DELETION SCREENING
AND
POINT MUTATION ANALYSIS
IN REGIONS
OF THE
DUCHENNE / BECKER MUSCULAR DYSTROPHY GENE.

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ΣΤΟΥΣ ΓΟΝΕΙΣ ΜΟΥ ΚΑΙ

ΣΤΟΝ ΑΔΕΛΦΟ ΜΟΥ.

Εσυ δεν θα μου πεις ευχαριστω,
οπως δε λες ευχαριστω στους χτυπους της καρδιας σου
που σμιλευουν το προσωπο της ζωης σου.

Ομως εγω θα σου πω ευχαριστω
γιατι γνωριζω τι σου οφειλω.

Γ. Ριτσος

You won't say thanks to me
just as you don't say thanks to your heartbeats
carving out the face of your life.

But I will say thanks to you
because I know what I owe you.

Y. Ritsos

SUMMARY

Duchenne muscular dystrophy (DMD) is the commonest dystrophy with a birth incidence of one in 3000-3500 males, and approximately one third of all cases result from a new mutation. Affected males present with progressive muscle weakness and die as a result of respiratory or cardiac involvement in their late teens or early twenties. Becker muscular dystrophy (BMD) is a mild variety of the disease, and the most useful criterion for differentiating the two types is the age when patients become wheelchair bound.

The gene is located on Xp21, and consists of more than 65 exons encoding a protein 3685 amino acids long, named dystrophin. About 65% of affected boys have a small submicroscopic gene deletion, heterogeneous in both the specific region and number of exons missing, that can be detected by Southern blot analysis using cDNA probes, and/or by sequence amplification with the polymerase chain reaction. Partial gene duplication accounts for the mutation in about 6% of the patients, while in the remainder point mutations are suspected

An aim of this project was to screen DMD/BMD patients for deletions in exons 30 to 47 of the dystrophin gene covered by cDNA probe 5b-7, on BglII digested DNA, and at the 3' end of the gene using the restriction enzymes Hind III and Bgl II in combination with cDNA probes 9, 10 and 11-14.

Seventeen DMD/BMD patients were studied with cDNA 5b-7 and one deletion was detected having the distal end at exon 44 while the proximal end was in exon 20, in the region of cDNA probe 4-5a. Five cDNA deletions were detected having both end-points in the distal part of the dystrophin gene in a panel of thirty-six DMD/BMD affected males showing no deletion or duplication with the rest of the cDNA probes. Also, two cases of deletions which started in the region of cDNA 8 were identified to extend in the region of cDNA 9, with one of the two having the 3' end into the region of cDNA 10. No deletions were found with cDNA 11-14. Taking advantage of the different size of deletions detected, some of the Bgl II genomic fragments were related to Hind III genomic fragments of known orientation, and the order of the Hind III fragments in the region of cDNA 10 was rearranged. Also, the correlation between deletion and phenotype was examined and found to fit the reading-frame model proposed to explain the clinical difference in severity between DMD and BMD patients. For diagnostic purposes the detection of the molecular pathology of the disease can confirm the diagnosis of DMD/BMD in sporadic cases and offer direct accurate prenatal diagnosis in the family without the necessity for linkage analysis that requires DNA samples from key relatives.

As Southern analysis takes five to ten days for results to be obtained assessment of the value and reliability of the polymerase chain reaction in performing deletion detection screening at multiple sites simultaneously was the next aim of the present study.

Simultaneous amplification by PCR of exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 of the dystrophin gene in 118 DMD/BMD DNA samples identified a mutation in 48.3% of the patients and detected 86% of the deletions previously revealed by Southern analysis. Discrepancies were not found between the results obtained by the two methods of analysis. On the contrary, the problem of weak hybridisation was circumvented in two cases allowing the accurate mapping of the deletion end-points. Postnatal-detection screening was also performed in two patients and both results were confirmed by Southern analysis. The ability of PCR to amplify DNA fragments from small starting amounts of not necessarily good quality DNA permitted the amplification of DNA extracted from haematoxylin and eosin stained tissue sections, as well as the detection of a deletion in an individual whose DNA failed to produce detectable signal by Southern analysis because of degradation. Speed, sensitivity, specificity and efficiency characterise the method of multiplex amplification with the polymerase chain reaction, and make it ideal in the analysis of mutations in routine clinical practice as an initial screen to detect the molecular pathology of the disease.

The final aim of this project was to scan regions of the DMD/BMD gene in affected males whose molecular pathology was still unknown, for new polymorphic sites or mutations that may account for the development of the disease and to compare and assess the different methodologies for mutational screening.

The techniques of single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and chemical cleavage of the mismatch (AMD) were applied on material generated by the polymerase chain reaction. PCR-SSCP analysis and PCR-DGGE analysis were applied on sequences flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 of the dystrophin gene in thirty-nine DMD/BMD patients. PCR-SSCP analysis identified a polymorphism at the distal part of intron 44, a known hot spot for recombination in the DMD/BMD locus. Asymmetric PCR and direct sequencing reveal a single nucleotide substitution (G to A), which was also present in nine of thirty-seven unrelated male controls screened by the same method. This sequence variation was identifiable by the DGGE approach, and it was also confirmed by the technique of chemical cleavage of the mismatch after hydroxylamine modification of the DNA. No other sequence alteration was detected by either PCR-SSCP analysis or DGGE. This was not an unexpected result considering the limited size of the region examined (approximately 3.5 kb of genomic sequence, 1500 base pairs of exonic sequence), compared to the genomic size of the locus (2 Mb).

PCR-SSCP analysis proved to be a rather simple and fast though isotopic method, with no specific requirement for special equipment. No false-positives were observed, but false negatives were not excluded. For the same length of fragments the non-isotopic PCR-DGGE approach for mutation detection can be used which however requires special equipment for the

generation of a temperature gradient in the gel. Also the identification of single-base mutations by means of DGGE is restricted to detection of mutations in low melting domains of the DNA fragments, unless a "GC-clamp" of up to 40 bp is incorporated in at least one direction during the amplification step. However, both methods have the disadvantage of failing to localise the variable site. The only information gained is whether there is at least one site of sequence variation in the region examined. This information can be obtained by the method of chemical cleavage of the mismatch. The main drawback of this method is the use of highly toxic, dangerous to handle and difficult to dispose of chemicals, but it does provide positional information on the mutation. Whatever the screening approach is, final confirmation requires sequencing of the corresponding area.

These screening strategies together with direct sequencing could be applied to detect the molecular pathology in DMD/BMD cases where multiplex PCR gave a normal result. An approach which uses transcribed cDNA from abnormal transcript of dystrophin in lymphocytes to reduce the DNA screening target from 2 MB to 14 kb allowing analysis without muscle biopsy has recently been described. In turn these results will provide further information for the molecular pathology of the disease and they will be of direct benefit for the improvement of genetic counselling within affected families.

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LIST OF ABBREVIATIONS

aa	amino acid
AMD	amplification mismatch detection
ATP	adenosine tri-phosphate
BMD	Becker Muscular Dystrophy
bp	base pairs
cDNA	complementary copy DNA
CFTR	cysti fibrosis transmembrane conductance regulator
CVS	chorionic villus sampling
dATP	2'deoxyadenosine 5'-triphosphate
dCTP	2'deoxycytodine 5'-triphosphate
dGTP	2'deoxyguanidine 5'-triphosphate
DGGE	denaturing gradient gel electrophoresis
DMD	Duchenne Muscular Dystrophy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double strand DNA
dTTP	2'deoxythymidine 5'-triphosphate
EDTA	ethylene diamine tetra-acetic acid
et al.	et alia
g	gram
H&E	Haematoxylin and Eosin
kb	kilo-base
l	litre
M	molar
Mb	mega-basepair
mRNA	messenger ribonucleic acid
n	nano
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PERT	phenol enhanced reassociation technique
RFLP	restriction fragment length polymorphism
rev/m	revolutions per minute
SCK	serum creatine kinase
SSC	standard saline citrate
ssDNA	single strand DNA
SDS	sodium dodecyl sulphate
SSCP	single strand conformation polymorphism

Taq	Thermus aquaticus
Tris	tris (hydroxymethyl) aminoethane
UV	ultra violet
Xp	short arm of the X chromosome
K	1000 rev/min

CHAPTER I

INTRODUCTION

1.1 Frequency of Duchenne Muscular Dystrophy.

Duchenne muscular dystrophy (DMD) is an X-linked recessive trait with a birth incidence of one in 3000-3500 males (Emery, 1988). Despite increasing awareness of the disease in recent years there has been no apparent increase in the number of reported cases. Some reduction in the incidence of Duchenne muscular dystrophy though, can be expected due to the increasing application of prenatal diagnosis, carrier detection and genetic counselling. Assuming that the male and female mutation rate for recessive X-linked lethal disorders is equal, and that the reproductive viability of the patients is virtually zero, the mutation rate is estimated to be 10^{-4} per generation and one third of all patients should result from a new mutation (Haldane, 1935).

