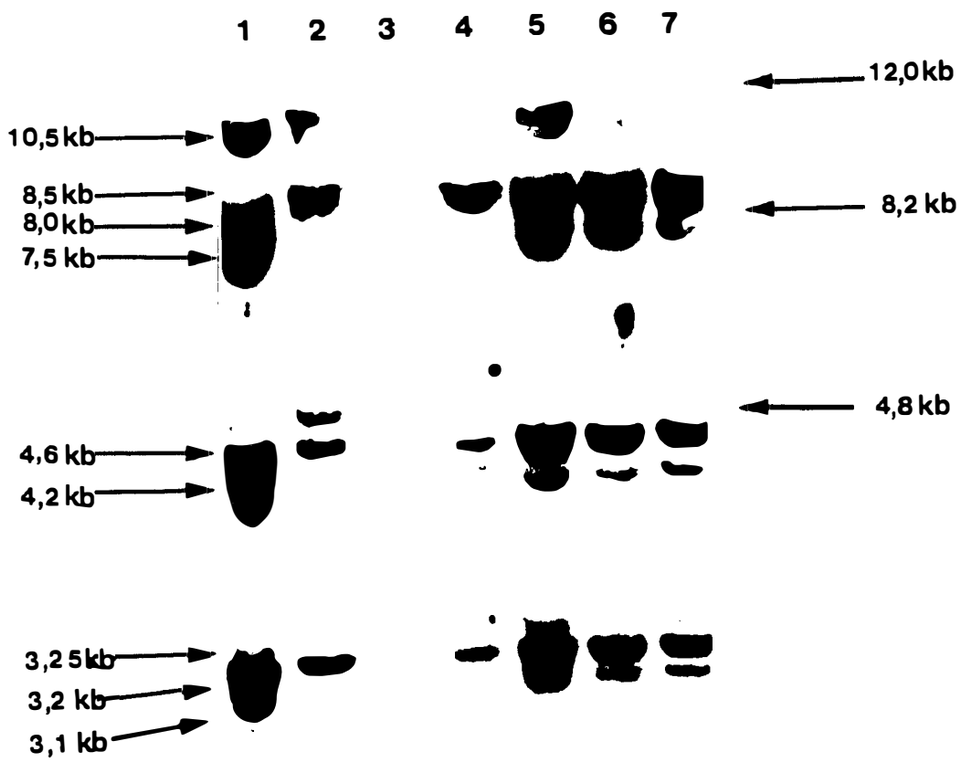

Figure III-2.

An autoradiograph showing the HindIII hybridisation pattern of cDMD1-2a. **Key to Fig.III-2**

The expected HindIII banding pattern of cDMD1-2a is shown in lane 1. The rare RFLPs are shown in lanes 2 and 3 (4,8/7,5kb), lane 3 (7,5/8,2Kb), and lanes 6 and 7 (8,2/12,0kb).

Subclone 5b - 6

EXON	30-33	34	35	36	37	38+39	40+41	42	43
BAND (kb)	18	1,8	0,4	1,3	1,5	6,1	6,2	4,2	11.0

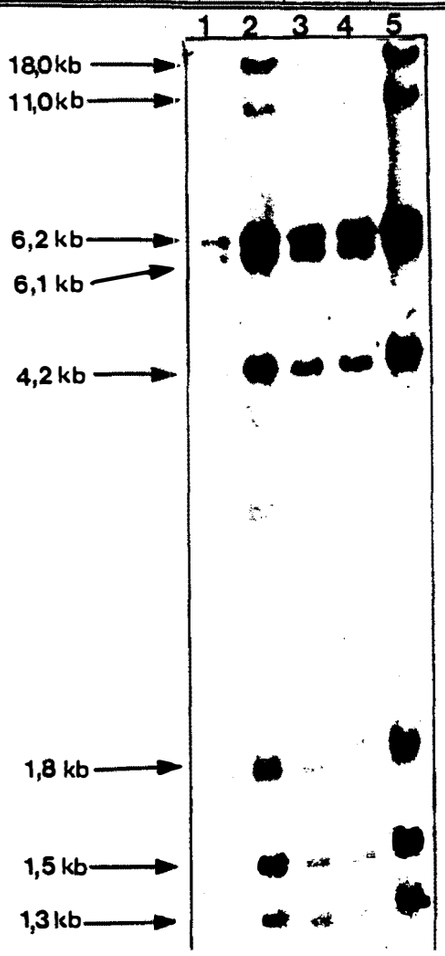

Figure III-3.

An autoradiograph showing the HindIII hybridisation pattern of cDMD5b-6. **Key to Fig. III-3.**

The DNA samples in lanes 1,2,3 and 5 show the normal hybridisation pattern of cDMD5b-6. The smaller bands are faint. The 0,45kb fragment was lost during electrophoresis, indicating that a shorter run was required. The absence of the 1,8kb and 18kb fragments in lane 4 indicates a deletion of exons 30 to 34.

Subclone 9

EXON	53	54	55	56	57	58
HIND III bands (kb)	7,8+1	8,3	2,3	1,0	6,0	8,8

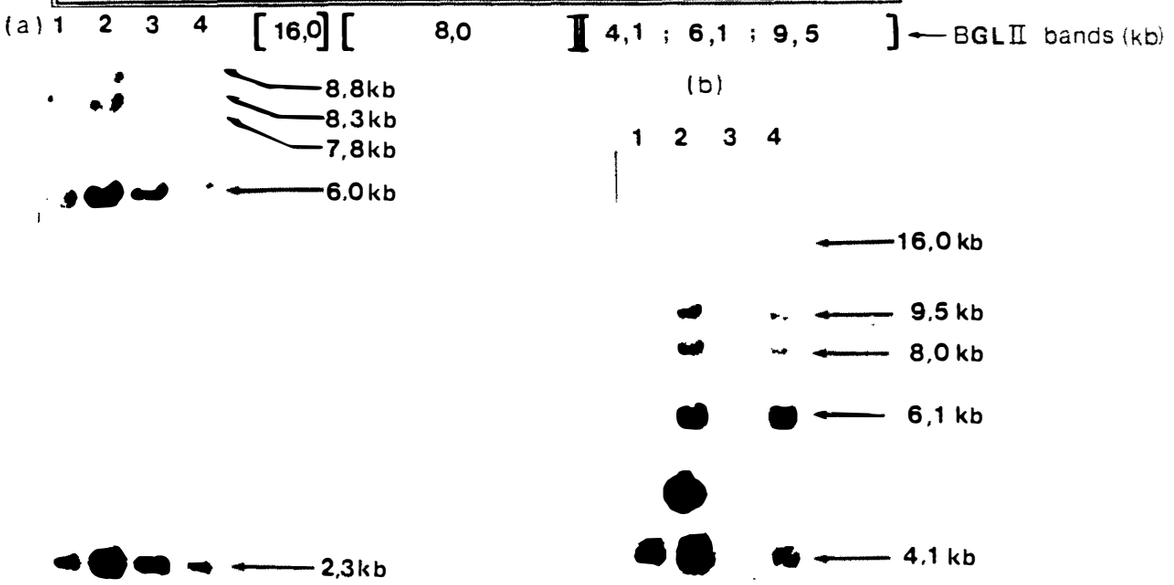

Figure III-4

Autoradiographs showing the Hind III (a) and BglII (b) hybridisation patterns of cDMD9.

The HindIII banding pattern (a) observed with cDMD9 consists of 7 hybridising fragments (lanes 2, 3 and 4). The two 1kb fragments co-migrate and cannot be resolved. In lane 1, the absence of the 8,3kb fragment was identified as an RFLP by the absence of altered fragments in the hybridisation pattern of a BglII digest of this sample ((b) lane 4).

The PCR

The PCR products yielded strong sharp bands on ethidium-bromide staining and were easily visualised. In this study, the two multiplex PCR kits (i.e. for each of the two hotspots) amplified a total of ten different exons within the DMD gene. The ten products ranged in size from 139 bp to 547 bp. None of the fragments overlapped in size and they were therefore easily resolved on 3% agarose gels (Fig. III-5).

3.3.2 Deletion frequency observed in South African DMD/BMD patients

In this study 110 unrelated males affected with DMD and 18 with BMD, were screened for deletions.

Genomic deletions

Genomic probes from the 5' and 3' mutation hotspots in the DMD gene detected deletions in 26 (24%) of 110 South African DMD patients screened. Fifteen of these deletions were detected by probes in the 5' genomic region spanning the DXS164 and DXS206 loci and 11 were detected with the P20 probe from the 3' region of the gene. The proportion of deletions detected by each genomic probe is summarised in Table III-4.

Deletions were detected in six (33%) of the 18 BMD patients; three in the 5' hotspot and two in the 3' hotspot of the dystrophin gene.

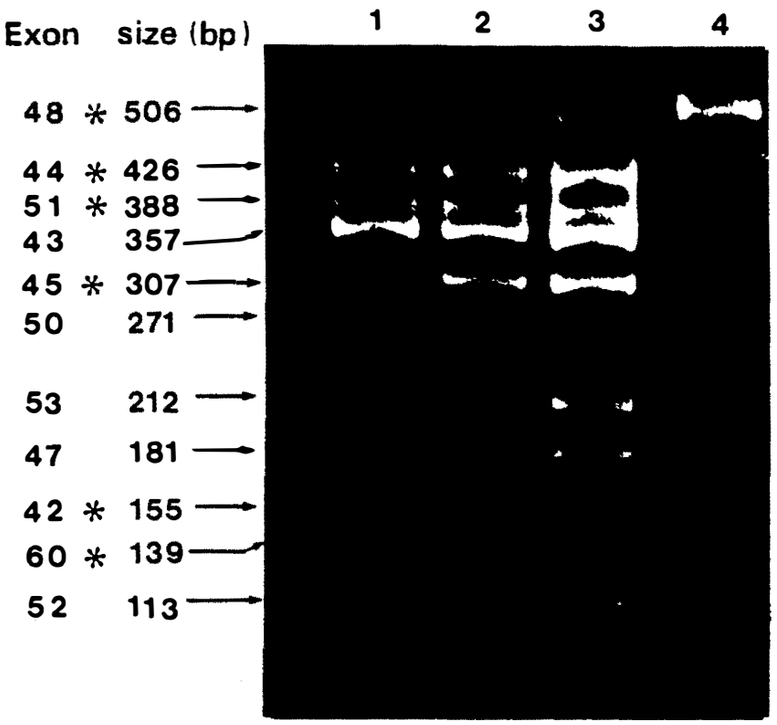

Figure III-5

Multiplex PCR products

Key to Fig.III-5

These results demonstrate the amplification of 11 exons in one reaction (primer sets obtained from C. Mathew, U.K.). The bands marked with an asterisk are those included in the multiplex kits of the present study. Lane 1 shows a deletion of exons 45 to 47. Lane 4 is a DNA control (i.e. all reagents except enzyme included)to monitor contamination.

Table III-4. Genomic deletions observed in the South African DMD/BMD patients. The number of deletions observed with the pERT84-10, pERT87 and XJ probes, are summarised.

	PATIENTS STUDIED	DELETIONS DETECTED	pERT 84-10	XJ	pERT 87	XJ AND pERT87	P20
DMD	110	26 (24%)	1	3	3	8	11
BMD	18	5 (28%)	0	1	1	1	2

cDNA deletions

cDNA probes were used to determine the endpoints of the 31 intronic deletions in DMD/BMD patients, detected with genomic probes, and to search for aberrant banding patterns in the remaining 97 patients in whom intronic deletions had not been detected. The distribution and size of the observed deletions are shown schematically in Fig. III-6.

In addition to those deletions identified by the genomic 5' probes, the cDNA probes spanning that region identified two deletions which involved exons 20-25. The cDNA probes spanning the 3' hotspot detected 29 DMD deletions and four BMD deletions. Of the 18 DMD deletions which had not been detected by the genomic probes, 17 had a breakpoint distal to the P20 intron and one deletion extended proximally from the P20 intron. Only one of the four BMD deletions detected with the cDNA probes, had not been detected by P20.

In this study, a total of 46 DMD deletions and six BMD deletions were detected using a combination of genomic and cDNA probes. Of the DMD deletions, 65% occurred in the 3' hotspot, the majority of which were detected by the probe cDMD8. Most of the deletions in the 5' region of the gene were detected by the probe cDMD1-2a. The other cDNA subclones detected a smaller number of deletions. These results are summarised in Fig. III-7.

Of the 46 DMD deletions detected, 11 had a 3' breakpoint between exons 52 and 53; 10 had an endpoint in the intron between exons 50 and 51; and three involved a single HindIII fragment (one of exon 51, one of exon 44 and one of exon 50). Two DMD deletions had a 3' breakpoint in the P20 intron and extended in a 5' direction; one involved exon 44 alone and one ended in intron 7. A large proportion of deletions in this study had a 5' endpoint within the P20 intron, between exons 44 and 45. The proportion of deletion endpoints relative to the introns of the 3' hotspot are shown in Fig. III-8.

Of the 18 deletions which were detected in the 5' hotspot, five (27.8%) had breakpoints in intron 7. The largest deletion observed in this study comprised 47 exons, stretching from exon 3 to exon 50.

Of the six BMD deletions, three were located in the 3' hotspot, two encompassed exons in the 5' hotspot and one deletion stretched from exon 14 in the 5' region to exon 51 in the 3' region.

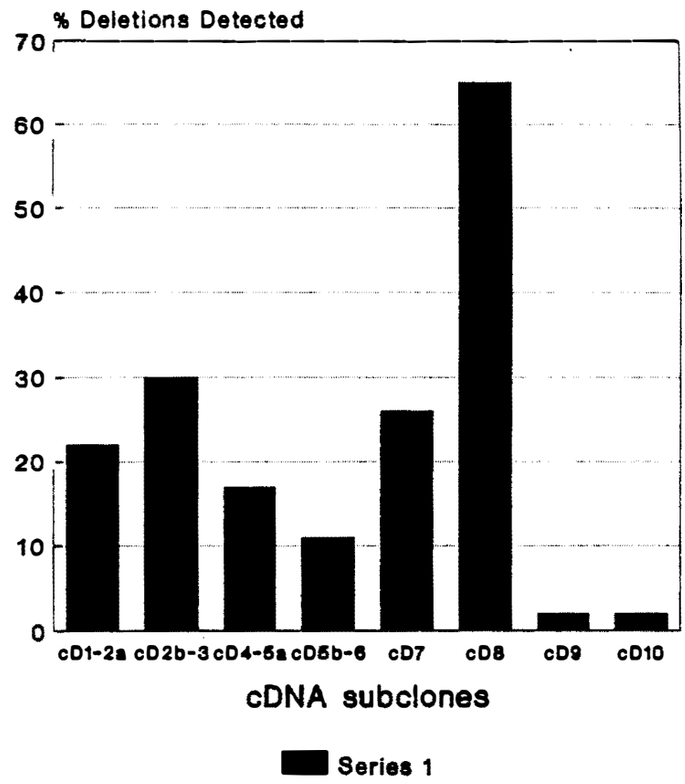

Figure III-7

The proportion of deletions detected by each cDNA subclone.

cD = cDMD

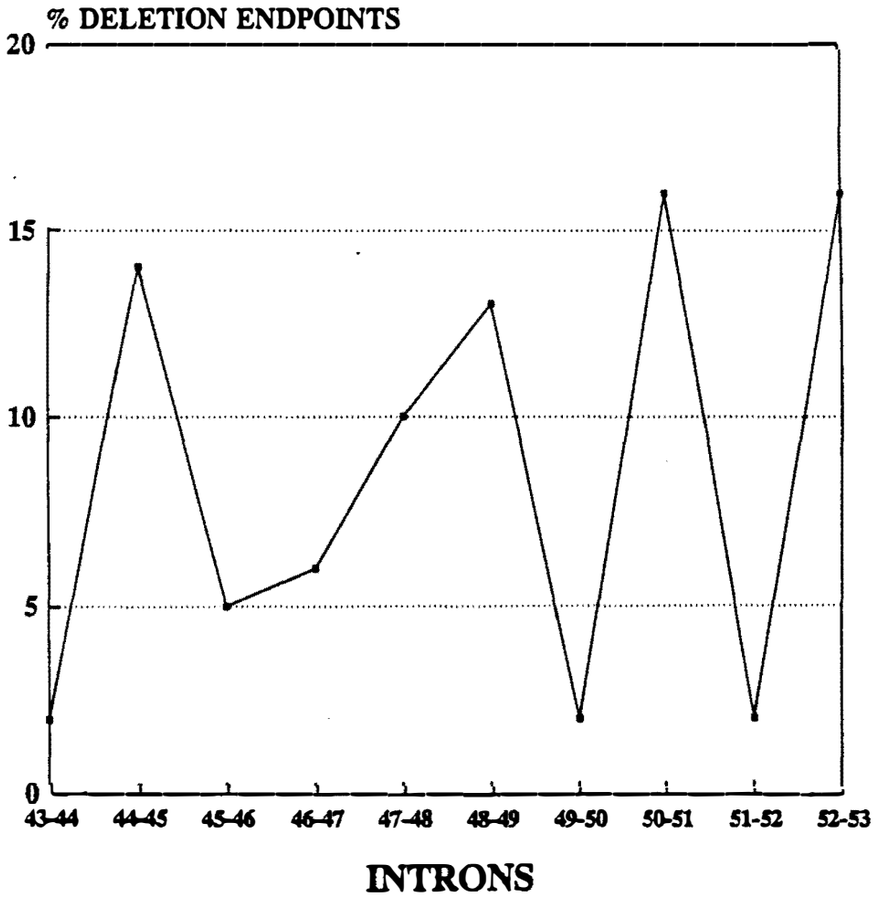

Figure III-8

A graphic representation of deletion endpoints with respect to the introns in the 3' deletion hotspot.

Key to Figure III-8

The units on the X-axis are shown in terms of the exons which flank the intron in question.

Screening for DMD/BMD deletions with the PCR

Of the 46 DMD and six BMD deletions detected by Southern blot analysis, 85% and 83% respectively, were detected by the PCR reactions. The PCR was particularly useful in patients who had deletions of the P20 intron and exon 45, because it resolved the 0,5kb HindIII fragment of exon 45 which is not clear on autoradiographs of Southern blot cDNA hybridisations. Those deletions which were not detected by PCR, encompassed exons for which the primer sets were not available in the reaction kits. The relevant primer sets available for PCR screening therefore represented 100% detection of deletions which had been detected by Southern analysis.

3.3.3 Ethnic distribution of deletions in South African patients

The observed deletion distribution and frequency in affected DMD and BMD males from the different South African ethnic groups, Black, Caucasian, Indian and Mixed Ancestry (as described in section 3.1) is summarised in Table III-5. (As stated earlier, for the purposes of this study all of the males investigated were unrelated).

Group 1 (Black):

Of the 39 DMD patients investigated in this group, 10 (25,6%) had detectable deletions in the dystrophin gene; 3 were identified with the 5' probes, and 5 deletions were detected

with the probes spanning the 3' hotspot. Two deletions were identified by probes from both hotspots. Deletions were not detected in either of the 2 BMD patients.

Group 2 (Caucasian):

Of the 23 DMD patients, 13 (56,5%) showed altered HindIII banding patterns. Four of these were seen when the blots were hybridised to probes from the 5' mutation hotspot. Nine deletions were recognised by the probes from the 3' region. Six of these involved exon 52.

Of the nine BMD patients, two had deletions in the 3' hotspot extending distally from P20 and one had a deletion in the 5' hotspot involving exons 10 to 20.

Group 3 (Indian):

Deletions were detected in 10 (47,6%) of the 21 DMD patients. The majority of the deletions were recognised by probes from the 3' hotspot. Four of these deletions had a 5' endpoint in the P20 intron, and three had a 5' endpoint involving exon 49. Only two patients had a deletion in the 5' region.

Deletions were detected in two of the three BMD patients; in one patient the deletion spanned exons 14 to 51, in the other exons 48 to 51 had been deleted.

Group 4 (Mixed Ancestry):

Deletions were apparent in 13 (48%) of the 27 DMD patients screened: four were detected in the 5' mutation hotspot; eight in the 3' hotspot; and one large deletion with probes from both regions. Five of the DMD deletions had an endpoint between exons 50 and 51, and four had an endpoint involving exon 52.

A deletion, involving cDMD1-2a, was identified in one of the four BMD patients.

Table III-5. The frequency and distribution of deletions in the dystrophin gene of South African DMD and BMD patients.

ETHNIC GROUP	NO. OF UNRELATED PATIENTS DMD BMD	NO. OF DELETIONS DMD BMD	5' DMD BMD	3' DMD BMD	5'-3' DMD BMD
BLACK	39	10 (26%)	3 (30%)	5 (50%)	2 (20%)
	2	0	-	-	-
CAUCASIAN	23	13 (57%)	4 (31%)	9 (69%)	0
	9	3 (44%)	2	1	-
INDIAN	21	10 (45%)	2 (20%)	8 (80%)	0 (8%)
	3	2 (67%)	1	-	1
MIXED ANCESTRY	27	13 (48%)	4 (31%)	8 (61%)	1 (8%)
	4	1 (25%)	-	1	-
TOTAL	**110**	**46 (42%)**	**28%**	**65%**	**7%**
	18	**6 (33%)**	**50%**	**25%**	**25%**

3.3.4 Genotype/Phenotype correlations in South African DMD/BMD patients

The deletion data and clinical phenotypes of the DMD and BMD patients are summarised in Tables III-6 to III-9. The deletions observed in the present study, and their effect on the reading frame in the two deletion-rich regions, are shown in Fig. III-9 and Fig. III-10. The translational reading frame of the first 60 exons in the DMD gene were taken from the literature [Koenig et al., 1988; Baumbach et al., 1989; Koenig et al., 1989].

Deletions in the 5' hotspot

All except two of the seven DMD deletions occurring between exons 1 and 22, disrupted the translational reading frame. The two exceptions were deletions of exons 10 to 13 (patient D-25) and exons 11 to 13 (patient D-3). The two BMD deletions (exons 3 to 9 in patient B-6, and exons 10 to 20 in patient B-3) maintained the reading frame.

Deletions in the 3' hotspot

All except three of the 25 DMD deletions within the 3' hotspot (between exons 44 and 52) obeyed the reading frame rule [Monaco et al., 1988]. These in-frame DMD deletions involved exons 45 to 47, 45 to 48 and exons 47 and 48 (in patients D-33, D-2 and D-13, respectively). The three BMD deletions

involving exons 44 to 52 (patients B-1, B-2 and B-4) maintained the reading frame. A large deletion, stretching from exon 14 in the 5' hotspot to exon 51 in the 3' hotspot, had no effect on the translational reading frame and resulted in a BMD phenotype (patient B-5).

Deletions with unknown frame shift

The exon-intron boundaries of some exons are unknown, and the effect of their absence on the translational reading frame could not be determined. These deletions included one with a 3' endpoint involving exon 59 (patient D-27), and one with a deletion ending in exon 25 (patient D-15).

DMD and Mental retardation

In the present study, 24% of the patients had a deletion involving exon 52. The mental capacity of these patients was not formally assessed, but was inferred from school performance.

Of the 12 patients with a deletion encompassing exon 52, nine attended conventional schools and were considered to be bright and intelligent. The other 3 DMD patients with similar deletions had some form of mental retardation.

Table III-6.

Genotype/phenotype correlation in Black DMD/BMD patients with a dystrophin gene deletion.

PATIENT NO.	FAMILIAL (F)/ ISOLATED (I)	EXONS INVOLVED	PHENOTYPE
D-1	I	7 - 19	DMD
D-2	I	45 - 48	DMD
D-3	F	11 - 13	DMD
D-4	I	44	DMD
D-5	I	10 - 44	DMD
D-6	I	7 - 20	DMD
D-7	I	3 - 21	DMD
D-8	F	45	DMD
D-9	I	13 - 43	DMD
D-10	1	50	DMD

Table III-7.

Genotype/phenotype correlation in Caucasian DMD/BMD patients with a dystrophin gene deletion.

PATIENT NO.	FAMILIAL (F)/ ISOLATED (I)	EXONS INVOLVED	PHENOTYPE
D-11	I	3 - 7	DMD
D-12	I	48 - 52	DMD
D-13	I	47 - 48	DMD
D-14	I	45 - 52	DMD
D-15	I	21 - 25	DMD
D-16	F	8 - 11	DMD
D-17	I	49 - 52	DMD
D-18	I	45 - 52	DMD
D-19	I	49 - 50	DMD
D-20	F	45 - 50	DMD
D-21	I	47 - 52	DMD
D-22	I	49 - 52	DMD
D-22	F	Pm - 1	DMD
B-3	F	45 - 47	BMD
B-2	I	45 - 47	BMD
B-3	F	10 - 20	BMD

Table III-8.

Genotype/phenotype correlation in DMD/BMD patients, of Indian origin, with a dystrophin gene deletion.

PATIENT NO.	FAMILIAL (F)/ ISOLATED (I)	EXONS INVOLVED	PHENOTYPE
D-24	F	45	DMD
D-25	I	10 - 13	DMD
D-26	I	49 - 50	DMD
D-27	F	49 - 59	DMD
D-28	F	45 - 52	DMD
D-29	F	49 - 50	DMD
D-30	F	45 - 52	DMD
D-31	I	20 - 25	DMD
D-32	F	45 - 47	DMD
D-33	F	51	DMD
B-4	F	48 - 51	BMD
B-5	F	14 - 51	BMD

Table III-9.

Genotype/phenotype correlation in DMD/BMD patients, of Mixed Ancestry, with a dystrophin gene deletion.

PATIENT NO.	FAMILIAL (F)/ ISOLATED (I)	EXONS INVOLVED	PHENOTYPE
D-34	I	5 - 11	DMD
D-35	I	48 - 50	DMD
D-36	I	6 - 20	DMD
D-37	I	48 - 50	DMD
D-38	I	45 - 50	DMD
D-39	I	50 - 52	DMD
D-40	I	3 - 50	DMD
D-41	I	52 - 53	DMD
D-42	I	7 - 36	DMD
D-43	I	47 - 50	DMD
D-44	I	3 - 7	DMD
D-45	I	45 - 52	DMD
D-46	I	48 - 52	DMD
B-6	F	3 - 9	BMD

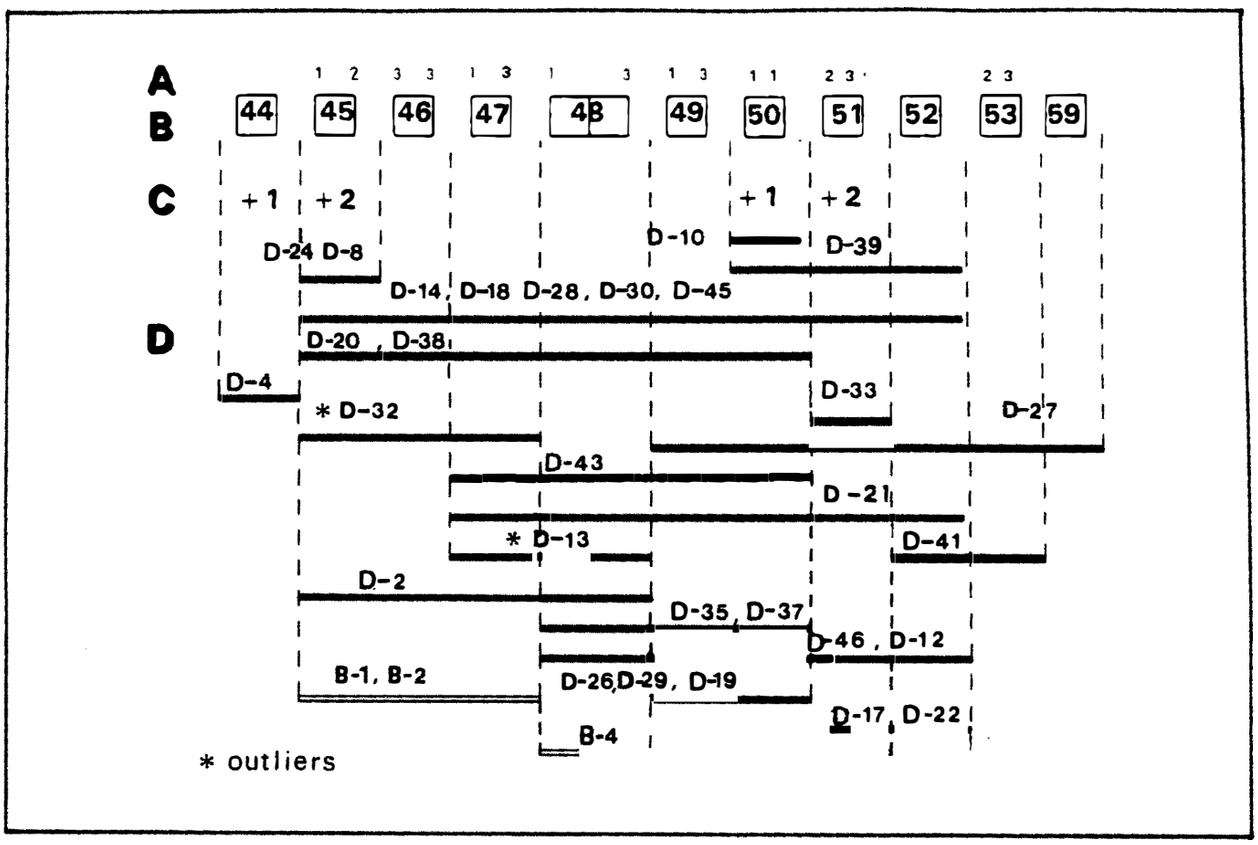

Figure III-10

DMD/BMD deletions with respect to the translational reading frame in the 3' hotspot.

Key to Fig. III-10

A= The position of the nucleotide within the codon, at the exon/intron boundary;

B= Exons in the dystrophin gene;

C= The frame shift resulting from a deletion of the exon;

D= DMD deletions (solid bars); BMD deletions (unshaded bars).

* indicate deletions which do not conform with the reading frame rule.