1.2 Clinical Features of Duchenne and Becker Muscular Dystrophies. Confirmation of the Diagnosis.

Duchenne Muscular Dystrophy (DMD) is named after Guillaume Benjamin Amand Duchenne or Duchenne of Boulogne as he signed himself. He was the first to study the histology of affected muscles from the same patient at different stages of the disease using his needle-harpoon to obtain biopsy specimens in life, and the first to talk about an X-linked inheritance pattern (Duchenne, 1868). The clinical features of the disease were presented in detail later by William R. Gowers(1879), who described what is nowadays usually referred as Gowers' manoeuvre.

Walking is delayed until at least 18 months in 56% of DMD and roughly a quarter do not walk until they are at least 2 years old, whereas of normal children the average age in learning to walk is about 13 months, and 97% are walking by 18 months (Emery, 1988).

The most obvious feature in the early stages of the disease is enlargement of the calf muscles which at least in part is due to an excess of adipose and connective tissue. However, such enlargement may also involve the masseters, deltoids, serrati anterior and quadriceps, and occasionally other muscles. Macroglossia is not uncommon. Muscle involvement is always bilateral and symmetrical. In general, in the early stages of the disease, the lower limbs are affected more than the upper limbs, and the proximal muscles more than the distal muscles. This pattern becomes less clear as the disease progresses. Later slight facial weakness often develops and the intercostal muscles also become affected, but sphincter control, chewing, and swallowing are never affected. This pattern of involvement results in several well defined

physical features associated with the disease such as the waddling gait which is due to pelvic girdle weakness and shortening of the Achilles tendons, and the classical Gowers' manoeuvre. Severe kyphoscoliosis and cardiac involvement are common. A proportion have some impairment of intellect. The reduction of IQ is not due to any lack of educational opportunity as a result of physical disability because it is not found in other diseases with comparable disability. A probable explanation is that the reduction in IQ is a direct effect of the mutant gene (Emery, 1988).

The age of onset is before school age in 90% of DMD cases, whilst the age when patients become wheelchair bound is before 12 years of age in 95%. Age at becoming confined to a wheelchair is significantly correlated with age at death. It seems that age of death after 15 increases roughly by one year for each year that a boy remains ambulant after the age of 7 up to the age of 10 or more (Emery, 1988)

Becker Muscular Dystrophy (BMD) was first clearly delineated by Becker in 1955. The distribution of muscle wasting and weakness is very similar to Duchenne muscular dystrophy. The serum creatine kinase (SCK) level is raised to levels found in boys with Duchenne muscular dystrophy even in the preclinical stage of the disease which may last 10 years or more, when there is no apparent muscle weakness. The age of onset is after the age of 5.3 years for 88.1% and 93.7% die after the age of 21.1 years (Emery, 1988). The most useful criterion for differentiating Duchenne muscular dystrophy from the milder form, Becker muscular dystrophy is the age when patients become wheelchair bound which is after the age of 12.4 years for 96% of males with BMD (Emery, 1988). Diagnosis of DMD/BMD based on the clinical characteristics can be further supported by measuring the serum level of creatine kinase, and by muscle biopsy.

The serum creatine kinase (SCK) activity is raised considerably, up to a hundred times, in patients with DMD/BMD even at birth and before the disease becomes clinically evident (Dreyfus et al., 1960). The most likely explanation for the very high SCK levels in DMD is that the enzyme originates in muscle and escapes into the serum. The decrease in functioning muscle tissue and reduction in physical activity when patients become confined to a wheelchair are probably responsible for the much lower SCK levels in the later stages of the disease (Emery, 1988).

Muscle pathology is altered before there are any obvious clinical manifestations of the disease. While in normal muscle the eosinophilic fibres are absent or very infrequent, they are increased in number in the affected tissue and they contain higher amount of intracellular calcium (Emery and Burt, 1980). At later stages and as the muscle fibres undergo necrosis and are phagocytosed, they are replaced by fat and connective tissue so that eventually only small

proportion of muscle tissue remains (Cullen and Mastaglia, 1980).

1.3 Presence of the DMD/BMD gene in Females.

Over twenty females have been described with DMD/BMD who have X-autosome translocations that break the X chromosome within band Xp21 (Boyd et al., 1986). There is a considerable variation in clinical severity in girls and it seems possible that the phenotype may well depend on the proportion of cells in which the derived X is active. Several of these translocations have been mapped with genomic probes throughout the DMD gene. Bodrug et al. (1989) localised four translocations: t(X;5), t(X;11), t(X;1) and t(X;21) to specific introns using a cDNA clone from the 5'end of the DMD/BMD gene. Several mechanisms have been proposed by which these translocations might arise. It is possible that a specific DNA sequence such as an Alu repeat, which is involved in a homologous exchange between elements on non-homologous chromosomes is required for translocation (Bodrug et al., 1989). Alternatively, the mechanism could involve a less defined factor such as a variation in chromatic configuration leading to an increased probability of rearrangement. This possibility is supported by the fact that some of the DMD translocation breakpoints involve autosomal regions coincident with known fragile sites (Boyd et al., 1986).

Females who carry the gene in heterozygous state (carriers) are essentially healthy. Only in about 2.5% of carriers is some degree of muscle weakness present as the result of inactivation of a higher proportion of those X-chromosomes bearing the normal gene (Norman et al., 1989). The detection of healthy female carriers is therefore a major problem. A woman with two affected sons or with one affected son and another affected close male relative is considered to be an obligate carrier, having a one in four chance of having a child with Duchenne muscular dystrophy. In cases of possible or probable carriers this genetic risk can be reduced by estimating the level of serum creatine kinase, which is known to be elevated in some mothers of sons with Duchenne muscular dystrophy (Emery, 1965). There are a few precautions in using the SCK levels such as: a) the effect of exercise (Hudgson et al., 1967). High SCK levels can occur in normal female subjects after prolonged exercise. b) the effect of pregnancy (Emery and King, 1971). The SCK levels are lowered by pregnancy making carrier detection unreliable. c) the effect of age (Lane and Roses, 1981). In normal premenarchal girls the SCK levels are also higher. When the SCK values for both controls and carriers are logged they have a normal distribution. Because the two curves overlap a proportion of carriers cannot be detected as the values fall both into the tail of the carrier curve as well as into the normal control curve. This could be explained by the occasional non-random inactivation of the X-chromosome carrying the normal allele, instead of the expected random inactivation of either X-chromosome as predicted by the Lyon hypothesis (Lyon, 1961). Carrier detection based only on elevated SCK levels gives a false negative result in about 40% of cases (Bullock et al., 1979). It is very

important therefore to assess the probability of a woman being a carrier using Bayesian statistics where a combination of pedigree information, SCK levels and when available DNA data, which are not affected by X-inactivation are taken into account. This is particularly difficult when only one affected male occurs in the family. It is believed that about one third of sporadic DMD patients arise as a result of new mutations and are sons of non-carriers (Davie and Emery, 1978)

1.4 Prenatal Diagnosis.

In the past a woman at risk of having an affected son with Duchenne or Becker muscular dystrophy could choose fetal sexing with selective abortion of any male fetus, and in this way was guaranteed a daughter who would not be affected. However that was unsatisfactory as at least half of the aborted fetuses would in fact be normal. The development of recombinant DNA technology has opened up an entirely different approach to prenatal diagnosis. Fetal DNA extracted from either fetal tissue obtained by chorionic villus sampling (CVS) at around ten weeks gestation, or from amniotic fluid cells obtained by transabdominal amniocentesis at about 16 to 18 weeks of gestation could be analysed using a closely linked DNA marker, or after the gene was isolated a gene specific probe to determine the probability of the fetus being affected.

Linkage analysis uses genetic markers in the form of restriction fragment length polymorphisms (RFLPs) detected by DNA probes with restriction endonuclease analysis. The RFLPs are naturally occurring genetic polymorphisms at the level of DNA sequence that can be used as genetic markers throughout the genome. This type of analysis tests the segregation, in a particular family, of DNA markers physically located near (flanking) or within the dystrophin gene (intragenic) and less for the gene mutation itself. Thus, the use of several markers in developing the haplotypes of many family members is necessary to increase the likelihood of a family being informative and reduce the probability of error due to crossing over. Xp21 probes have an estimated recombination rate of 5% (Davies, 1986; Old and Davies, 1986).

Several disorders such as various congenital myopathies and spinal muscular atrophy, with a different pattern of inheritance (mostly autosomal), together with the autosomal recessive limb girdle muscular dystrophy of childhood can mimic Duchenne muscular dystrophy introducing a potential pitfall in the prenatal diagnosis of the disease. It is obvious that counselling results can be erroneous in those families without an obvious X-linked family history or without a firm clinical diagnosis of DMD or BMD, based solely on linkage analysis.

Non-paternity is another potential pitfall when RFLP analysis is used for carrier detection. In forty-seven families studied by Bakker et al. (1988) non-paternity was encountered in about 5% of the cases. The use of a large number of RFLP probes can detect RFLP alleles that do not fit in the expected mendelian inheritance pattern.

Therefore for diagnostic purposes the detection of the molecular pathology of the disease has great advantages compared to RFLP analysis as: a) the diagnosis of DMD or BMD in sporadic cases can be confirmed, b) key relatives essential for linkage analysis do not have to be involved in the investigation, and c) a reliable prenatal diagnosis can be performed witout the added complication of genetic recombination, always present when RFLPs are used.

1.5 Mapping and Isolation of the Duchenne and Becker Muscular Dystrophy Gene.

Three separate lines of evidence conclusively mapped the DMD/BMD gene to band p21 of the short arm of the X chromosome (Xp21).

An early clue as to the specific location of the gene, came from the study of rare cases of girls with elevated SCK levels and muscle pathology consistent with the diagnosis of Duchenne muscular dystrophy, who had a reciprocal translocation between an autosome and the X-chromosome. Different autosomes were involved in the translocations but the breakpoint on the X-chromosome was always in the region of Xp21. The most likely explanation was that the translocation in some way disrupted the normal gene at Xp21 giving a DMD/BMD phenotype (Boyd, 1986).

Second, two DNA sequences (DXS7 and DXS9) from cloned fragments derived from an X genomic library, were isolated by Dr. K. Davies' group and found to be genetically linked to DMD (Davies et al., 1981 and 1983). Subsequently it was shown that Becker Muscular Dystrophy was linked at roughly similar genetic distances to DMD indicating that the two diseases could either be allelic or the two loci very close together in the same region of the X-chromosome (Kingston et al., 1983 and 1984, Wilcox et al., 1985).

The third piece of evidence came from a boy with DMD, but who also exhibited chronic granulomatous disease, McLeod syndrome and retinitis pigmentosa, whose Xp21 band appeared to be smaller in size by high resolution chromosome banding. Molecular studies confirmed a small interstitial deletion in this region (Francke et al., 1985).

The DNA represented in the sub-bands Xp211, Xp212 and Xp213 is around 5000 kb, 2000 kb and 4000 kb respectively (Emery, 1988). The chromosomal region involved in some way with DMD/BMD muscular dystrophy would therefore appear to span more than a thousand kilobases of DNA.

Since the biochemical basis for DMD/BMD was totally unknown a positional cloning

approach based on its chromosomal position, was employed to isolate the gene. Dr. Kunkel's group in Boston (1985) approached the problem by extracting DNA from the patient described by Francke et al.(1985). The DNA was then sheared by sonication for the production of DNA fragments with irregular ends. DNA from a 49,XXXXY lymphoblastoid cell line was cleaved with the restriction enzyme MboI. The two sets of fragments were then mixed and heated in order to disassociate the DNA strands. These were then allowed to reassociate in the presence of phenol (phenol enhanced reassociation technique-PERT). Under these conditions and with patient's DNA in excess, most of the reassociated molecules would have sheared ends and a few would be hybrid molecules with one sheared end and one MboI sticky end. However those sequences in the control deleted from patient's DNA would not hybridise with the patient's DNA. They would hybridise between themselves and therefore, consist of perfectly reassociated molecules with two MboI ends. Only these could be ligated into an appropriately cleaved plasmid and be cloned. In this way a library of cloned sequences (referred as PERT probes), corresponding to the portion of DNA deleted in the affected boy, was produced. These detected several RFLPs closely linked to the DMD locus and also small deletions in a proportion of affected boys. pERT87 was shown to be deleted in 7% of affected males (Kunkel et al., 1986).

Another approach involved isolation of a translocation junction fragment from the DNA of a female with an X;21 translocation. In this translocation the autosomal breakpoint had occurred in a block of tandemly repeated ribosomal genes on the short arm of chromosome 21. Segments of the already cloned ribosomal RNA gene were used as probes to isolate the X chromosome junction fragment (XJ) at the translocation breakpoint and therefore, sequences related to the DMD/BMD gene. The X chromosomal part of the clone (XJ 1.1) detected deletions in some male DMD patients and defined an RFLP closely linked to the DMD gene (Ray et al., 1985).

The localisation of the DMD/BMD gene on the short arm of the X-chromosome and the relative positions of some genomic probes are shown in figure 1.1.

The identification of mRNA transcribed from coding DNA segments of the gene in the muscle tissue, where this gene is predominantly expressed, proved difficult. The relatively low abundance of the protein in muscle and the small size of the exons necessitated the approach of cross-species conservation to be applied. The entire cDNA for the Duchenne gene was isolated by Koenig et al (1987), and shown to contain more than 65 exons. The protein product of the Duchenne gene was named dystrophin