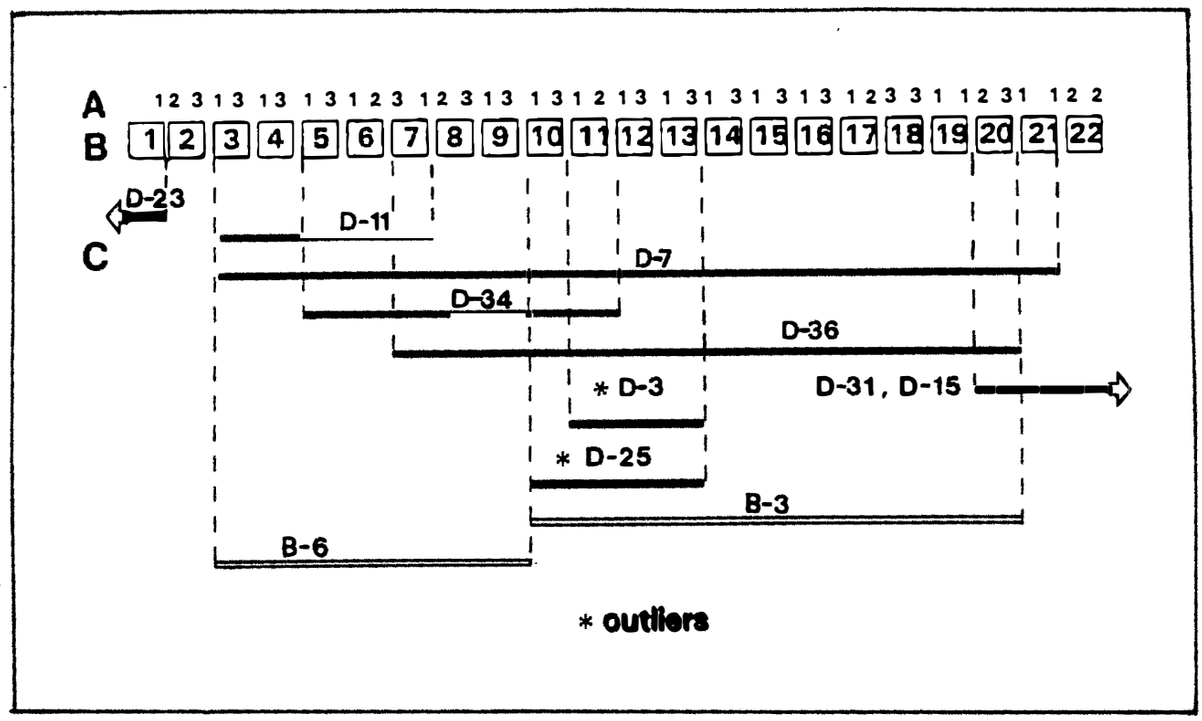

Figure III-9

DMD/BMD deletions with respect to the translational reading frame in the 5′ hotspot. The first 22 exons of the gene are shown.

Key to Fig. III-9

A= The position of the nucleotide within the codon, at the exon/intron boundary;

B= Exons in the dystrophin gene

C= DMD deletions (solid bars); BMD deletions (unshaded bars).

* indicate deletions which do not conform with the reading frame rule.

3.4 DISCUSSION OF DYSTROPHIN GENE DELETIONS IN SOUTH AFRICAN DMD/ BMD PATIENTS

3.4.1 Frequency and distribution of observed deletions

A total of 46 (42%) deletions were identified in 110 unrelated South African DMD patients. This deletion frequency is consistent with published values of 40% to 60% in other populations, using similar methodology [Koenig et al., 1987; Darras et al., 1988; Sugino et al., 1989; Passos-Bueno et al., 1990].

Of the 46 deletions detected in the present study, 65% had a breakpoint in the 3' hotspot whereas only 28% were detected in the 5' hotspot. This distribution corroborates previous reports [Gillard et al., 1989; Baumbach et al., 1989]. The remaining deletions were large and involved genomic regions stretching from the 5' hotspot to the 3' hotspot. The clustering of breakpoints in the P20 intron (22%), in the intron between exons 50 and 51 (22%), and in intron 7 (9%), was similar to that shown in other studies [Bartlett et al., 1988; Darras et al., 1988; Forrest et al., 1988; Gilgenkrantz et al., 1989; Gillard et al., 1989; Hart et al., 1989].

In the present study, only one deletion was observed in the promoter region of the dystrophin gene. This paucity of deletions in the promoter has been observed by other investigators and could be due to the selective pressure for

the conservation of a consensus sequence [Boyce et al., 1991; Vitiello et al., 1992].

The deletion frequency observed in the 18 BMD patients was much lower (33%) than that reported by other investigators (about 60%) with the same set of cDNA probes [Gilgenkrantz et al., 1989; Liechti-Gallati et al., 1989; Lindlof et al., 1989]. A possible explanation for this observation could be that a few patients with unclassified or intermediate forms of muscular dystrophy may have been included in the present study, even though the diagnosis of DMD and BMD was substantiated at an expert level. This unrecognised heterogeneity would contribute to the reduced deletion frequency observed in the South African BMD patients. This explanation may have some validity in view of the diagnostic difficulties which are inherent in the muscle dystrophies [Norman et al., 1989; Arikawa et al., 1991].

3.4.2 The ethnic distribution of DMD/BMD deletions

The deletions observed within each South African ethnic population was not unique to that particular group. The deletion frequency for each ethnic group differs significantly, however, from the combined frequency for affected individuals of all groups.

The low rate of observed deletions occurring in the Black population (less than 30%) might be due to other intragenic

mutations, lying outside the known deletion hotspots. The phenotype in the Black patients in whom deletions were not detected, is identical to that in other groups and non-allelic heterogeneity seems unlikely. It could be suggested that the rare autosomal recessive (AR) form of DMD [Salih et al., 1983; Francke et al., 1989; Vainzof et al., 1991] which is phenotypically indistinguishable from classical X-linked DMD may be more prevalent in the Black population. If so, this would have the net effect of reducing the frequency of X-linked DMD in this ethnic group even further. However, as discussed with respect to the increased DMD frequency in the Indian population (see section 2.2.3), if an AR form of DMD was indeed present in this population, it would be expected that affected females would be encountered. The fact that 39 unrelated affected Black males have been examined, whereas not a single female with a DMD-like phenotype has been seen, argues against the presence of AR DMD in any significant frequency in the South African Black population.

3.4.3 Genotype/ Phenotype correlations in South African patients.

In the present study there was no apparent correlation between the size of a deletion in the dystrophin gene, and the progression and severity of the disorder. A deletion of a single exon (D-33) results in the same severe phenotype as a deletion of 8 exons (D-28). This observation is consistent with the findings of other investigators [Darras et al., 1988;

Gilgenkrantz et al., 1989; Hart et al., 1987]. As stated previously, the reading frame model [Monaco et al., 1988] was proposed to explain the effect of different deletions in the dystrophin gene on the severity of the resultant clinical phenotype. The phenotypes resulting from 90% of the deletions reported in this study conform with the reading frame rule. Deletions which disrupt the translational reading frame result in the DMD phenotype and deletions maintaining the frame result in BMD. Exceptions to the reading frame theory are well documented [Malhotra et al., 1988; Gillard et al., 1989; Covone et al., 1991]. In the present study the four exceptions to the reading frame theory were DMD deletions which maintained the reading frame.

All of the BMD deletions detected in the present study, occurred in a genomic region (exons 10 to 60) which is known to code for the rod-domain of dystrophin [Beggs et al., 1991]. The rod domain consists of a number of repeat units and appears to be of low functional importance since "in-frame" deletions involving parts of it result in variable phenotypes [Love et al., 1990; Arahata et al., 1991; Beggs et al., 1991]. This would possibly explain why a large "in-frame" deletion, encompassing more than 30 exons coding for this domain, resulted in a BMD phenotype in a South African patient (patient B-5 is alive, but confined to a wheelchair, at 41 years of age).

DMD and Mental retardation

In the present study no correlation was observed between deletions encompassing exon 52 and the mental capacity of the DMD patient. However, as stated previously, the intelligence of the South African patients was inferred from their school performance. For that reason, these present findings cannot confirm or refute the observations of other investigators. Furthermore, the numbers in the South African study are small, and as Rapaport et al. [1991] suggest, it would require a formal assessment of intelligence and a larger deletion study in order to confirm an association between exon 52 and the intelligence quotient in DMD patients.

CHAPTER 4

POLYMORPHISM FREQUENCIES IN THE DYSTROPHIN GENE OF MEMBERS OF THE SOUTH AFRICAN POPULATION

4.1 INTRODUCTION

Deletion screening provides an accurate means of testing for the mutation causing DMD and BMD and it is a definitive diagnostic criterion in more than 60% of affected males. However, deletions are not easy to detect in carrier females as their corresponding non-deleted gene masks the effect of the mutation. In this regard, the application of recombinant DNA technology in the form of polymorphism analysis, provides a reliable alternative to deletion screening. This methodology also facilitates the identification of females carrying the defective dystrophin gene in the families of non-deletion patients.

Polymorphisms are neutral base pair variations in the DNA sequence between individuals which occur about once every 500 base pairs [Botstein et al., 1980]. If these variations alter the normal recognition site of a restriction enzyme they result in different lengths of DNA being produced (with the enzyme) which can be detected by recombinant DNA techniques. RFLPs are non-pathogenic and are inherited in a Mendelian fashion. If a DNA marker is cloned from a region flanking or containing an RFLP site which is closely linked to a disease locus, a particular form of the polymorphism which is

recognised by the marker, will always be inherited (i.e. co-segregate) with the disease gene. Co-segregation will change if crossing-over has occurred between the marker and the disease locus during meiosis. The closer the two loci, the less the chance of crossing-over between them. In this way the inheritance of marker RFLPs which are closely linked to the DMD gene can be tracked within families.

The use of cloned DNA segments, which are tightly linked to the DMD gene, have radically improved the previously imprecise methods of carrier identification and prenatal diagnosis (i.e. CPK analysis and foetal sexing) [Kingston et al., 1985; Goodship et al., 1988; LeRoy et al., 1988].

In addition to RFLPs, polymorphisms for linkage analysis are provided by PCR-formatted simple sequence dinucleotide repeat polymorphisms which occur every 30-60kb in the human genome [Weber and May, 1989]. These repeats occur in blocks of up to 26 repeat units, block size varying between individuals. Since the length of the repeat block is polymorphic and cosegregates in a Mendelian fashion, these have proved very useful for linkage studies. Dinucleotide $[CA]_n$ repeats identified within the dystrophin gene have significantly increased the polymorphic information available for tracking haplotypes associated with DMD or BMD (as outlined in Chapter 5) [Clemens et al., 1991; Feener et al., 1991; Oudet et al., 1991].

Informative haplotype-based analysis requires the mother of an affected male individual to have different alleles of the marker on her two X-chromosomes. This heterozygous genotype permits the differentiation of her normal and disease-associated alleles and the subsequent tracking of these alleles within the family, thereby facilitating identification of female carriers. For this reason, the markers which are to be used for predictive testing should ideally have a high polymorphic content within the target population. It has been shown that the polymorphic information content for DNA markers varies between the different South African ethnic groups in other genetic disorders [Greenberg et al., 1991]. For this reason it was necessary to establish allele and heterozygote frequencies in each of the local populations for potentially usable polymorphisms in the dystrophin gene and examine their applicability in the specific ethnic groups.

4.2 METHODS AND MATERIALS FOR POLYMORPHISM ANALYSIS

4.2.1 RFLPs analysis

Eighty unrelated normal control females from the Black, Caucasian, Indian and Mixed Ancestry populations, were used for RFLP analysis. Analyses of $[CA]_n$ repeats were completed for 100 unrelated females from each population. Allele frequencies and heterozygosity frequencies were determined and polymorphism information content (PIC) values were calculated according to the method of Botstein et al. [1980].

4.2.2. Dinucleotide length polymorphism analysis

Oligonucleotide primers for amplification of $[CA]_n$ repeats, by the PCR, in introns 44 and 49 were taken from Clemens et al. [1991]. Primer sequences for the $[CA]_n$ repeat in the 3' non-coding region of the gene were as described in Beggs and Kunkel [1990b] and are listed in Table IV-1.

The PCR method used was as described in section 3.2.3. For $[CA]_n$ analysis, the PCR volumes were scaled down to a total volume of 20ul and 0,1ul of a-dCTP^{32} was added to each reaction. For visualisation, 2ul of the PCR product was mixed with 3ul of loading buffer containing formamide, xylene cyanol and bromophenol blue (Appendix B) and heated to 92°C for 3mins. The samples were loaded onto an 8% denaturing polyacrylamide gel containing 50% urea (Appendix B) and electrophoresed at 2kV for

2 hours. The gels were dried on a vacuum drier and autoradiographed at -70°C overnight.

In order to determine the allele sizes of the dinucleotide repeats, termination products of a sequencing reaction and an end-labelled molecular weight marker were loaded on each gel. The size of the sequencing product was estimated from the molecular weight marker and the $[CA]_n$ allele sizes could be estimated according to the bases in the sequencing products (Fig. IV-1).

Table IV-1. Oligonucleotide primers used to amplify dinucleotide repeat sequences in the dystrophin gene.

3' = 3' untranslated region of the dystrophin gene.

F = Forward primer R = Reverse primer

POSITION OF $[CA]_n$ REPEAT	5' PRIMER SEQUENCE 3'	PCR PRODUCT
Intron 44	F-TCCAACATTGGAAATCACATTTCAA R-TCATCACAAATAGATGTTTCACAG	From 174bp
Intron 49	F-CGTTTACCAGCTCAAAATCTCAAC R-CATATGATACGATTCGTGTTTTGC	From 227bp
3'	F-GAAAGATTGTAAACTAAAGTGTGC R-GGATGCAAAACAATGCGCTGCCTC	From 129bp

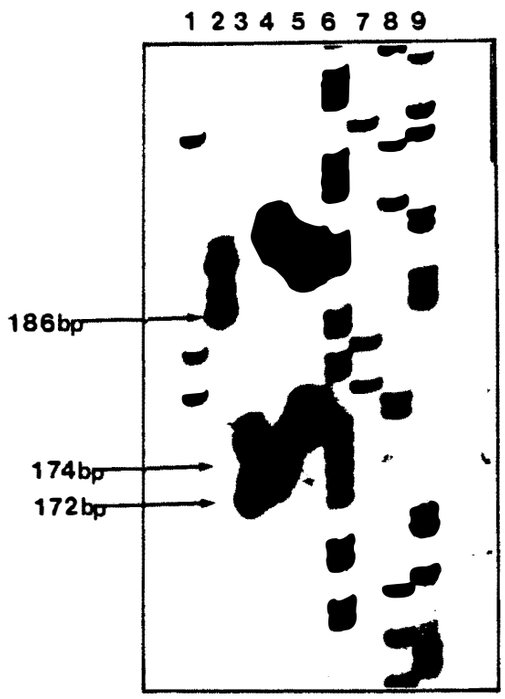

Figure IV-1.

Sizing of $[CA]_n$ polymorphisms.

Key to Fig. IV-1

Sequencing reaction products- lanes 1 and 6-9.

$[CA]_n$ polymorphisms - lanes 2-5.

The end-labelled phage lambda molecular weight marker which was used to size the nucleotides in the sequencing reaction, occurred lower down on the autorad.

4.3 RESULTS OF POLYMORPHISM ANALYSIS IN THE SOUTH AFRICAN STUDY

4.3.1 RFLP analysis

The allele frequencies and PIC values in each of the South African ethnic groups are compared to those published previously for Caucasians [Davies et al. 1987] in Table IV-2.

The allele frequencies for the polymorphisms analysed in South African Caucasians were generally similar to the reported figures. The allele frequencies in the Mixed Ancestry group were similar to that of the Caucasians for the 754/PstI, pERT87-8/TaqI, and C7/EcoRV polymorphisms. However, differences in the polymorphism frequencies of the probes pERT87-15, XJ1.1 and pERT87-1, were observed between the two groups. Gene frequencies for the Indian female controls were not markedly different from that of the Mixed Ancestry and Caucasian groups. In the Black population, the allele frequencies for the probes 754 and XJ1.1 are reasonably informative (0,5>PIC>0,25), whereas for the other probes the minor alleles were either rare (pERT87-1, C7, pERT87-15 and pERT87-8) or absent (P20). In the latter population group, however, pERT87-8 recognised a third TaqI allele (1,9kb) which was not observed in the other local populations investigated. This variant was, however, present at a similar frequency in a Black community of North America [Schwartz and Barjon, 1987]. This 1,9kb allele was not associated with DMD since it

was also present in the DNA of unaffected males. The Caucasian, Indian and Mixed Ancestry groups were reasonably informative (0,5>PIC>0,25) with pERT87-8 for the 3,8/2,7;1,1kb allele system, whereas in the Black group the 3,8kb allele was rarely seen. Heterozygosity with the pERT87-8 probe is markedly increased (PIC approaching 0,5) in the Black population by the presence of the 1,9kb allele.

All of the South African ethnic populations in this study were reasonably informative (0,5>PIC>0,25) for the 5' flanking marker 754. However, the C7 polymorphism was less informative in the Black, Caucasian and Mixed Ancestry groups (0,25<PIC<0,3) but considerably higher in the Indian controls (PIC approaching 0,5).

4.3.2 Results of dinucleotide length polymorphism analysis

PCR artifacts, which have been reported in the literature [Clemens et al., 1991] during the amplification of dinucleotide repeats have not been a factor in this study. Shadow bands visible on the autoradiographs of PAGE gels were considerably fainter than the allelic polymorphic bands.

Heterozygosity frequencies of the $[CA]_n$ polymorphisms used in the present study, are listed in Table IV-3. Of the 100 females screened, 75% were heterozygous for at least one of the short tandem repeats (STR). The different South African

ethnic groups showed variations in the degree of polymorphism and the allele sizes. The most polymorphic locus was STR49, with a total of 17 alleles ranging in size from 201bp to 236bp. At this locus a heterozygosity of between 75% (Indian females) and 83% (the other ethnic groups) was observed. At the STR44 locus, 12 alleles, ranging from 170bp to 190bp, were observed.

The informativity for the STR44 alleles was lower than for STR49, with the Black, Caucasian and Mixed Ancestry populations having 74% to 80% heterozygosity. Only 55% of the Indian females were heterozygous at this locus.

The dinucleotide repeat in the 3' untranslated region of the dystrophin gene, with only 7 alleles, was the least polymorphic locus used in this study. The South African Indian females exhibited only four of the seven alleles and were the least informative (10% observed heterozygosity). The Caucasian females also had a low heterozygosity in this region (36%), whereas the Mixed Ancestry and Black populations were highly polymorphic (60% to 77% heterozygosity).

Table IV-3 . Heterozygosity frequencies of $[CA]_n$ length polymorphisms observed in the South African study. The heterozygosity frequencies observed in the previously reported Caucasian populations are listed.

Key to Table IV-3

STR= short tandem repeats; N= number of alleles observed; S.A.= South African frequencies; N.A.= North American frequencies as reported by Clemens et al. [1991] (STR44 and STR49) and Beggs and Kunkel [1990] (3' untranslated region).

ETHNIC GROUP	STR49		STR44		3' REGION	
	N=17 (SA)	N=19 (NA)	N=12 (SA)	N=12 (NA)	N=7 (SA)	N=4 (NA)
BLACK	83%	-		-	77%	-
INDIAN	75%	-	55%	-	10%	-
MIXED ANCESTRY	83%	-	74%	-	61%	-
CAUCASIAN	83%	93%	76%	87%	36%	-
TOTAL	**82%**	**-**	**69%**	**-**	**50%**	**50%**

4.4 DISCUSSION OF ALLELE AND HETEROZYGOSITY FREQUENCIES OBSERVED IN THE SOUTH AFRICAN POPULATION

4.4.1 RFLP frequencies

In the present study, several of the RFLPs which were analysed had different allele frequencies in the four ethnic groups. This information is valuable for the genetic management of the DMD and BMD families. For example, the P20 RFLPs are not useful for linkage studies in the Black population, since they are monomorphic for the RFLP whereas the peculiar pERT87-8 allele (1,9kb) which is present only in the Black population, provides an increased informativity in this group alone. Similarly, the minor allele for the pERT87-1/MspI RFLP in Caucasians is the major allele in the Black population.

The RFLPs analysed in the present study are reasonably informative for family studies. Additional polymorphic markers will have to be tested in the Black population, however, in order to increase the informativity in the 3' region of the gene.

4.4.2 $[CA]_n$ length polymorphisms

The polymorphic information obtained with $[CA]_n$ repeat sequences has significantly improved the scope of linkage analysis in the South African population. The heterozygosity frequencies in the STR49 and STR44 loci in South African

Caucasians are consistent with the frequencies observed for North American Caucasians [Clemens et al., 1991]. The heterozygosity frequency in the 3' untranslated region, of the South African Caucasian population is much lower (36%) compared to that in the reported American study (50%) [Beggs and Kunkel, 1990]. However, the overall heterozygosity frequency at this locus was consistent with the reported figure of 50%, for all of the South African females investigated.

In the present study, the Black population was very informative for all of the dinucleotide length polymorphisms used, exhibiting between 77% (3' untranslated region) and 83% (intron 49) informativity. The Mixed Ancestry, Indian and Caucasian females were highly polymorphic for the intron 49 alleles but were less informative for the STR44 and 3' untranslated region.

Overall, in the South African population, the dinucleotide polymorphisms are significantly more abundant than RFLPs and are a powerful tool for haplotype analysis. This is particularly important in view of the high risk of recombination across the DMD gene which contributes to inaccuracies in predictive diagnosis of DMD and BMD (see Chapter 5). The larger the number of polymorphic loci used in haplotyping, the greater the chance of detecting a recombination event in the gene.

The high degree of polymorphism and heterozygosity at the $[CA]_n$ loci makes this technology well suited to complement RFLP analysis in DMD and BMD families. This is particularly important in members of the Black population where there is a low informativity for RFLPs of genomic probes in the 3' region of the gene. A further factor in favour of $[CA]_n$ analysis is the fact that it utilises PCR which is rapid and cost-effective, and is particularly useful when the quantity of biological material is small (as in material for prenatal diagnosis). It is forseeable that this technology might replace Southern blot hybridisations for haplotype analysis, as more dinucleotide repeats are identified within the dystrophin gene.

CHAPTER 5

CARRIER STATUS DETERMINATION AND PRENATAL DIAGNOSIS FOR DMD AND BMD IN SOUTH AFRICA

5.1 INTRODUCTION

The detection of deletions in 42% of affected individuals in the major ethnic groups in South Africa, permits prenatal diagnosis in their families, with an accuracy approaching 100%. Deletion detection is feasible regardless of pedigree structure or the availability of key family members. Deletion analysis has proven to be especially useful for prenatal diagnosis in the families of sporadic patients, when uncertainty about the origin of the mutation prevents accurate carrier risk estimations.

The identification of females carrying DMD deletions is difficult since the deleted (or null) allele is masked by the normal allele on a Southern blot. In these circumstances, dosage studies by densitometry are required to detect the reduced intensity of the hybridising bands. As stated previously (see section 3.3.1), however, dosage studies require highly reproducible Southern blots which are not always possible to achieve. These procedures are therefore considered unreliable for detecting heterozygosity for a normal and a deleted allele in a female [Darras et al.1988; Kimber et al. 1989].

Alternative methods using Southern analysis for identifying deletion carriers involve either the analysis of junction fragments bordering the deleted region or the analysis of polymorphisms flanking the deletion. Junction fragments are generated when a deletion breakpoint is sufficiently close to an exon to yield a hybridising fragment of altered size. Although uncommon, these junction fragments are extremely useful for detecting female deletion carriers as they are not masked by the corresponding allele of the non-deleted X-chromosome. The normal fragment as well as the fragments of altered mobility can therefore be visualised as two separate bands on electrophoresis [Darras et al.,1988; Lindlof et al.,1989; Asano et al.,1991].

Junction fragments are rarely detected because most known deletions remove complete exons. A reliable alternative for identifying deletion carriers is haplotype analysis using RFLPs adjacent to the missing genomic region. By tracking the segregation of alleles in and around the deletion locus, a haplotype which is associated with the disorder in a particular family can be established. Once the disease-haplotype has been determined and the carriers identified, their genotypes at the deletion locus can be used to confirm or negate their carrier status. Carrier risk estimations based on the results of polymorphism analysis are discussed in the next section.

In the absence of a detectable deletion, haplotype analysis using multiple polymorphisms within and flanking the dystrophin gene, is probably the most reliable means of determining carrier risks and diagnosing DMD or BMD in male foetuses [Forrest et al., 1987; Cole et al., 1988; Kelly et al., 1990]. Linkage analysis using haplotypes has also proved very useful in kindreds in which affected males are deceased, but where an unaffected male sibling is available [Darras et al., 1987; Chen et al., 1988]. In the latter circumstance, carrier risk estimations are based on the segregation of the normal alleles. Carrier detection and prenatal diagnosis by haplotype analysis is not always precise, however, due to a number of factors including the following:

(i) Uncertainty in haplotype assignments due to the unavailability of key family members;

The ideal family structure for genetic investigation and counselling is one in which the disorder is transmitted through several generations and all of the key members are available. The ideal pedigree for molecular investigations is shown in Fig. V-1. Family members who would be important for accurate testing are listed below:

* **the patient (III-1)** - to define the mutation or to determine the haplotype associated with the disease.

* **a second affected male relative (III-3)** - to confirm the molecular findings in the proband.

* **unaffected male relatives (II-4, III-2)** - to ascertain the normal marker alleles.

* **obligate carriers of the defective DMD gene (II-3, II-6)** - to track the defective X-chromosome and confirm its mode of transmission.

* **potential gene carriers (II-7, III-4)**- these females who are at risk of carrying the defective gene, will be concerned with their potential carrier status particularly if they are contemplating pregnancy.

* **both parents (I-1, I-2/ II-5, II-6) of the potential carriers** - in order to establish the paternal and maternal alleles in their daughter and to track the transmission of the defective DMD gene.

* **the maternal grandparents of the affected male (I-1, I-2)** - so that the origin of the defective dystrophin gene and the phase of the disorder can be determined.

This ideal pedigree is seen very rarely, however, and the most common situation is a family in which either the father of the potential carrier is unavailable or the key affected individual is deceased or the disorder has occurred sporadically within the family. These circumstances introduce complications into the application of DNA investigations for genetic counselling.

(ii) recombination across the DMD gene

As a result of the large size (more than 2Mb) of the dystrophin gene [Burmeister and Lehrach., 1987, Monaco and Kunkel, 1988] the possibility of crossing-over occurring between polymorphic loci and the mutation site is increased and complicates accurate carrier risk estimations [Bakker et al., 1985; Darras et al., 1987; Prior et al., 1989].

The frequency of recombination across the entire DMD gene has been estimated to be about 12% [Chen et al., 1989; Abbs et al., 1990]. The frequency of crossing-over is therefore much higher across the DMD gene (6cmo/Mb) than that established for the whole X-chromosome (1,2cmo/Mb) [Drayna and White, 1985]. For this reason, carrier risk estimations by segregation analysis using a single intragenic marker, has an error rate of about 12%. The use of the two closest flanking markers, 754 and C7, in linkage studies, reduces this error to about 4%, which is the probability of a double recombination event occurring between the flanking markers. The use of multiple polymorphisms within and flanking

error rate to less than 1,5% (which is the probability of a double recombinant event ocurring within the gene) [Abbs et al., 1990].

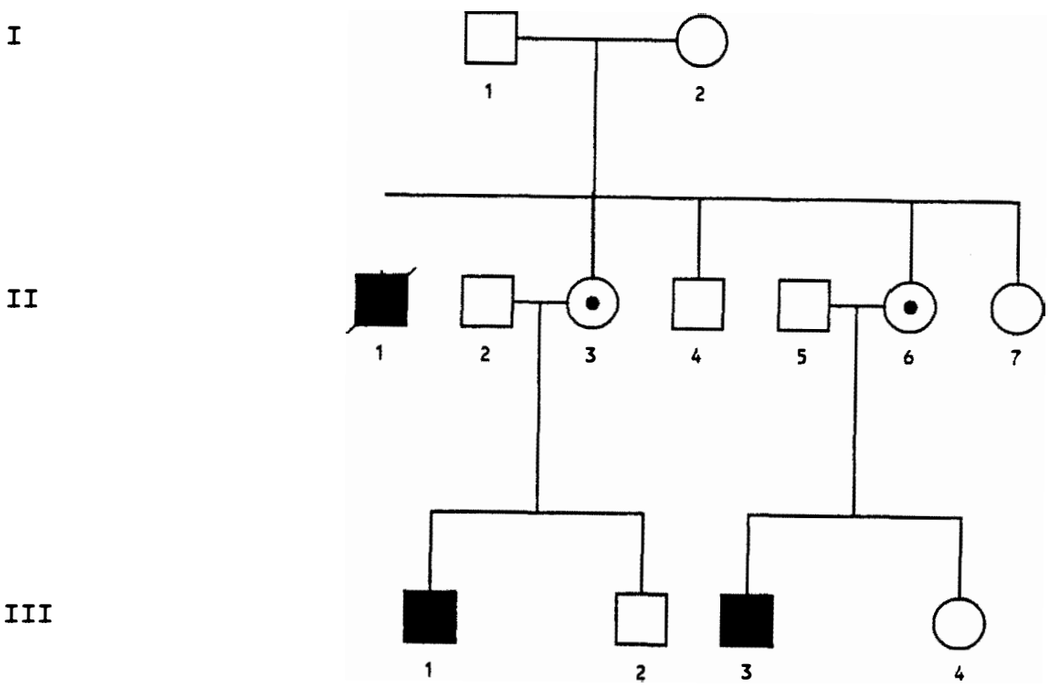

Figure V-1

Pedigree of an ideal family for haplotype analysis.

(iii) Uncertainty of the origin of the mutation in sporadic patients and the possibility of germline mosaicism.

New mutations

As discussed previously, about 30% of DMD patients have new mutations [Haldane, 1935]. This hypothesis is based on the assumption that mutation rates in male and female gametes are equal [Kunkel et al., 1986]. Although evidence for increased mutation rates in male gametes has been shown [Danieli and Barbujani, 1984; Roses, 1988; Passos-Bueno et al., 1990], most centres counselling DMD families on the results of DNA-based investigations, accept that about 70% of the mothers of DMD patients are carriers of the defective gene [Hodgson et al., 1989].

Germline mosaicism

In kindreds with an isolated patient, the origin of the dystrophin gene mutation is uncertain, since it might be new (i.e. arising in the patient) or transmitted through the germline of his mother or one of his grandparents. Carrier status in these circumstances cannot be resolved by linkage studies, but may be facilitated by the detection of a deletion. It would be expected that heterozygosity for the normal alleles of a deleted marker negates the chance of a female being a deletion carrier. It has been reported, however, that heterozygosity for the normal alleles of a

deleted marker in the lymphocytic DNA of the patient's mother, does not imply her non-carrier status, i.e. an apparently non-carrier female can have more than one affected son [Bakker et al., 1987]. This unexpected recurrence of the disorder is thought to be due to mosaicism of a deletion arising from unequal meiotic recombination in the female germline. These germline mutations are not detectable by the conventional DNA-based strategies and result in the inaccurate estimation of the carrier risk of a female who has both normal alleles at the deletion locus. Due to this phenomenon of gonadal/germline mosaicism, the risk of DMD recurring in a family where the mother is apparently not a deletion carrier, has been estimated to be between 7% and 25% [Bakker et al., 1987; Hall, 1988; Claustres et al., 1990; van Essen et al., 1992].