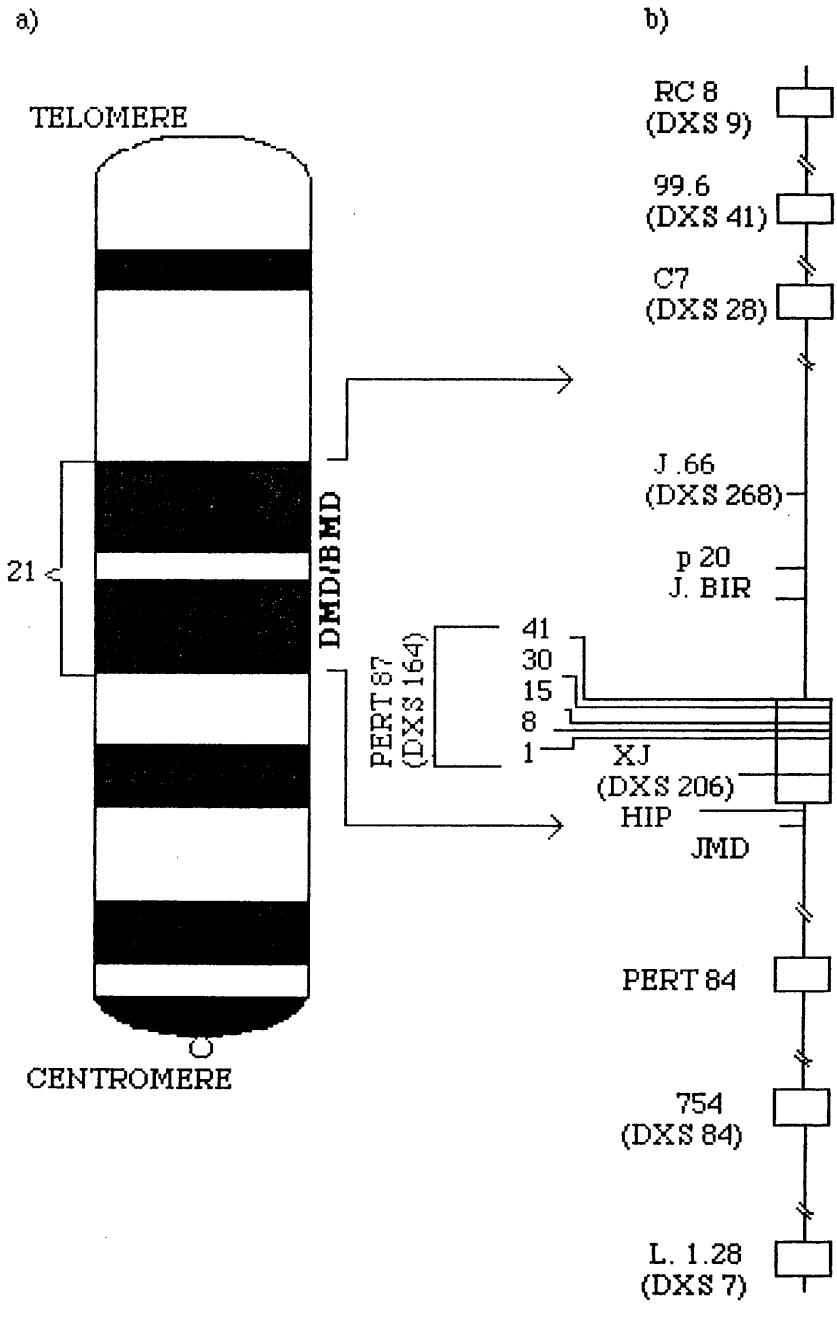


FIGURE 1.1: Schematic representation of the DMD/BMD gene. a) Ideogram of G bands on the short arm of the X chromosome. Localisation of the DMD/BMD locus on Xp21. b) The order of several genomic probes within and around the DMD/BMD gene (Davies et al., 1983); Kunkel et al., 1986; Chen et al., 1989; den Dunnen et al., 1989).

1.6 Dystrophin - The Protein Product of the Duchenne Muscular Dystrophy Gene.

The complete sequence of the human DMD cDNA has been determined and shown to consist of more than 65 exons with a mean size of 200 bp and a mean size for introns of 35 kb. The open reading frame which is followed by a 2.7 kb segment with no apparent coding potential, encodes a protein 3685 aa long, with a predicted molecular weight of 427 kd that has been identified and named dystrophin (Hoffman et al.,1987).

Dystrophin appears to be formed by four different domains: an N-terminal "actin-binding" domain, a middle domain formed by 26 repeats in tandem, a cysteine-rich domain, and a less characterised COOH domain. The 240 aa N-terminal domain has been shown to be conserved with the actin binding domain of a-actinin (Hammonds, 1987). The entire dystrophin coding sequence contains 26 repeat domains organised in tandem. They extend from the end of the NH_2 "actin-binding" domain to the beginning of the cysteine-rich domain. The length of the repeats ranges between 88 and 126 aa with rather loose homology between them. The middle domain of dystrophin probably adopts a rod shape about 150 nm in length. The cysteine-rich segment is similar in part to the entire COOH domain of the Dictyostelium a-actinin, while the 420 aa C-terminal domain of dystrophin does not show any similarity to previously reported proteins (Koenig et al.,1988).

Dystrophin is predominately hydrophilic throughout the entire molecule as no substantial hydrophobic stretch of aa was found that could account for a signal peptide segment or a membrane spanning segment. In 1989, Campbell and Kahl suggest a linkage between dystrophin and an integral membrane glycoprotein in the sarcolemma which acts as a "dystrophin receptor". Recent results demonstrate that dystrophin is associated with a 156 kd dystrophin-associated glycoprotein by way a 50 kd, 43 kd, and 35 kd transmembrane glycoprotein complex, and suggest that dystrophin serves as a specialised link between the actin cytoskeleton and components external to the sarcolemmal membrane (Ervasti and Campbell, 1991).

In the human embryo, dystrophin appears from eight weeks of development onwards, first in the sarcoplasm of the extremities of the myofibres between the most distal nuclei and the myotendinous junction. At 17 weeks it has accumulated throughout the myotubular sarcoplasm, with sarcolemmal staining appearing first in slow switch type I fibres (Ginjaar et al., 1989). The amount of dystrophin transcript appears to vary in different tissues. If the amount present in skeletal muscle and heart is defined as 100% then to 5-10% is present in smooth muscle, 1-2% in brain cortex, kidney, lung and less than 1% in liver placenta, spleen, fibroblasts and hepatoma cells (Chelly et al., 1988).

The human dystrophin transcript has different first exons in brain and muscle, indicating that dystrophin expression can be differentially regulated in these tissues by usage of distinct promoters (Nudel et al., 1989). The 5'-untranslated region of brain mRNA appears highly conserved in evolution which is not the case for the muscle 5'-untranslated region. At the other hand the 3' end of the dystrophin transcript can be alternatively spliced to create numerous isoforms differing at their carboxyl domains allowing dystrophin to interact with different proteins in brain than it does in muscle (Feener et al., 1989).

The biochemical defects responsible for DMD/BMD are abnormalities of dystrophin. Dystrophin comprises 2% of sarcolemmal proteins and 5% of the sarcolemmal cytoskeleton which supports the role of the dystrophin-glycoprotein complex in maintaining skeletal muscle architecture. Absence of dystrophin thus may compromise the integrity and flexibility of the sarcolemma, leading to either mechanical damage or alteration in specific calcium regulatory mechanisms of the sarcolemmal membrane (Ervasti and Campbell., 1991).

Immunoblot characterisation and immunofluorescence localisation of dystrophin can offer accurate information regarding the molecular weight and relative abundance of the protein contained in a patient's skeletal muscle by using a small amount of tissue derived from a muscle biopsy specimen. In normal muscle dystrophin is visualised by immunofluorescence as a continuous thin ring of staining at the periphery of every muscle fibre and by immunoblotting is detected as a 400 kd, low abundance protein. Studies where DMD, BMD and patients with unrelated disorders were tested for the presence of dystrophin revealed a very strong correlation of clinical diagnoses with the type of dystrophin abnormality. Duchenne muscular dystrophy patients showed complete or almost complete absence of dystrophin, while BMD patients had clearly abnormal dystrophin and patients with unrelated diseases showed normal dystrophin pattern (Hoffman et al., 1988; Arahata et al., 1989). Immunoblot analysis of dystrophin in BMD patients could be useful in the prediction of their genetic defect as lower molecular weight dystrophin implies an in-frame deletion, while higher molecular weight dystrophin implies an in-frame duplication.

The abnormal size and/or reduced quantity of dystrophin in BMD patients could be interpretated as partial integration and protection of mildly abnormal dystrophins and this may explain the clinical differences between these two disorders. Also quantitative abnormalities of dystrophin result in a more severe clinical phenotype than do qualitative abnormalities (Hoffman et al., 1988).

1.7 Investigation of the Molecular Pathology of Duchenne and Becker Muscular Dystrophy.

The availability of both probes flanking the Duchenne gene and intragenic genomic DNA

probes, followed by dystrophin cDNA probes has enhanced the ability to detect the molecular pathology of the disease in many cases using Southern blot analysis.

Intragenic deletions appear to be the most common gene defect leading to DMD/BMD. Bartlett et al. (1988), reports a 23% deletion detection rate using probes XJ-1.1, JBir and cloned sequences of the DXS164 locus (PERT probes), which increases to 39% when the p20 probe was also applied. The recombination rate between the disease locus and these probes was calculated to be 5 to 6%. Application of the XJ2.3, pERT87-15, pERT87-30, JBir, p20 and J66 intronic probes in a deletion screening of 223 unrelated DMD/BMD patients revealed a deletion in about 25% of the cases (Gilkenkrantz et al., 1989). 43% of the deletion detected in DMD patients and 78% of these detected in BMD patients involved the p20 sequence.

Regions of the DMD locus were cloned and used as cDNA probes (Koenig et al., 1987). The cDNA probes 1-3, 4-5a, 5b-7, 8 and 9 -14 cover the entire cDNA with the exception of 50 bp at the 5' end, figure 1.2. The last one can generate probes 9-10, 11, 12a and 12b-14 after cutting with Hinc II (Liechti-Gallati et al., 1989). The subprobe 12b-14 corresponds to the 3' untranslated sequences of the transcript of the last exon.