(iv) the inclusion of sporadically affected patients with the autosomal recessive form of DMD

The rare autosomal recessive (AR) form of DMD exists in about 8-12% of sporadic male patients who were diagnosed as having classical DMD, by conventional methods mentioned in section 1.1 [Francke et al., 1989; Vainzof et al., 1991]. The only way of identifying AR mutations is by screening the patients for dystrophin abnormalities, since the absence of a family history of the disorder cannot confirm X-linked inheritance, and the absence of a detectable deletion in the dystrophin gene does not negate the diagnosis of classical DMD. The distinction between the AR and X-linked forms of DMD have

important implications for carrier risk estimations in female relatives of the patients concerned, and may be provided by dystrophin protein analysis. However, since this technology is invasive and expensive, requiring muscle biopsy specimens, for logistical reasons, protein analysis was not implemented in the present study. Because of the possibility of misdiagnosing AR DMD as X-linked DMD, DNA-based carrier testing and prenatal diagnosis in families with an isolated DMD patient, in whom a deletion is not detected, is subject to an error rate of 8-12%.

5.2 METHODOLOGY FOR CARRIER STATUS DETERMINATION AND PRENATAL DIAGNOSIS

5.2.1 Diagnostic strategy

DNA from the patient and his family members was extracted and processed according to conventional procedures (described in the Appendix A). The probes and polymorphisms used in the family studies were those discussed in section 4.3. The diagnostic strategy was as follows:

1. DELETION SCREENING by cDNA hybridisation in the patient and his unaffected brother. The samples were digested with HindIII for screening with cDMD8, cDMD7, cDMD1-2a and cDMD2b-3. If these probes did not detect a deletion in one of the hotspots, screening continued with the following probes: cDMD4-5a, cDMD5b-6, cDMD9 and cDMD10. The same HindIII Southern blot could be probed successively with all of these probes and yielded discernable results each time. In the event of a single band being deleted in the patient's hybridisation pattern, BglII-digested DNA was probed with the cDNA subclone to determine whether the absence of the hybridising band was due to an RFLP or a deletion.

2. SOUTHERN ANALYSIS in the patient's mother, to find informative markers, was undertaken concurrently with deletion screening. The DNA samples were routinely

digested with the following enzymes: TaqI for screening with XJ1.1, pERT87-8, and pERT87-15; EcoRV for screening with P20, pERT87-1 and C7; and PstI for screening with 754.

3. HAPLOTYPE ANALYSIS in the family, using markers which detected a dystrophin deletion in the patient, and/or which were informative for linkage studies.

When molecular investigations were requested urgently, DNA from all of the key family members, necessary for the risk estimations, was probed simultaneously in order to maximise the number of markers which could be tested within the time limit.

5.2.2 Carrier risk estimations

Carrier risks in a family with DMD or BMD may be assessed on the basis of pedigree analysis, CPK estimations and DNA-based haplotype analysis. However, as mentioned previously, pedigree analysis in the family of a sporadic patient is a complicated matter and CPK levels can fluctuate due to disease processes or developmental changes in females [Hodgson et al., 1989]. For these reasons, carrier estimations based on pedigree analysis and CPK levels alone, are erroneous. In contrast, DNA-based investigations in families with a definite history of DMD/BMD are more accurate and are subject predominantly to the error of recombination events (discussed in section 5.1).

In this study, because of the reported uncertainty about the rate of recombination within the DMD gene, it was decided to allocate a 5% error in carrier risk estimations, based on the 5% recombination rate initially shown between 5' genomic probes and the mutation locus [Kunkel et al., 1985; Worton et al., 1985].

Carrier estimations in families with more than one affected relative

In a family in which members from more than one generation have the disorder, a female who has an affected son as well as other affected relatives, must be an obligate carrier. DNA analysis will not change this status and it follows that her daughters have a 50% chance of inheriting the defective dystrophin gene from their mother.

In these families, if a female inherits the same DMD haplotype as her affected brother, she has a >95% risk of being a carrier (taking into account the <5% error in analysis if recombination has occurred between the marker and the mutation site). Conversely, if the sister inherits the opposite alleles to her affected brother, the likelihood of her carrying the mutated gene is equal to the chance of crossing-over having occurred between the marker and the mutation (which is less than 5%).

If a deletion is detected in a familial patient, the presence of both normal alleles at the deleted locus in his sister, implies that she is not a carrier. Where a female is not heterozygous when it is expected that she should be because of her parents' genotype, it may be assumed that she had inherited the deleted allele and her risk of being a carrier is estimated to be close to 100% (this risk estimation can be altered, however, if the father in the molecular investigations is not the female's biological father. Incorrect paternity was not an issue in the present study). In some instances the parents' genotypes are identical, and the presence of one allele in their daughter could imply that she is either homozygous for the normal allele, or hemizygous i.e. carrying the deleted allele. In these circumstances, RFLP analysis using probes from the region flanking the deletion site will facilitate risk estimations: if the female has consistently inherited the same genotype as her affected brother, the risk of her having inherited the deletion is determined by the chance of crossing-over between the marker loci and the deletion site. Her risk of being a carrier is therefore greater than 95%; if the female has inherited the opposite alleles to her affected brother, her carrier risk is reduced to less than 5%.

Carrier risk estimations in families with a sporadic patient

As discussed previously, the mother of a sporadic affected male, has a theoretical risk of 70% of carrying the mutated

gene. On a basis of pedigree data each of her daughters therefore have a 35% risk of having inherited the mutation.

If the patient and his sister inherit the same maternal alleles, the sister's risk of being a carrier is increased to 53-57% (taking into account the 30% chance of the patient having a new mutation and the 8-12% chance of him having AR DMD). Multiple elevated CPK estimations in the female would increase her risk of carrying the dystrophin gene mutation to 70%. If her CPK level is within the normal range, her carrier risk is reduced to 20-25% (which is the probability of a carrier having a normal CPK level). Alternatively, if the patient and his sister have inherited opposite maternal alleles, the sister's carrier risk is reduced to less than 5% (i.e. the chance of crossing-over occurring between the marker loci and the mutation site).

In the family of a patient with a deletion, heterozygosity for both normal alleles in his sister would reduce the sister's carrier risk to the background risk for females in the general population. As mentioned above, where the patient's sister is not heterozygous when it is expected that she should be because of her parents' genotype, it may be assumed that she had inherited the deleted allele and her risk of being a carrier is estimated to be close to 100%.

In the case of the mother of a patient with a deletion, the reason for her homozygosity at the deleted locus (i.e. whether it is due to both normal alleles or to the presence of the null allele) could not be determined because of the limitations of dosage studies (see section 3.3.1). In this instance increased CPK levels would increase her carrier risk. CPK levels within the normal range would not change the initial risk estimation of 70% (i.e. from pedigree data). If the patient's mother is heterozygous for the normal alleles of the marker which is deleted in her son, her carrier risk is reduced to 7-25% (based on the recurrence risk due to germline mosaicism).

The above approach was used in the opposite sense if the familial/sporadic patient was deceased but a normal male sibling was available for molecular investigations. Carrier risk estimations were then based on the segregation of the normal alleles i.e. risks would be reduced if the potential carrier inherited the same haplotype as her unaffected brother, and increased if she inherited the opposite haplotype.

5.3 STUDIES OF SELECTED FAMILIES TO DEMONSTRATE THE DIAGNOSTIC STRATEGY FOR CARRIER DETERMINATION AND PRENATAL DIAGNOSIS

DNA analysis in specific families which illustrate the benefits and limitations of the molecular advances, are presented in this section.

5.3.1 Carrier detection by RFLP analysis

As discussed previously, deletion detection in females can be difficult. DNA studies in families 1 and 2 demonstrate the use of RFLP analyses to identify female deletion carriers.

Family 1 (DMD)

The pedigree of this family is shown in Fig. V-2. Genealogical data indicated that the females I-2, II-4 and II-6 were obligate carriers of DMD as they had produced affected sons. Each of their daughters (II-8, III-1 and III-3) therefore have a 50% risk, prior to DNA analysis, of carrying the defective DMD gene.

A deletion was detected in the proband, III-4, using intragenic probes (XJ1.1, pERT87-8 and pERT87-15) from the 5' end of the gene. Analysis of polymorphisms at the deletion loci revealed that the patient's mother, II-6, and his maternal aunt, II-4, were homozygous for the markers. On the

On the basis of the pattern of inheritance in this family, it was deduced that these females must have been carrying the deletion and that they were hemizygous (i.e. having one normal and one null allele) at this locus. The patient's aunt, II-8, also lacked heterozygosity for the normal alleles of these markers. Her carrier status remained uncertain until it was shown that her daughter, III-5, had inherited her respective paternal alleles but not the obligatory maternal alleles at the deleted regions. III-5 must therefore have inherited the null alleles from her mother. Similarly, III-3 had inherited the deletion from her mother, II-6. III-6 was heterozygous for the normal marker alleles at the deleted loci and clearly could not be carrying the deletion.

Molecular investigations in this family have increased the carrier risks of the patient's aunt (II-8) and sister (III-3) from 50% (based on pedigree data) to almost 100%. Similarly, the carrier status of the patient's cousin III-5 was increased and the risk of her sister III-6 was reduced to that of the general population.

Family 2 (BMD)

The pedigree of this family is shown in Fig. V-3. Pedigree data indicated that the female I-8 was an obligate carrier of the defective BMD gene since she had an affected brother, I-5, and two affected sons, II-4 and II-7. The affected male, I-5, had procreated, and his daughter, II-2, must have inherited

his X-chromosome carrying the abnormal gene and was thus an obligate carrier of the mutation.

Using intragenic probes from the 5' end of the gene, a deletion was not detected in the affected male, II-4. RFLP studies using multiple polymorphisms (754/PstI, XJ1.1, pERT87-1/MspI, pERT87-8/TaqI and C7/EcoRV) however, identified the haplotype associated with the disorder. At the loci where the obligate carrier female, I-8, was heterozygous for normal marker alleles, she transmitted the same haplotype to her unaffected son, II-8, and to her daughter, II-10. Therefore, the likelihood of II-10 being a carrier of the abnormal gene was reduced to less than 5%.

Retrospective screening of the affected male's DNA with the P20 probe and the cDNA subclones identified a deletion in the 3' hotspot of the gene. Although densitometric assessment of the hybridising bands was not possible and there was no informative RFLP at the deleted loci, prenatal diagnosis, by deletion screening, could be offered to the obligate carrier, II-2, for future pregnancies, and to her maternal aunts and their daughters, who had not been tested in this study and who are at risk of carrying the defective gene.

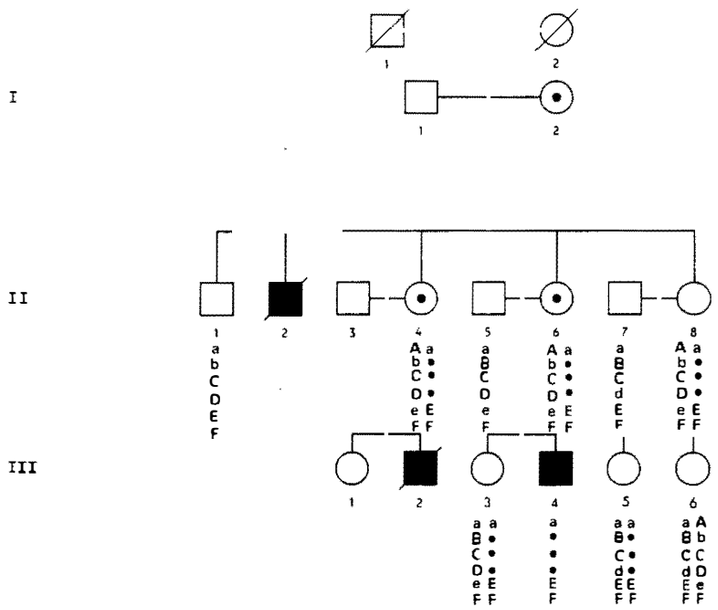

Figure V-2

Pedigree of Family 1.

The following polymorphisms are denoted

Aa = 754/PstI; Bb = XJ1.1/TaqI alleles (3,1;3,8)

Cc = pERT87-8/TaqI; Dd = pERT87-15; Ee = P20/EcoRV;

Ff = C7/EcoRV; • deleted alleles

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

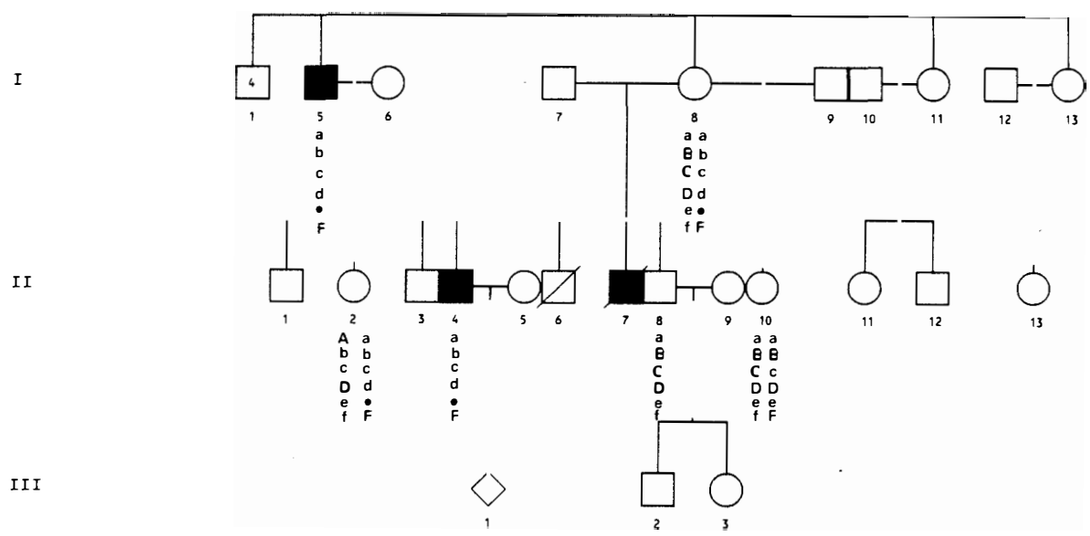

Figure V-3

Pedigree of Family 2.

Key to Fig.V-3

The following polymorphisms are denoted

Aa = 754/PstI; Bb = XJ1.1/TaqI alleles (3,1;3,8)

Cc = pERT87-1/MspI; Dd = pERT87-8/TaqI; Ee = P20/EcoRV;

Ff = C7/EcoRV; • deleted alleles.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

5.3.2 Uncertainty in carrier risk estimations due to new mutations and germline mosaicism

As mentioned previously, in families of sporadic DMD patients, predictions of carrier status in female relatives lack precision due to the difficulty in identifying the origin of the mutation.

In the present study, in families 3 and 4 where the affected males were apparently sporadic, it is theoretically possible that germline mosaicism could result in the recurrence of the disorder.

Family 3 (DMD)

The pedigree of this family is shown in Fig. V-4. The affected male, III-1, is the only known DMD patient in this family and he might therefore have a new mutation. Alternatively, his mother, II-3, might carry the mutated DMD gene.

DNA screening with the P20 probe detected a deletion in the 3' hotspot of the dystrophin gene. RFLP analysis with this probe showed that the patient's mother, II-3, was heterozygous for both marker alleles and was therefore not carrying the deletion in her lymphocytic chromosomes.