The percentage of deletions in both DMD and BMD patients increased after the application of cDNA probes for screening. Darras et al., (1988a) reported an overall deletion detection rate of 66% with cDNA probes. cDNA probe 1-3 detected 29% of the deletions found, while probe cDNA 8 alone detected 57%. No deletions were confined to the segment covered by probe cDNA 9. Five of the six deletions in the 1-3 region had a breakpoint within the intron separating exons 7 and 8, and seven of fifteen deletions in the central part of the gene had a break between two specific Hind III fragments (4.1 kb and 0.5 kb) which suggests a deletion breakpoint cluster within a confined region, the p20-containing large intron. No duplications were identified. A similar result (65%) was reported by den Dunnen et al., (1989). 53% was the deletion frequency resulted from a screening study of thirty French patients using the cDNA probes (Lucotte et al., 1989). This percentage was slightly smaller (42.8%) in Japanese families (Sugino et al., 1989). No detectable deletions or duplications were found at the intragenic sequence between DMD 9 and the 3' end of the gene. The frequency of cDNA probe deletions observed in the Scottish DMD patients (110 pedigrees) was 65% (Cooke et al., 1990). One duplication was detected too. In addition, 10 deletions and one duplication were recognised out of 13 pedigrees with definite X-linked BMD. Most of the deletions were concentrated in the area of cDNA probes 7 and 8 or with the genomic probes p20 and GMGX11, and a large number of patients had their deletion endpoints in the intron containing p20.

In all deletion studies performed a heterogeneity in both the number of exons and the

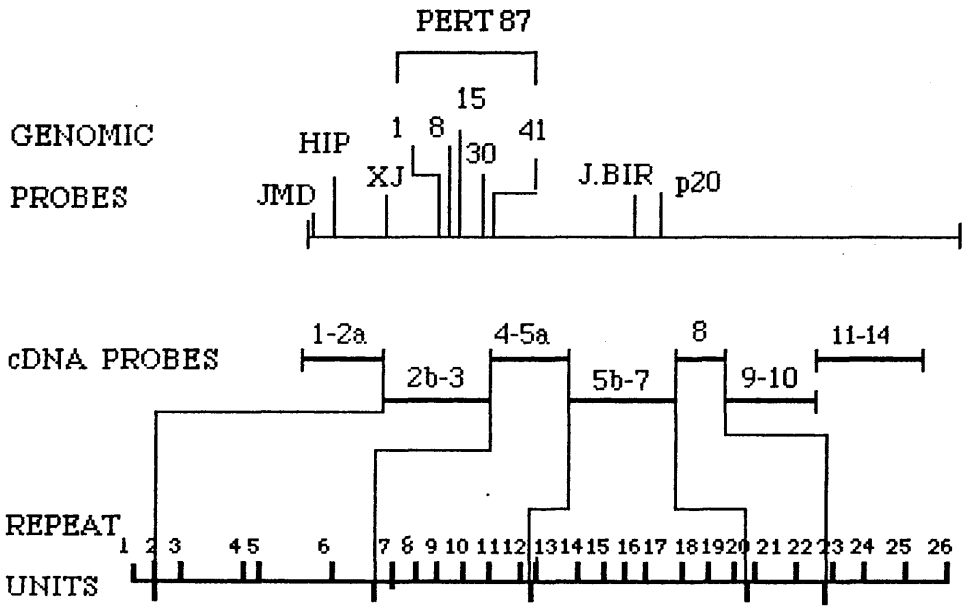


FIGURE 1.2: Schematic drawing of the dystrophin cDNA probes. The relative position of several genomic probes and the distribution of the repeat units relative to the cDNA subclones (Koenig et al., 1988) are shown.

specific region deleted in patients with DMD is observed. Individually deletions vary greatly in length and they clearly have a non-random distribution. Intragenically, the deletions seem to be localised preferentially to two regions: one containing exons of the first two kilobases of the cDNA (detected by cDNA probe 1-2a), and the second, larger one, associated with exons around the middle part of the cDNA, detected by cDNA probes 7 and 8. Within the latter, so-called hot spot region, there are three main locations for deletion breakpoints originating in introns: i) between the exons correspond to the genomic Hind III fragments 4.1 kb and 0.5 kb (cDNA 5b-7), ii) between the 0.5 kb and 1.5 kb (cDNA 5b-7), and iii) between 10kb and 1.25 kb (cDNA 8). The probe p20 has also been localised close to the 0.5 kb Hind III fragment, (cDNA 5b-7), (Liechti-Gallati et al., 1989). Deletions are rare in the area between p87 and Jbir DNA probes (3.0-5.8 kb) and at the 3' end of the cDNA distal to the J66 (DXS268) region beyond 9.5 kb (den Dunnen et al., 1989). Deletions at the 3' end of the gene may exist but if they cause a milder, non-progressive myopathy the diagnosis of DMD/BMD would be unsuspected.

In 1988 Chamberlain and his co-workers exploited these observations to develop a simple plus/minus deletion detection assay for the DMD/BMD gene based on the recently developed method of polymerase chain reaction (Saiki et al.1988). In this method sequences flanking deletion prone exons of the gene were simultaneously amplified via PCR. Any of these regions absent from patient DNA failed to amplify and were immediately identified via agarose gel electrophoresis of the reaction products. In 37% of the cases a deletion was detectable via this approach eliminating the necessity for Southern analysis for the identification of the molecular pathology of the disease.

The extent of deletions found in patients is not related in a simple manner to the severity of the phenotype. Small deletions can lead to both severe DMD and mild BMD. Also different pedigrees with the same apparent deletion show widely differing phenotypes. In addition substantial variations in clinical severity exist within individual pedigrees where each affected subject will presumably have the same deletion at this point of resolution. A molecular mechanism to explain the clinical difference in severity between DMD and BMD patients who bear partial deletions of the same gene locus was proposed by Monaco et al. (1988). The model is based on the breakpoints of intragenic deletions and their affect on the translation of triplet codons into amino acids of the protein product.

Eukaryotic genes are organised in genomic DNA with exons encoding open reading frames (ORF) of nucleotide triplet codons for aa. Exons are separated by non-coding sequences called introns that contain translational stop codons, and exon:intron borders can begin and end in any of the three positions of the triplet code for aa. For normal gene function two exons in series along the chromosomal DNA must have triplet codon breakpoints that maintain the correct

translational ORF during the splicing of intron sequences to form the mature mRNA. An intragenic deletion that connects genomic exons that maintain an ORF of triplet codons in the mRNA after splicing, would translate into a protein with an interstitial deletion of aa corresponding to the deleted exons. By contrast, intragenic deletions that frameshift the ORF after intron splicing would truncate the protein during translation of the mRNA due to an eventual stop codon. Therefore adjacent exons that can maintain an ORF in the spliced mRNA despite a deletion would give rise to the less severe BMD phenotype, and predict the production of a lower molecular weight, semifunctional dystrophin protein. Adjacent exons that cannot maintain an ORF because of frameshifted triplet codons would give rise to the more severe DMD phenotype due to the production of a truncated, nonfunctional dystrophin protein. This hypothesis has been supported by a number of studies (Koenig et al, 1989, Speer et al., 1990, Thibodeau et al., 1989). Nevertheless, there are cases of nonconformity to the model. Cases of BMD patients have been reported where the reading frame was found to be disrupted (Malhotra et al., 1988; Gilkenkrantz et al., 1989; Speer et al., 1990), or deletions of exons 3 to 7 resulting in mild phenotype (Malhotra et al., 1988), whereas large in-frame deletions of more than 30 exons and deletions of the first exon result in DMD. For these cases an alternative mechanism might exist to generate a protein with partial function. Further analysis of cases where the phenotype is in agreement with the reading frame hypothesis for the correlation of different dystrophin domains to the pathophysiology of DMD and BMD, assuming that the dystrophin protein is synthesised as predicted by the deletion analysis, showed that insertial deletions in the N-terminal domain and in both the first 13 and the last 8 repeats result in BMD while terminal deletions of both cysteine rich and C-terminal domains result in DMD (Koenig et al., 1989).

Partial gene duplication accounts for the mutation in about 6% of patients with DMD or BMD (Hu et al., 1990). Evidence of gene duplication in the DMD locus was first provided by Bertelson et al. in 1986, who reported a DMD patient with a double intensity hybridisation band detected with the intragenic genomic probe pERT87-15. Although that the great excess of deletions over duplications may be due to mechanisms that generate deletions without the generation of a concomitant duplication, the difficulty of detecting a doubling of a hybridisation intensity on Southern blot analysis compared to the absence of a hybridisation signal, can contribute to the ascertainment bias.