Linkage analysis using intragenic probes flanking the deleted locus (i.e. 754, XJ1.1, pERT87-8, pERT87-15 and C7) showed

that the proband had inherited his grandpaternal haplotype. The mutation therefore did not originate in his grandmother, I-2. Since the patient's mother definitely inherited a normal allele from her father at the deleted P20 locus, the mutation could not have been transmitted through his germline. The deletion must therefore either have had its origin in the germline of II-3, or in the affected proband.

In this family, the absence of a detectable deletion in the lymphocytic DNA of the potential carrier, II-3, does not imply the integrity of the corresponding gene in her germline. The estimate of a 7-25% recurrence risk for the patient's mother was based on this factor.

Despite the fact that carrier status could not be determined accurately, prenatal diagnosis by deletion screening in a male foetus could be offered with an accuracy approaching 100%.

Family 4 (DMD)

The pedigree of this family is shown in Fig. V-5. The male, III-1, is the only affected member in the family. DMD in this person could therefore be the result of a new mutation, or the result of a mutation transmitted through his maternal germline.

No deletion was detected in the DMD gene of the patient, III-1. RFLP analysis was undertaken to determine the carrier risks

of his mother (II-2), maternal aunt (II-4) and maternal grandmother (I-2). The patient's grandmother, I-2, was not informative for RFLP studies whereas her daughters, II-2 and II-4, were heterozygous for the normal alleles of multiple intragenic probes (754, pERT87-8, pERT87-15, P20 and C7). At the loci of the informative markers, the affected proband, III-1, and his sister, III-2, both inherited the grandpaternally-derived chromosome from their mother.

DNA studies showed that the disease-associated haplotype was derived from the proband's maternal grandfather, thereby reducing the risk that his grandmother, I-2, carried the defective gene. The possibility that the mutation originated in the grandfather's germline increased the carrier risk of the patient's mother, II-2, and her sister, II-4. The carrier risk of the patient's sister, III-2, was increased because she inherited the same maternal alleles as her affected brother.

When the haplotype of the patient's four year-old male cousin, III-3, was examined, it was found that he too had inherited his grandfather's haplotype. A thorough examination of the boy by an experienced clinician, confirmed that this child was not affected with DMD. His serum CPK levels were also within the normal range. It could therefore be postulated that II-4 was not a carrier.

These results indicate that the mutation could have its origin in the germline of the patient's grandfather (I-1) or in the

patient's mother (II-2), or in the patient. In the former two situations, the patient's sister has a high-risk of being a carrier. If the affected male had a new mutation, neither his mother nor sister, would be carriers.

Due to the uncertainty about the origin of the mutation in this family, accurate carrier assessment could not be offered to either the mother or sister of the affected male. Their risks were estimated to 53-57% (probability of a new mutation and of the patient having AR DMD) as outlined earlier, and they were offered counselling prior to any future pregnancies. Should these potential carriers require accurate carrier testing, dystrophin analysis in a muscle biopsy specimen would be recommended to confirm or negate their carrier status (see section 1.5). DNA analysis has, however, reduced the carrier risks of the patient's grandmother and aunt on his mother's side to less than 5%.

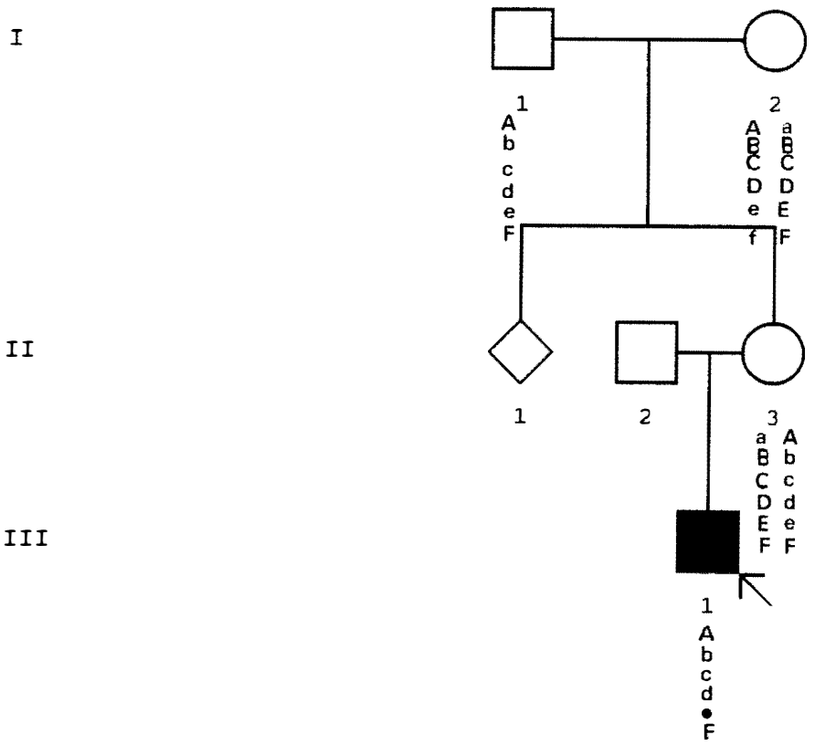

Figure V-4

Pedigree of Family 3.

Key to Fig.V-4

The following polymorphisms are denoted

Aa = 754/PstI; Bb = XJ1.1/TaqI alleles (3,1;3,8)

Cc = pERT87-8/TaqI; Dd = pERT87-15/TaqI; Ee = P20/EcoRV;

Ff = C7/EcoRV; • deleted alleles.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

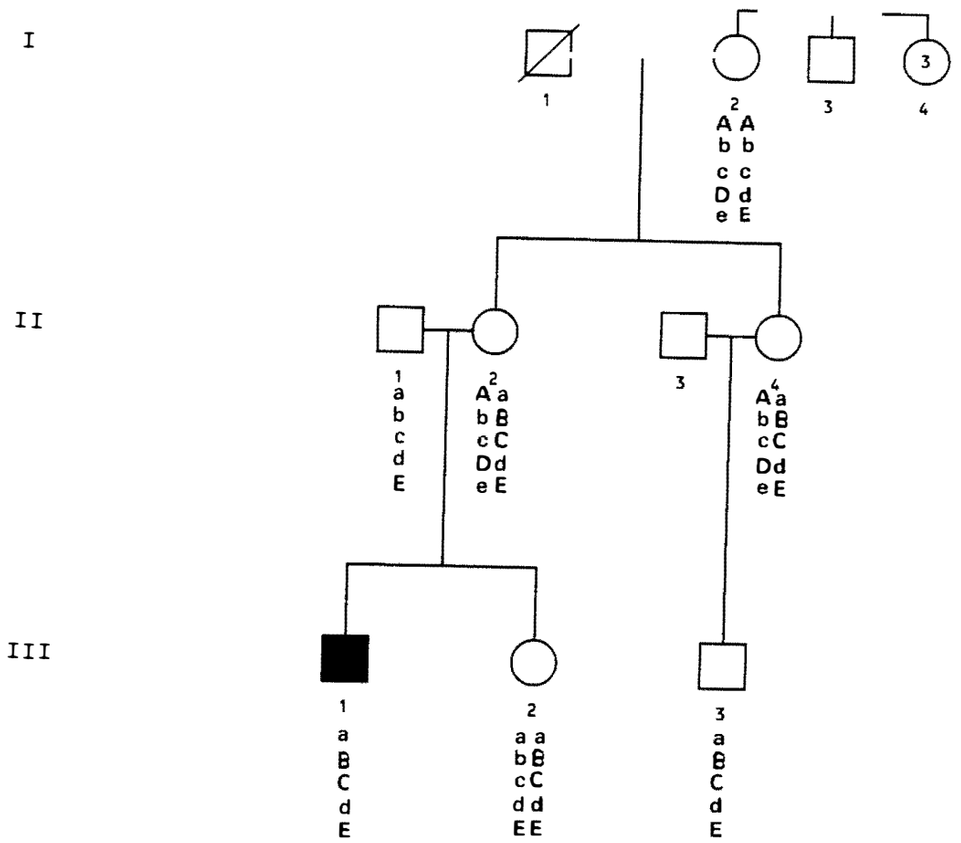

Figure V-5

Pedigree of Family 4.

Key to Fig.V-5

The following polymorphisms are denoted

Aa = 754/PstI; Bb = pERT87-8/TaqI; Cc = pERT87-15/TaqI;

Dd = P20/EcoRV; Ee = C7/EcoRV.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

5.3.3 Carrier detection by exclusion

Carrier testing is often requested by females who have a deceased affected brother. In these circumstances, an unaffected male sibling is essential for linkage analysis as he represents the marker alleles associated with the normal DMD gene. The following family exemplifies this situation.

Family 5 (DMD)

The pedigree of this family is shown in Fig. V-6. The mother (I-2) of the deceased patients, II-5 and II-10, is an obligate carrier of the defective DMD gene since she had two affected sons. Her daughters, II-2 and II-7, therefore have a 50% risk of carrying the DMD gene.

I-2 was heterozygous for the normal alleles of a number of intragenic markers as well as the flanking probes. Her daughters, II-2 and II-7, had both inherited the opposite maternal haplotype to their unaffected brother, II-8. Their other male sibling, II-2, was not available for molecular studies.

Pedigree data shows a clear history of DMD in this family and DNA analysis showed that the sisters, II-2 and II-7, had not inherited the normal gene; their carrier risks were thus increased to >95% (i.e. subject to possible error due to recombination). These females were therefore obligate carriers

of the mutated DMD gene. In instances such as these it would be useful to examine haplotypes of other unaffected males in the family as the findings would strengthen the evidence for determining the phase of the disorder.

Deletion analysis was not considered in this family because of the problems experienced in obtaining reproducible results for densitometric analysis (see section 3.3.1). Similarly, in families in which the deceased brother was a sporadic patient, DNA studies would be inconclusive because of the possibility of new mutation or germline mosaicism. In these latter circumstances, it cannot be assumed that the haplotype which is not associated with normality, segregates with the disorder. Analysis of dystrophin in a muscle biopsy specimens, would prove most useful for confirmation of carrier status.

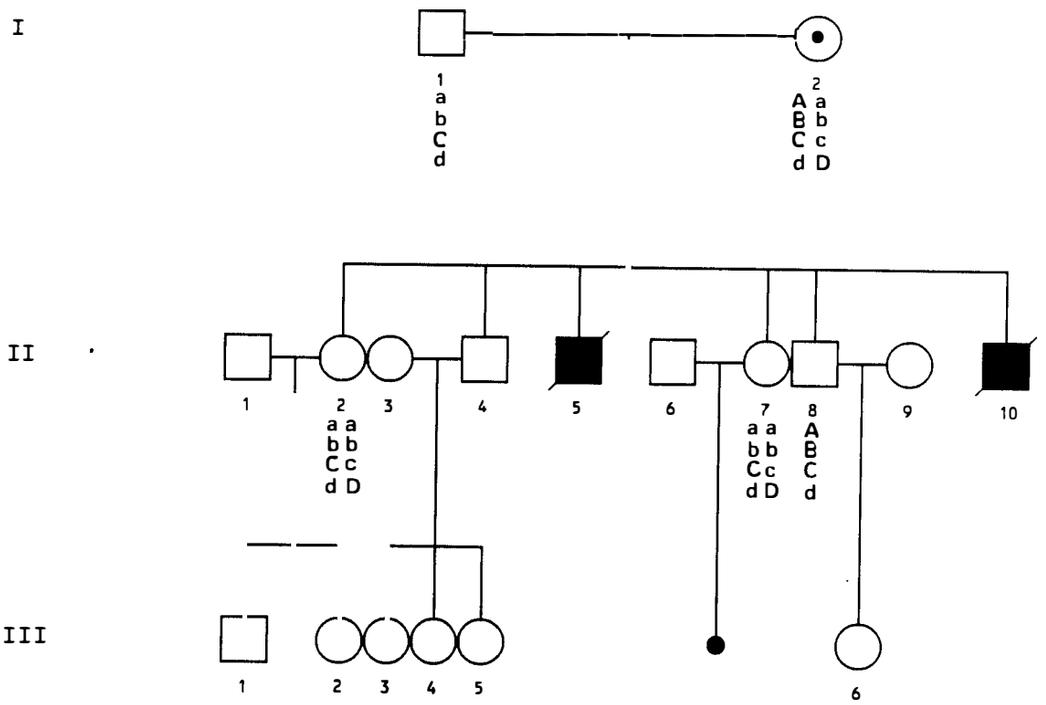

Figure V-6

Pedigree of Family 5

The following polymorphisms are denoted

Aa = XJ1.1/TaqI; Bb = pERT87-15/TaqI; Cc = P20/EcoRV;

Dd = C7/EcoRV.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

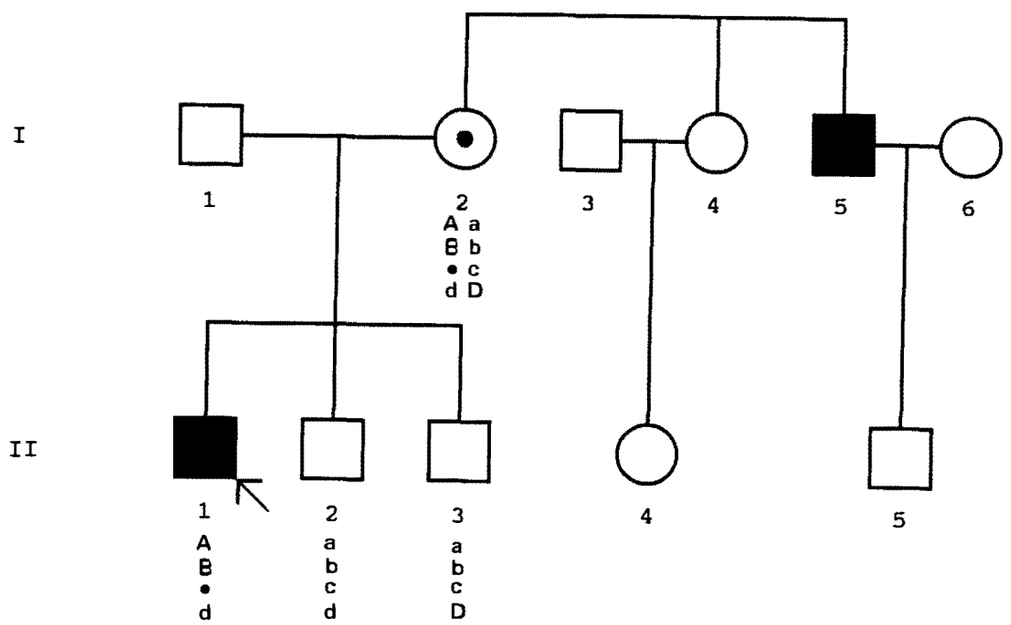

Figure V-7

Pedigree of Family 6.

The following polymorphisms are denoted

Aa = 754/PstI; Bb = pERT87-15/TaqI; Cc = P20/EcoRV;

Dd = C7/EcoRV; • deleted alleles.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

5.3.4 Presymptomatic diagnosis

In some instances, a pregnant mother does not wish to undergo invasive tests such as amniocentesis and chorion villus sampling for prenatal diagnosis, but requests molecular investigations for DMD or BMD in her newborn son. In other instances, a concerned mother notices symptoms in her young son. The latter situation is demonstrated in the following family.

Family 6 (BMD)

This family was referred for molecular investigation because the mother (I-2) of an affected male, II-1, suspected that her younger son, II-3, was exhibiting symptoms of BMD (Fig. V-7). Pedigree data indicated that the mother (I-1) of the 11 year-old patient (II-1), was an obligate carrier since she had another affected relative. Her unaffected 10 year-old son (II-2) and 5 year-old son (II-3) in whom the possible diagnosis of the disorder was being questioned, were also available for investigation.

Analysis with DNA probes detected a deletion in the 3' hotspot of II-1's dystrophin gene, encompassing the P20 intron and exon 48. This deletion was not detected in the two younger brothers of II-1. RFLP analysis using two intragenic markers, pERT87-15 and P20, and a flanking 5' marker, 754, with which the affected male's mother was heterozygous for both normal

alleles, indicated that II-1 had inherited the opposite marker alleles to his two brothers (II-2 and II-3). The patient's mother (I-2) was also heterozygous for the alleles of the 3' flanking marker, C7. At this locus, the patient and his unaffected brother, II-2, had the same marker allele. Since II-2 had consistently inherited the opposite alleles of other markers in this investigation, this inconsistency at the C7 locus indicates that a recombination event had occurred between the BMD mutation and C7. This recombination event was not, however, an issue in this family since it did not occur in the child in whom the diagnosis is being questioned (i.e. the patient's youngest brother, II-3).

Molecular investigations in this family revealed that the youngest son does not have the same mutation or the same maternal haplotype as his affected brother, and it is therefore unlikely that he has the disorder. It has recently been reported, however, that different mutations resulting in the DMD/BMD phenotype, have been detected in affected males within the same family [Miciak et al., 1992]. Although this is a rare occurrence, it was deemed necessary to recommend a thorough clinical appraisal of the youngest son in order to resolve this situation. A reliable alternative for confirmation of BMD in this young child, would be a muscle biopsy for conventional histological studies and if possible, dystrophin analysis. These techniques would unequivocally confirm or refute the diagnosis of the disorder in the boy.

5.3.5 Difficulties with haplotype assignment due to the unavailability of key family members

In DMD/BMD kindreds the unavailability of family members for molecular investigation is often a limiting factor in carrier risk estimations, regardless of the presence of a detectable deletion. The following families demonstrate the situations in which this problem arises:

Family 7 (DMD)

In this family (Fig. V-8) the mother (I-2) of the affected male, II-1, was deceased. I-2 was an obligate carrier of the faulty DMD gene by virtue of her relationship to the affected individuals, I-3 and II-1. The patient's sister, II-2, therefore has a 50% risk of carrying the mutated DMD gene.

Using intragenic and flanking markers (754, pERT87-8, pERT87-15 and C7) a haplotype could be established for the patient (II-1), his father (I-1) and his sister (II-2). The interpretation of the haplotypes is fraught with ambiguities, however, because the mother's genotype at each locus is uncertain. In this instance, RFLP studies in an unaffected male sibling would provide the missing information required to determine whether or not the sister (II-2) has inherited the maternal haplotype which is associated with the disorder.

Since II-2's father was available for study, the identification of his haplotype in his daughter facilitated the determination of her maternal haplotype. DNA analysis using a number of different markers from the dystrophin gene showed that II-2 had the same maternal alleles as her affected brother. Based on the DNA results, however, no conclusions can be drawn about II-2's carrier status as her mother might have been homozygous for all the polymorphisms investigated. In this latter situation the normal and disease-associated alleles would be the same. CPK levels in II-2 were not available for integration into carrier risk estimates.

For the reasons mentioned above, prenatal diagnosis in future pregnancies in II-2, based on haplotype analysis, would be equally ambiguous if a male foetus inherited his mother's maternal chromosome. Alternatively, if the maternal alleles in the potential carrier (II-2) were different to the patient's haplotype, she would not be a carrier of the DMD gene, unless a recombination event had occurred (which has a probability of less than 5%).

Family 8 (DMD)

The pedigree of this family is shown in Fig. V-9. The patient (II-1) must have inherited the defective DMD gene through his maternal line, because he has affected male cousins on his mother's side of the family. The patient's sister, II-2, therefore had a prior carrier risk of 50%. When II-2 requested

carrier testing, both parents were deceased, and the extended family was not available for molecular studies.

Molecular investigations were undertaken on the premise that although carrier testing could not be offered because of the unavailability of both parents, in the event of a deletion being detected in the male patient (II-1), prenatal diagnosis could be offered in future pregnancies. Carrier status risks would be unchanged, however, because of the limitations of dosage studies. If no deletion was detected in the patient, DNA studies would also be inconclusive for prenatal diagnosis.

The situation shown in family 8, would also apply in families with a sporadic affected child. In those kindreds, however, further complications are introduced in carrier risk estimations by the possibility of a new mutation.

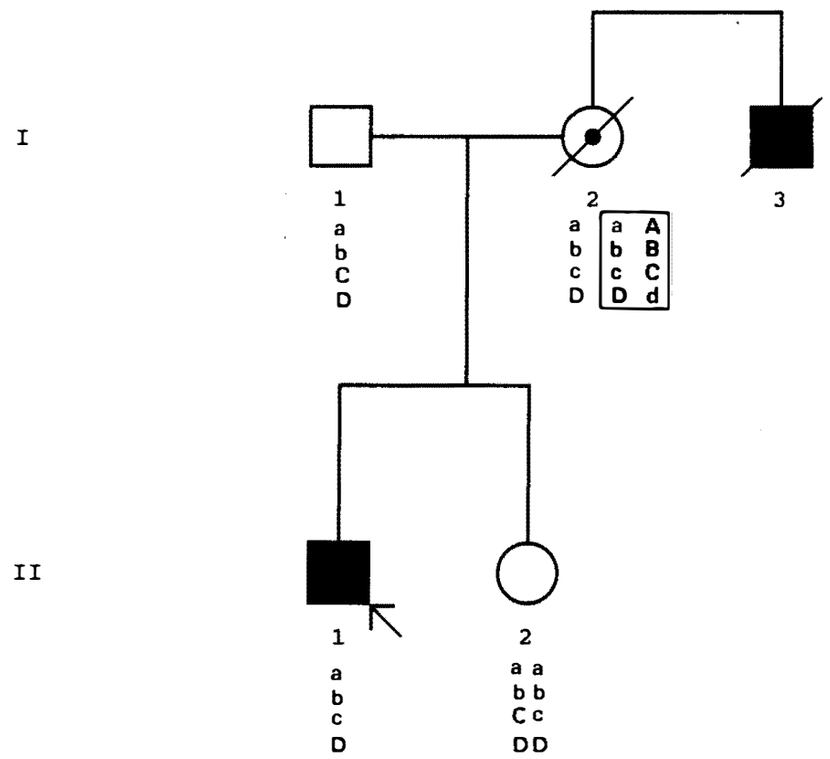

Figure V-8

Pedigree of Family 7.

The following polymorphisms are denoted

Aa = 754/PstI; Bb = pERT87-8/TaqI; Cc = pERT87-15/TaqI;

Dd = C7/EcoRV.

In each notation the upper case indicates the presence of the enzyme cutting site and lower case indicates its absence.

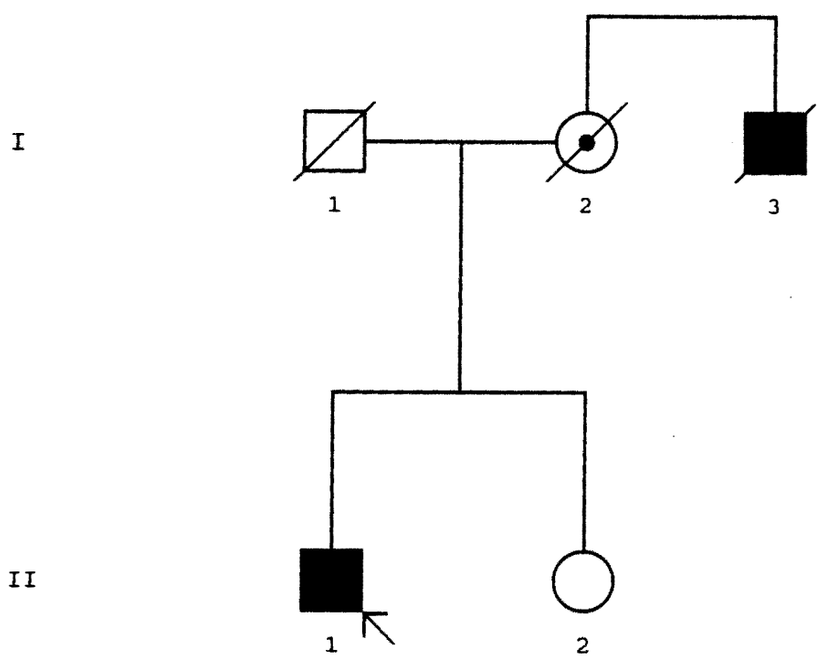

Figure V-9

Pedigree of Family 8.

5.4 THE EFFICIENCY OF DNA-BASED INVESTIGATIONS FOR THE GENETIC MANAGEMENT OF FAMILIES

Carrier risk estimations

Using the available molecular technology viz. deletion screening by Southern analysis and PCR, and haplotype analysis of RFLPs and length polymorphisms, carrier risk estimations have been provided on 230 women in families with one or more affected male relatives.

In 48 families with more than one affected relative, 43 women had an initial risk of 50% of being heterozygous gene carriers. DNA analysis reduced the risks of 16 of these females to less than 5% when they were shown to have the opposite maternal allele to their affected male relative. Conversely, a further 19 had their risk increased to more than 95%. Of the 71 second-degree female relatives in whom carrier testing was undertaken, 25 had their carrier risks increased to more than 95% when it was shown that they shared a common maternal haplotype with their affected relative. Conversely, 21 second-degree relatives had their risks reduced to less than 5% when it was shown that they inherited the opposite maternal chromosome. DNA studies were inconclusive for the remaining 25 females owing to the unavailability of key family members.

In 50 families with only one affected male, the carrier status

of the mother and sisters of the patient, was estimated to be between 53% and 57%. The risks of 63 second-degree female relatives were reduced when: (i) it was shown that the maternal grandfather's chromosome was segregating with the disorder (10 females); (ii) it was shown that they inherited the opposite maternal haplotype to the affected male (17 females); (iii) it was shown that their affected male relative and his unaffected brother shared common maternal alleles, indicating that the patient had a new mutation (20 females); (iv) it was shown that they were heterozygous for an RFLP at a locus where the patient had a deletion (16 females).

DNA analysis was inconclusive in 48 female relatives of sporadic patients in which risks were estimated to between 53% and 57% and in 36 others due to the factors mentioned in Chapter 5, including non-informativity of probes and the unavailability of key family members.

Prenatal Diagnosis

Prenatal diagnosis was requested by seven potential carriers of the abnormal DMD gene whose risks had been increased to 95% after molecular investigations. In three pregnancies the foetus was female and the pregnancy carried to term.

In three other pregnancies, DNA from male foetuses was screened for a deletion which had been detected in the dystrophin gene of the previously affected probands. All of

these male foetuses inherited the same maternal allele as their affected relative, as well as the deletion. In one of these diagnostic procedures, PCR was undertaken to identify a deletion in the 3' hotspot of the dystrophin gene. Although the deletion was detected by the PCR, the results were not released until they were confirmed by Southern blot analysis. The three sets of parents were advised that their foetuses had a 95% probability of having the disorder, and they all elected to terminate the pregnancies. Histological examination of muscle specimens from two of the abortuses showed changes compatible with the presence of a muscular dystrophy. The third family did not wish confirmatory tests to be undertaken on their aborted foetus.

In the fourth family, in which a deletion had not been detected, RFLP analysis with intragenic and flanking markers showed that the male foetus had inherited the opposite maternal alleles to his affected sibling. The foetus was therefore estimated to have a 95% probability of being unaffected. The parents accepted this finding as an indication that their child was not affected with DMD and the pregnancy is currently being carried to term.

CHAPTER 6

CONCLUDING REMARKS

The high reported incidence of Duchenne muscular dystrophy presents a major socio-economic problem in South Africa. Prior to this study, genetic management of South African families with DMD and BMD was based on increased CPK estimations (for identification of females carrying the defective gene) and the termination of male foetuses. This approach had considerable limitations due to the non-specificity of CPK estimations and the possibility of terminating a normal male foetus. In the past seven years, many advances in the understanding of the molecular basis of DMD and BMD have been made which significantly improve the approach to the management of affected kindreds.

The estimation of the frequency of DMD and BMD in the four major South African ethnic groups has revealed an increased prevalence in the Indian population and a low prevalence in the Black population. Furthermore, the frequency of BMD in the Caucasian population with respect to the observed DMD frequency, is unexpectedly high. These results might indicate the heightened awareness of the X-linked muscle disorders in some members of the South African population, compared to others. If so, there is a need for educating the general population about the implications of DMD and BMD and the measures which can be taken to prevent their recurrence within families. This is particularly important in view of the high medical costs involved in caring for an affected child.

A factor stemming from education and relating to the laboratory procedures, is the need for "at risk" female relatives to be investigated before they become pregnant. Experience gleaned from this project indicates that the conventional and reliable method of Southern analysis for deletion screening and linkage analysis, can be time-consuming and prone to technical problems. Although PCR technology is superior to Southern analysis regarding speed and its ability to detect mutations in very small quantities of DNA, the initial expenditure for primers is high. For this reason, Southern analysis is currently the method of choice in this centre and PCR analysis is used to supplement it where possible. Sufficient time is therefore required to allow for technical hitches and to permit the identification of as many informative markers as possible (in order to reduce error rates which are inherent in linkage analysis). For these reasons, a woman who is already pregnant when requesting carrier testing for the first time, subjects the laboratory to unnecessary time constraints. As a result, the likelihood of finding multiple informative markers for minimizing errors, due to recombination events, in haplotype analysis, is reduced.

Carrier risk predictions by linkage analysis and deletion screening is most accurate in families with more than one affected relative. In families with a deletion, the predictions are almost 100% accurate. In families in which a deletion is not detected, RFLP analysis is reliable but has

a low error rate as a result of the unusual characteristics of the dystrophin gene. Imprecise carrier predictions in the families with a definite familial pattern of inheritance in this study, were usually due to the unavailability of key family members for molecular investigations.

In families with an isolated case of DMD or BMD, carrier risk predictions were more variable. The major obstacle in the accuracy of carrier testing in these kindreds, was the uncertainty of the origin of the mutation and, to a lesser extent, the existence of the AR form of DMD. For this reason, DNA studies were inconclusive (i.e. risk estimates of 53-57%) for almost 50% of the potential carriers in the families with a sporadic patient in the present study. Further complications were introduced by the 7-25% risk of recurrence in pregnancies of females who did not carry a deletion in their lymphocytic chromosomes, as a result of gonadal mosaicism. It is thus forseeable that, due to these complications, linkage analysis will not prevent the birth of affected boys in all of these kindreds. Improved prospects for these families may be provided by dystrophin analysis in muscle biopsies from potential carriers and from "high risk" foetuses. Dystrophin analysis is expensive, however, and due to financial constraints, was not possible to implement in this study.

In overseas centres, computerised programs are being used to determine final carrier risk estimates, based on pedigree data, CPK levels and DNA analysis. In the present study, for

logistical reasons, computed risks were not integrated with the results of the DNA analysis. However, for a large proportion of the South African population, due to religious beliefs, moral attitudes and family influence, numerical carrier risks are not an exclusive factor when decisions are made about prenatal diagnosis and the termination of pregnancies. The small number of requests for prenatal diagnosis in this study, is a possible reflection of this situation.