In the rest of the DMD/BMD cases point mutations can account for the development of the disease. Point mutations can either eliminate splicing sites, generate novel splicing sites, or activate cryptic ones. They can also cause a frame shift in the ORF, or introduce an Amber stop codon. So far, the majority of base changes have been identified by the restriction fragment length polymorphism (RFLP) approach, which measures DNA sequence alterations due to a loss or gain of a restriction enzyme cleavage site, or to variation in length caused by deletion or

insertion. However, many single base changes do not alter a restriction enzyme cleavage site and therefore cannot be detected by the RFLP approach. Since detection and localisation of single base substitutions within long DNA sequences, such as the DMD gene, are impractical by complete sequence determination, other methods have been developed. Denaturing gradient gel electrophoresis (Myers et al., 1985), chemical cleavage of the mismatch (Cotton et al., 1988), and single-strand conformation polymorphism (Orita et al., 1989a), are some of those developed for an initial detection screening of unknown mutations in a gene. The employment of the polymerase chain reaction (PCR) has facilitated their application a lot and therefore this technique will be discussed first.

1.8 Basic Methodology of the Polymerase Chain Reaction (PCR).

The polymerase chain reaction (PCR) is an *in vitro* method for amplifying a specific DNA fragment of defined length and sequence from small amounts of a complex template, by as much as 10^8-fold, in a matter of hours. The method was first described by Saiki et al.in 1985

PCR amplification involves: a) two oligonucleotide primers that flank the DNA segment to be amplified, and b) repeated cycles of heat denaturation of DNA to obtain single stranded templates, followed, first by annealing of the primers to their complementation sequences, and then by a pulse of DNA synthesis with DNA polymerase. The primers are designed to hybridise to opposite strands of the target sequence and are oriented with their 3'ends facing each other, so that DNA synthesis by the polymerase proceeds across the region between them, effectively doubling the amount of that DNA segment. Since the extension products are also complementary to and capable of binding the primers, successive cycles of amplification continue to double theoretically the amount of DNA synthesised in the previous cycle. Thus, the overall increase in this number is exponential. A schematic diagram of PCR is presented in figure 1.3.

1.8.1 Reaction Components.

A PCR mix includes the DNA to be used as a template, a pair of primers specific for the target sequence, the DNA polymerase, buffer and deoxyribonucleotide triphosphates (dNTPs).

The DNA to be used as a template can be isolated from any biological source. It can be obtained from cells (Kawasaki, 1990a), sperm (Gyllensten, 1990) or surgical biopsy tissue sample (Wright and Manos, 1990). Even DNA extracted from embedded archival tissues or

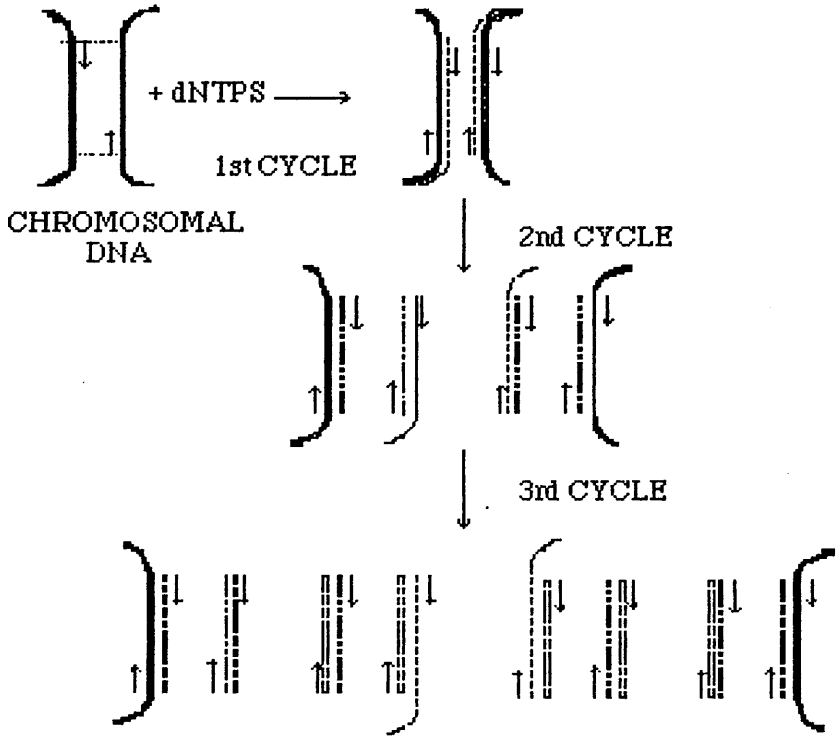


FIGURE 1.3: Scheme summarising the procedure of polymerase chain reaction (PCR). The products at the end of the three initial PCR cycles are shown. The two anti-parallel strands of chromosomal DNA are indicated. The direction of the transcription is 5' to 3'. The primers are indicated by arrows. PCR products are represented by dashed lines.

from specimens of extinct animal species is suitable for amplification (Pääbo, 1990). RNA can also be amplified using the polymerase chain reaction method(Kawasaki, 1990b). The amplification of RNA sequences is preceded by a reverse transcription step, resulting in the generation of single-stranded DNA, which is then converted into double-stranded DNA. Occasionally, the template sequences themselves prevent their successful amplification because of a stable intramolecular secondary structure, as in the case of high G+C content in the target region. This can be overcome by using the nucleotide analogue 7-deaza-dGTP or in combination with dGTP, which destabilises intrastrand folding without impairing the Watson-Crick base pairing between strands (Innis, 1990).

The primers are added at the beginning of the reaction in 10^6-fold to 10^7-fold stoichiometric excess depending on the original concentration of target sequences. Increase of this ratio can cause the appearance of 46-50 bp products called primer dimer. A primer dimer can appear in the case of the two primers overlapping at their 3'end. The length of the primers is usually about 20 to 30 bases. The design of successful PCR primers tends to be empirical, but some general rules can be followed such as: a) selecting primers with an average GC content, and a random base distribution. Primers with stretches of polypurine, polypyrimidines, or other unusual sequences are usually best avoided. b) avoiding primers with AT and GC rich regions. c) the substantial secondary structure should be checked using the available computer programmes. d) checking the primers against each other for complementarity (Saiki, 1989). Sequences not complementary to the template can be added at the 5'end of primers since they do not significantly affect the efficiency of the amplification. These sequences become incorporated in the double-stranded PCR product and provide a means of introducing restriction sites, specific mutations at specific positions in the DNA, or regulatory elements at the ends of the amplified target sequence (Scharf et al., 1986).

A number of different DNA polymerases can be used for the amplification of the target sequence in the third step of the cycle. Originally the Klenow fragment of Escherichia coli (E. coli) DNA polymerase I was used (Mullis and Faloona, 1987), but the unmodified DNA polymerase I, the DNA polymerase of the phage T4, or the modified T7 DNA polymerase can also be applied (Teinor-MacLachlan, 1988 cited in Vosberg, 1989). The disadvantages of these enzymes is their heat liability, therefore fresh enzyme has to be added in each cycle after the DNA denaturation step.

Currently the most frequently used enzyme is a heat-stable polymerase isolated from the archiobacterium Thermus aquaticus (Taq), which has the advantage of surviving extended incubation at 95^0C, and therefore it does not need to be replaced in each cycle. Besides simplifying the reaction, this has also allowed the development of automatic equipment for PCR. In addition the higher optimum temperature of this enzyme significantly increases the

specificity, yield and length of targets that can be amplified, by minimising any non-specific hybridisation of the primers to the target due to partial homology with random or related, but not identical sequences (Saiki, 1988). The activity of the enzyme can be affected by other compounds that might be included in the reaction depending on the source of template, such as heparin and iron that has been found to inhibit the enzyme, ethanol which has no effect up to 10%, and SDS, 10% inhibition at 0.01% (Saiki, 1989).

The buffer provided by the manufacturer for the specific Taq polymerase is adequate for most applications. It usually contains: a) 50 mM KCl, as higher concentrations inhibit the reaction. b) 10 mM Tris-HCl, HCl-neutralised Tris (hydroxymethyl)aminomethane pH=8.3 at 25^0C, so that the pH will be 7.4 to 7.5 at 72^0C extension temperature, the optimum for Taq polymerase. c) $MgCl_2$, as the enzyme is magnesium dependent. Usually the $MgCl_2$ concentration is 1.5 mM, but it seems that different primers display different Mg^{2+} optima, with ranges varing between 1.2 and 2.5 mM. d) 0.1% (v/w) gelatin. Gelatin is recommended over bovine serum albumin because it is less likely to coagulate during the denaturation step, and is readily sterilised in an autoclave. Sometimes dimethyl sulfoxide (DMSO) is included in the reaction as it reduces the secondary structure of the DNA. However, many investigators have noticed that DMSO can be slightly inhibitory to the polymerase and decrease the yield of the amplification product (Gelfand, 1989).