The findings in this thesis show that the available molecular technology is useful for many members of the South African population. A molecular genetic service for identifying carriers and for prenatal diagnosis of DMD and BMD has proved to be feasible and, if utilised widely, will reduce the impact of these disorders for many families.

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APPENDIX A

1. *Processing of genomic DNA*

1.1 Extraction of genomic DNA from different tissues

The procedure followed was essentially as described by van der Plas et al. [1984], except where otherwise stated. DNA was isolated from whole blood or skin fibroblasts as outlined below.

i) DNA extraction from lymphocytes

30mls of red cell lysis buffer was added to 10mls of whole blood and the mixture allowed to stand for 30 minutes. The white cells were then pelleted by centrifugation at 10,000rpm for 10mins at 4°C and resuspended in white cell lysis buffer. Protein was digested by incubating the lysed nuclei with 100ug/ml proteinase K in the presence of 10ug/ml of SDS at 42°C overnight. This procedure was repeated for 1hr at 42°C. Cellular debris was then precipitated from the solution by the addition of saturated sodium acetate followed by vigorous mixing. The clear supernatant containing the nucleic acids was removed from the layer of debris after centrifugation at 6000rpm for 15mins. The DNA was precipitated by the addition of an equal volume of isoproponol, washed twice with 70% ethanol and dried on a vacuum drier. The DNA was suspended in TE buffer or water.

ii) Fibroblasts and Amniocytes

2 X 75ml flasks of confluent fibroblasts or 2 X 25ml flasks of amniocytes were trypsinized and resuspended in 10ml and 3ml respectively, of cold phosphate buffered saline (PBS). The cells were then homogenised and the same procedure followed as for lymphocytes.

1.2 Determination of concentration and purity of DNA

The DNA concentration was calculated from the absorbance in water at 260nm (1 OD unit = 50ug/ml). The ratio of the 260nm to the 280nm absorbance values was used as a criterion of purity, a value greater than 1,55 being acceptable for digestion with restriction enzymes.

1.3 Restriction Enzyme Digestion

Restriction enzymes and their reaction buffers were purchased from Boehringer Mannheim, BRL or Amersham. 7,5ug to 10ug of DNA was digested with 30 units or more of enzyme using the restriction buffer and reaction conditions recommended by the supplier. The amount of enzyme added never exceeded 1/10th the total volume, to avoid inhibiting the reaction. The enzyme was added in two equal volumes separated by a 2hr incubation at the optimum temperature. In the case of Msp1 and EcoRV, three aliquots of enzyme was required for complete

digestion. After the first aliquot of enzyme, the reaction was left at 37°C. Four hours later, the second aliquot of enzyme was added and the reaction left to proceed at room temperature overnight. The third aliquot of enzyme was then added and the reaction left at 37°C. In order to check for complete digestion, 0,25ug of the digest was electrophoresed on a mini agarose gel.

1.4 Agarose gel electrophoresis

Digested DNA samples containing 3ul of loading buffer were electrophoresed for 16 - 18hrs (for the resolution of DNA fragments between 1,2kb and 6,6kb) or for 20 - 24hrs (for resolving fragments greater than 7,0kb) at 60V. For resolving fragments greater than 6,6kb, samples were run in a 0,6% horizontal agarose gel containing 0,5ug/ml of ethidium bromide, in 1 x TBE buffer. A 1ug sample of bacteriophage lambda DNA digest with HindIII was run concurrently as a molecular weight marker. The DNA was visualised and photographed on a ultra-violet transilluminator and the migration of the marker fragments were recorded.

Failure of the DNA digest to leave the well in the agarose gel during electrophoresis, indicates incomplete digestion with the restriction enzyme. In those cases where the DNA failed to digest with the enzyme, the DNA was cleaned with phenol, followed by a

chloroform/octanol wash. Sodium acetate was added and the DNA precipitated with absolute ethanol. The DNA was washed with 70% ethanol, dried under vacuum and reconstituted.

1.5 DNA Transfer

Initially Hybond N nylon membrane supplied by Amersham was standardly used for DNA transfer and the buffer used for transfer was 20 x SSC. More recently, Hybond N+ has become the membrane of choice and the recommended transfer buffer is 0,4M NaOH [Amersham]. A blotting bridge was prepared by placing a glass plate, the size of the gel, on top of four small glass vials in a glass dish containing 0,4M NaOH. A wick was cut of 3MM chromatography paper which was laid on the glass so that the ends of the paper dipped into the transfer buffer. The wick was wetted with 0,4M NaOH and topped with three sheets of chromatography paper, the size of the glass plate, which had been soaked in 0,4M NaOH. The gel was then layered carefully onto the bridge and covered with a sheet of Hybond N+. Two layers of wet chromatography paper was placed on top followed by a pack of paper towels and a glass plate. A cover brick was used to weight down the paper towel and the blot was left at room temperature overnight or at 4°C over the weekend.

In those instances when DNA fragments larger than 6,6kb were to be transferred, before blotting the DNA was acid-nicked by gently shaking the gel in 0,25N HCl for 15mins, followed by a 30min wash in 0,4M NaOH. The nylon membrane was rinsed briefly by floating on a layer of 3 x SSC covered with saran wrap and stored at 4°C until hybridisation.

2. Processing of plasmid DNA

The cDNA subclones were obtained in lyophilised form. The dried cells were resuspended in Luria-Bertani (LB) broth and an aliquot amplified in LB overnight. Other probes were received either as stab cultures which were processed as described in Maniatis, or as small amounts of DNA which were transformed into competent E.coli cells as described below.

2.1 Preparation of Competent cells

Initially, competent E.coli cells were prepared by inoculating 100ml of LB broth with 1ml of an overnight culture of the E.coli strain HB101. The LB broth was vigorously shaken at 37°C until the absorbance at 550nm read 0,5 (about 3hrs). The culture was chilled for 10mins, then centrifuged at 7 000rpm for 5mins at 4°C. The cell pellet was gently resuspended in 12,5ml of ice cold 50mM CaCl /10mM Tris pH 8. After 15mins on ice, the suspension was centrifuged at 7 000rpm. The cell pellet

was resuspended in 1,7ml of ice cold CaCl/ Tris solution an stored.

More recently, competent cells prepared by the method of Chung and Miller [1989] was found to be more efficient and became the method of choice.

All pipette tips and plastic tubes were chilled before use. Bacterial cells were grown in LB broth for about two hours at 37°C with the appropriate antibiotic until an OD 600 = 0,3 to 0,6. The cells were pelleted by centrifugation at 7000rpm for 5mins at 4°C. The cell pellet was resuspended in 10ml of chilled TSB and incubated on ice for 10mins. The cells were then either stored at -70°C or transformed immediately.

2.2 Transformation

A 0,1ml aliquot of competent cells were added to 100pg of plasmid DNA and the suspension left on ice for 15mins. 0,9ml of TSB, warmed to 37°C, was added with 20mM glucose and the cells incubated on a shaker at 37°C for 1hr to allow the antibiotic resistent gene to be expressed. Aliquots (50ul, 100ul and 200ul) were plated on agar plates containing the appropriate antibiotic to select transformed cells.

2.3 Preparation of glycerol stocks

A single bacterial colony was suspended in 10ml of LB broth containing the appropriate antibiotic and cultured overnight on a shaker at 37°C . 0,15ml glycerol was added to 0,85ml of the overnight culture and the mixture thoroughly vortexed. The glycerol stocks were stored at -70°C and refreshed annually.

2.4 Isolation of Probe DNA

(Initially, a modification of the Greenway and Dale method [1983] was followed).

The host bacteria were amplified by culturing 10ul of a glycerol stock in 5ml of LB broth overnight on a shaker at 37°C. The culture was further amplified in 600ml LB broth overnight, shaking vigorously at 37°C. The cells were pelleted by centrifugation at 7 000rpm for 2 hrs at 2°C. The cell pellet was resuspended (on ice) in a solution containing 25% sucrose/ 0,05M Tris. The cells were lysed by the addition of a 3mg/ml solution of lysozyme in 25% sucrose/0,05M Tris. 0.25M EDTA was added and complete lysis effected by by gradual addition of a Triton/DOC solution. After a 20min incubation on ice, the suspension was mixed and NaCl added to a final concentration of 0,2M. Protein contaminants were pelleted by centrifugation at 15 000rpm for 45mins at

4°C. The clear supernatant was accurately measured and 1 X T.E. added to a volume of 30ml or 60ml. 0,04% ethidium bromide was added and caesium chloride (CsCl) was added to a density of 1,6g/ml. the circular plasmid DNA was separated from the chromosomal DNA by centrifugation at 50 000 rpm for 20hrs at 18°C in a Beckman ultracentrifuge. The lower plasmid DNA band was visualised under ultraviolet light and removed. The ethidium bromide was extracted with amyl alcohol and the CsCl removed by overnight dialysis in 1 X T.E. at 4°C. Two volumes of cold ethanol was added and the plasmid DNA allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation at 15 000rpm for an hour at 4°C, the pellet dried and dissolved in water to yield a concentration suitable for labelling with radioisotopes.

More recently, a rapid kit method, Qiagen, has replaced the above procedure. The protocol described in the Qiagen booklet was followed.

After amplifying bacterial cells overnight, the cells were pelleted by centrifugation at 7000rpm for 30mins and resuspended in solution 1. The suspension was gently mixed with solution 2 and incubated at room temperature for 5mins. A solution of 2,5M Potassium Acetate pH4,8 was then added followed by immediate mixing to avoid precipitation of potassium dodecyl sulphate.

The mixture was centrifuged at 15000rpm for 30mins at 4°C. The clear supernatant was applied to an anion-exchange minicolumn containing a silica gel with a particle size of about 100um. This permits a wide range for elution and thus yields up to 90% recovery of probe DNA. The column had been equilibrated with solution 3. The supernatant was washed with a low salt solution and the DNA eluted with solution 4. The DNA was precipitated with isopropanol at room temperature for 2mins and centrifuged for 30mins at 15000rpm. The DNA was washed with 70% ethanol, vacuum dried and dissolved in water.

2.5 Extraction of Probe Insert

Plasmid DNA was digested with the appropriate enzyme to release the insert. The DNA fragments were separated on an agarose gel containing ethidium bromide. The insert was visualised on a u-v transilluminator and excised with a surgical blade, trimming close to the band. The band was placed in an eppendorf tube, the bottom of which had been pierced with a needle and plugged with glasswool and the agarose block frozen at -70°C for 3hrs. The tube was spun at 15 000rpm for 5mins to allow the DNA to elute out of the gel matrix and through the glasswool into a larger tube.

The DNA was precipitated with 1/10 th volume of 3M sodium acetate and 2 volumes of absolute ethanol, washed twice with 70% ethanol, vacuum-dried and reconstituted in water.

2.6 Labelling of probes with radioisotopes

a) The multiprime DNA labelling system of Amersham was used.

50ng of double-stranded probe insert (in a total volume of 10ul) was denatured by boiling for 3mins and rapid chilling for 10mins. The following reagents were added to the 50ng of DNA:

20ul nucleotide buffer
10ul primer
7,5ul a-P32 dCTP (3 000 Ci/mmol)
4ul Klenow enzyme
H_2O to a total volume of 100ul

The reaction was left to proceed overnight at room temperature and was stopped by separating the bound radioisotope from the unbound. This was done by layering the reaction mixture onto a 3ml sephadex G-50 column. The fractions were eluted by washing the column through with 200ul aliquots of 3 x SSC. In this way, ten fractions were collected and the radioactivity measured in a scintillation counter (Packard Instruments). The first peak of

radioactivity was pooled (fractions 3-6) and used for hybridisation.

b) The alternative method used for labelling plasmid containing the probe insert, was the Nick Translation method as described by Amersham.

The following solutions were added to a tube:
10ul probe DNA (0,25ug -0,5ug)
5ul buffer
2,5ul nucleotide buffer
2,5ul enzyme
5ul water

The reaction was allowed to proceed at 15°C for 1-2hrs, then stopped by adding the reaction mixture to a sephadex G-50 column as decribed above.

2.7 Preannealing of probes

Plasmids and cosmids containing repeat sequences were preannealed to human placental DNA or Cot DNA to minimise cross hybridisation.

Before hybridisation, the labelled probe was prehybridised to sonicated human placental DNA (250ul of a 2,5mg/ml solution) for 2hrs at 65°C.

2.8 Southern-blot Hybridisations

The procedure followed was essentially that of van den Plas et al [1984]. Modifications were as described by Johnson et al [1984] who showed that non-fat dried milk (Blotto) could be used as an efficient and economical substitute for Denhardt's solution.

Southern blots were prewashed at 65°C for 2-16hrs in a solution of 1M sodium chloride/ 1% SDS/ 0,25% Blotto/ 5% dextran sulphate/ 100ug/ml sonicated salmon sperm.

The prewash was replaced with 10ml of fresh prewash containing freshly denatured labelled probe and hybridisation was allowed to proceed at 65°C for 1-2 days. After hybridisation, the blots were washed with the following solutions:

3 x SSC/ 0,5% SDS	10mins/R.T.
3 X SSC/ 0,5% SDS	30mins/65°C
0,6 X SSC/ 0,3% SDS	30mins/65°C

At this stage the blot was laid out on a sheet of plastic and the radioactivity signal monitored with a geiger counter. An increased signal warranted a last stringent wash in 0,1 x SSC/0,5% SDS for 5-10mins. The filter was sealed between plastic sheets and exposed to Dupont Cronex-4 X-ray film using intensifying screens, at -70°C for 2-5 days.

2.9 Removal of labelled probes

The bound probe was removed by incubating the membranes for 30mins in 0,4M NaOH at 45°C, followed by neutralisation in a solution of 0,1X SSC/ 0,1% SDS/0,2M Tris HCl pH 7,5. The membranes were sealed between plastic and stored at 4°C until further use.

APPENDIX B

Loading buffer for agarose gels

0,25% bromophenol blue/40% (w/v) sucrose in water

Loading buffer for polyacrylamide gels

0,25% bromophenol blue/0,25% xylene cyanol FF/40% (w/v) sucrose

Luria-Bertani broth

0,2% glucose/1% tryptone/0,5% NaCl/0,5% yeast

Lysis buffer (Red cell)

320mM sucrose/ 1% (v/v) triton X-100/ 5mM $MgCl_2$/10mM TrisHCl pH7.6

Lysis buffer (White cell)

25mM EDTA pH8.0/ 75mM NaCl

PCR buffer (1 X)

50mM KCl/10mM Tris pH8.4/1,5-2,5mM $MgCl_2$

Polyacrylamide gels (denaturing)

6,5-8% acrylamide/bis-acrylamide solution

50% urea/1 X TBE

Sephadex G-50

5% sephadex G-50 in water. Autoclave and cool.

SSC (20 X) pH 7.0

3M NaCl/ 0,3M sodium citrate

T.B.E. (10 X) pH8.0

0,89M Tris/ 0,89M Boric acid/ 0,02M EDTA

T.E. (100 X) pH8.0

1M TrisHCl/ 0,1M EDTA

Triton/DOC

0,5% Triton/ 50mM TrisHCl pH8.0/ 6,25mM EDTA/ 0,4% sodium deoxycholate

T.S.B. buffer

LB broth containing 10% polyethylene glycol (MW=3,350) , 5% dimethyl sulphoxide and 20mM Mg^{2+}(10mM $MgCl_2$ + 10mM $MgSO_4$)