1.8.2 Thermal Cycling Parameters.

PCR is performed by incubating the samples at three different temperatures corresponding to the three different steps in the amplification cycle. During the denaturation step the temperature of the reaction should reach at least 90^0C for strand separation to occur.

The temperature applied for primer annealing depends a lot on the length and GC content of the primers - the lower the GC content, the lower the optimal temperature of the reaction. Raising of the annealing temperature usually helps in cases where a non-specific amplification is observed. Long incubation at the annealing temperature is not necessary, as hybridisation occurs almost instantaneously because of the very large molar excess of primers present in the reaction mix.

The optimum temperature for primer extension varies with the enzymes used. The incubation time for this step depends on the length of the DNA segment being amplified. The extension rate for the Taq polymerase at temperatures between 75 and

80^0C has been found to approach 150 nucleotides/second/enzyme molecule (Gelfand, 1989). Prolonged extension time increases the amount of non-specific amplification products.

1.8.3 The Fidelity of DNA Polymerase.

The final PCR product cannot be considered a unique entity, as even a discrete DNA band on an agarose gel may contain a variety of DNA molecules differing from the original genetic information by one or more nucleotides. In cases of direct characterisation of the amplified population by DNA sequence analysis or nucleic acid hybridisation, random errors in nucleotide sequence that may be produced during PCR are of little concern. However, in some PCR applications involving the characterisation of individual DNA molecules or rare molecules present in a heterogeneous population, or for any PCR application that begins with a small amount of starting material, the fidelity (error rate per nucleotide) of PCR is an important consideration. Observed error frequencies during PCR vary more than 10-fold, from $\sim 2 \times 10^{-4}$ to $< 1 \times 10^{-5}$ (assuming a constant efficiency per cycle), depending on the precise DNA sequence and the in vitro conditions of DNA synthesis (Eckert and Kunkel, 1991).

A variety of changes in DNA sequence can occur during enzymatic amplification such as single base-substitutions, resulting from the misincorporation (insertions or deletions) of an incorrect dNTP during DNA synthesis. The frequency of deletion mutations is sequence dependent and is increased in repetitive DNA sequences (Kunkel, 1990).

High-fidelity DNA synthesis conditions are those that exploit the inherent ability of polymerases to discriminate against errors. The efficiency of proofreading (3' to 5' exonucleolytic removal of 3' terminal mispairs to regenerate the starting DNA configuration), which is an important error discrimination mechanism, is not present in most commercially available thermostable DNA polymerases. For those enzymes that are proofreading deficient, the in vitro reaction conditions can significantly influence the polymerase error rates. To maximise fidelity any type of deoxynucleotides triphosphate pool imbalance should be avoided, reactions should contain a low $MgCl_2$ concentration, not in large excess over the total concentration of dNTP substrates, and reactions should use short synthesis times, low dNTP and enzyme concentration. Also, prolong incubation of DNA at high temperatures during PCR should be avoided as it produces DNA damage, such as deamination of cytosine to produce uracil, and spontaneous base release resulting from hydrolysis of the N-glycosylic bond. Finally, the total number of cycles should be kept to the minimum necessary to produce a feasible amount of product DNA for the desired use. It should be kept in mind that reactions that optimise fidelity may be relatively inefficient for amplification and therefore, the conditions generating more DNA product are not necessarily the best (Eckert and Kunkel, 1991).

1.9 Advantages of the Polymerase Chain Reaction.

The extent to which genetic properties or gene activities can be studied in molecular terms depends very much on the number of copies of DNA or RNA molecules available to warranty analysis by current methods; usually between 10^5 and 10^6 DNA or RNA molecules must be available for a single analytical test (Vosberg, 1989). A PCR reaction, due to its efficiency, specificity, and sensitivity facilitates the handling of nucleic acids by expanding the number of target sequences while at the same time reducing the background of sequences that are not wanted.

Although the theoretical upper limit of the number of product molecules is 2^n, where n is the number of cycles, under normal experimental conditions this value is not obtainable. The exponential accumulation of PCR amplification products is not an unlimited process. Firstly, the actual product does not appear till the end of the third cycle. Secondly, a level of amplification is reached where the amount of template molecules is higher than can be accepted by the enzyme, which at the same time is loosing some of its activity because of repeated heating; and thirdly the amounts of primers and product are the same and therefore the probability of annealing either the two strands of the template, or the template with the primer is the same. When this occurs (plateau effect), the efficiency of the reaction starts to decline, and the amount of PCR product accumulates in a linear rather than an exponential manner. The efficiency of the reaction declines also in these cases where because a heat-labile DNA polymerase is used, and there is a need for adding new enzyme in each cycle, a gradual change in the assay composition takes place, which affects the catalytic activity of the enzyme. Therefore a more realistic efficiency is 85% per cycle (Saiki et al., 1985).

The specificity of a PCR reaction can be affected by factors such as: a) the component concentration, b) the time of denaturation, renaturation and extension, c) the annealing temperature, and d) the primers. The ratio of dNTPs, primers, metal cation and enzyme is very important and if the concentration of one is changed, the others need to be adjusted. The specificity of the Taq DNA polymerase can be affected by the time allowed for the primer extension step and by the quantity of enzyme used in the reaction. For amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, there is an optimum concentration of Taq DNA polymerase, usually 1-4 units/ 100 µl reaction. Any increase in the amount of enzyme beyond this level can result in great production of non-specific PCR products, and reduced yield of the desired target fragment. Other approaches to enhance specificity are a partial fractionation of crude preparations, or the use of nested sets of primers. Nested sets of primers involve one round of repeated synthesis with one set of primers, and a second round with primer sequences located between the primers used in the previous round. Although the method works on genomic DNA as a whole, or on crude mixtures of total cellular

RNA, a high degree of DNA or RNA complexity has the disadvantage of reducing the specificity and also the efficiency of the reaction

It is still undetermined what the length-limits are in the amplification of target sequences. The Taq DNA polymerase can amplify genomic target sequences of up to 2.0 kb; longer fragments can also be synthesised, but the efficiency and the yield of the amplified product is reduced (Saiki, 1988).

The polymerase chain reaction is a method of very high sensitivity. A target sequence, which is present only once in 10^5 to 10^6 cells can be detected by amplification. At the cellular level, it has been shown that the RNA content of a single cell is sufficient for sequence specific amplification (Saiki et al., 1988).

The sensitivity of the method necessitates great care to avoid amplifying DNA contaminants. Minute contamination of benches, or frequently used laboratory equipment with template molecules from various sources, including previous amplifications, can lead to a false positive signal, since the product of the amplification serves as a substrate for the generation of more product. Therefore, several precautions such as, physical separation of PCR preparations and products, use of gloves and positive displacement pipettes, and sterilisation of all plastic consumables and solutions, should be taken in advance, in order to reduce the number of false positives.

1.10 Single - Strand Conformation Polymorphism (SSCP). Principle and Practice.

The method of single-strand conformational polymorphism developed by Orita (1989a), can be described as a mobility shift analysis of single-stranded DNA on neutral polyacrylamide gel electrophoresis.

The mechanism by which dissociated DNA strands separate in gel electrophoresis is not known. A difference in the molecular weight is apparently not a reason since the purine L-strand of a DNA moves faster than the pyrimidine-rich H strand. A hypothesis is, that when the two complementary strands separate, each strand folds on itself into one predominant semi-stable conformer prescribed by its primary structure. This interaction need not be comprised entirely of Watson-Crick base pair. Thus, complementary strands of the same size but of different nucleotide sequence acquire different conformation and be differentially retarded by the gel matrix during electrophoresis. (Maxam and Gilbert, 1980). Because of its high resolving power, polyacrylamide gel electrophoresis can distinguish conformational changes caused by subtle sequence differences such as a single base substitution in a several-hundred-base

fragment. The steps in the SSCP process are indicated in figure 1.4. At present, it is not possible to predict the shift of electrophoretic mobility induced by the mutation. Conversely, measurement of the mobility of already known mutated sequences can be an empirical approach to the prediction of higher-order structure of single-stranded nucleic acids.

Initially the method was applied on DNA restriction fragments, denatured in alkaline solution , run on a neutral polyacrylamide gel, and transferred to a nylon membrane. The mobility shift due to a nucleotide substitution was detected by either hybridisation of a nick-translated DNA fragment, or with RNA copies synthesised on each strand of the DNA fragment and used as probes (Orita, 1989a). The restriction enzyme digest and the hybridisation step were omitted in a later modification of the technique where specific regions of genomic sequences were labelled, and amplified simultaneously by using labelled substrates in the polymerase chain reaction with a high efficiency (Orita., 1989b). This improvement proved particularly important as it permits the use of a thin polyacrylamide gel which provides higher resolution and also allows the application of a steep voltage gradient without serious Ohmic heating, so that the time required for electrophoresis is shortened.

The detection of mutations depends on the conformational changes of the single-stranded molecule induced by the mutation, and therefore, sensitive to physical environment in the gel. The temperature during the electrophoresis is shown to affect the mobilities of separate strands as expected since their conformation is determined by the balance between thermal fluctuation and weak local stabilising forces derived from short intrastrand base pairing and base stackings. For this reason little Ohmic heating and efficient cooling are important (Orita, 1989b).

It has been found empirically that the presence of glycerol in low concentrations in a gel usually improves the separation of mutated sequences (Orita, 1989b). Although the reason for this is unknown, a hypothesis is that glycerol because of its weak denaturing action on nucleic acids, partially opens the folded structure of single-stranded nucleic acids so that more surface area of the molecule is exposed, and thus there is more chance for the acrylamide matrix to distinguish the structural differences caused by the mutation (Hayashi, 1991).

It has also been known that complementary single strands are better separated in gels with low cross-linking. The extent of cross-linking is expressed by %C, a ratio of the percent concentration of N,N'-methylenebisacrylamide to the concentration of total acrylamide monomer. At 5%C, the gel is the most rigid and has minimal pore size at any given total acrylamide concentration. A gel with a lower %C is more soft, has remarkably increased pore size, and seems to be more sensitive to conformation (Hayashi, 1991).

Short fragments, less than 400 bp are better suited for detection of mutations in the SSCP gel (Hayashi, 1991). Alternatively, longer fragments can be amplified using labelled radioactive

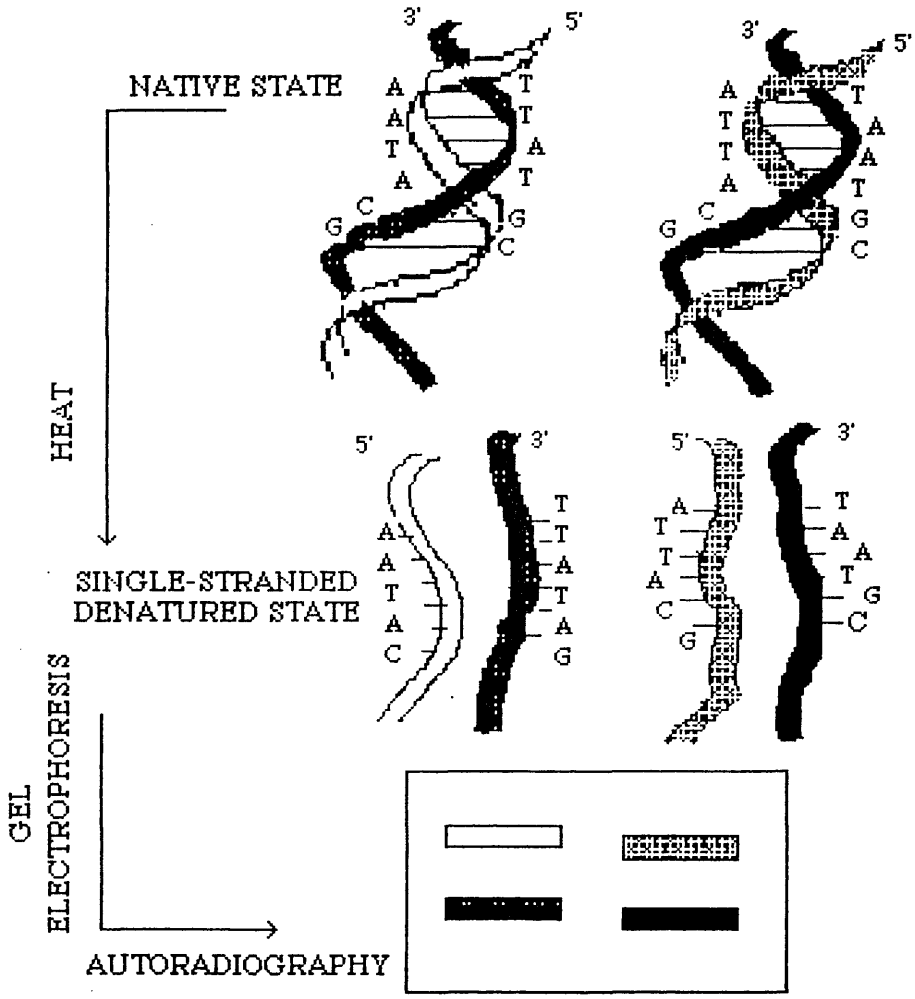


FIGURE 1.4: Illustration of single-strand conformation polymorphism (SSCP) protocol strategy.

deoxynucleotides, and the uniformly labelled PCR products are then digested by restriction enzymes. Samples are then denatured and applied to non-denaturing polyacrylamide gel.

1.11 Advantages of PCR-SSCP Analysis..

Simplicity is the major advantage of PCR-SSCP analysis, as mutations are detected by the presence of shifted bands rather than by the absence of signal. Therefore, failure in the PCR does not lead to false-positive results.

Another advantage is that it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments without the necessity of them being present in the recognition site of a restriction endonuclease, or in a region detected by a particular probe. As nucleotide substitutions have been estimated to occur every few hundred base pairs in the human genome (Cooper et al., 1985), SSCP could provide many genetic markers. It may seem. that PCR-SSCP analysis is much more sensitive to the replication errors that occur during the PCR. However, since the errors are assumed to occur randomly, and as not a single DNA molecule is used, this should not interfere with the results.

The amount of product required in PCR-SSCP analysis is much less than in other techniques in which products are detected by staining. Therefore, reactions can be optimised for high-fidelity DNA synthesis and not for the production of an impressive amount of amplification product. This means that volumes of the reaction and concentrations of substrates can be reduced for lower running cost and less radioactive hazard.

Most mutations in various sequence contexts seem to be detected. It has been estimated that the sensitivity of PCR-SSCP analysis (probability of detecting at least one strand shifted) is more than 99% in 100- to 300-base-long strands, and 89% in 300- to 450-base pair fragments using gels with 5-10% glycerol (Hayashi, 1991). Further accumulation of data are necessary though to estimate accurately the rate of detection of this technique.

1.12 Amplification and Mismatch Detection Analysis (AMD).

Amplification and mismatch detection analysis is a combination of PCR and the chemical mismatch method (Cotton et al., 1988) for the detection of sequence variation in a DNA fragment. The region of interest from the genomic DNA of the individual to be examined and from a reference sample are amplified with one of the two radioactively labelled. A mixture of the PCR products is denatured and then allowed to renature to form hybrid duplexes. Any variation between the "probe" sequence and the other strand in the duplex will disrupt base-

pairing; this site is sensitive to specific chemical modification by either hydroxylamine or osmium tetroxide. Hydroxylamine hydrochloride and osmium tetroxide react specifically with pyrimidine bases in DNA. Hydroxylamine at pH 6 modifies the C5=C6 double bond in cytosine which then labilises the ring to internal rearrangement and cleavage (Rubin and Schmid, 1980). Osmium tetroxide modification is a thymine-specific reaction in which the C5=C6 double bond is oxidised (Friedmann and Brown, 1978). Double-stranded DNA is attacked very slowly, while single-stranded regions are readily modified. At the mismatch site, the secondary structure of the DNA is disrupted and it appears single-stranded and therefore, susceptible to rapid modification. The DNA strand can then be cleaved at the site of modification with piperidine and analysed by denaturing polyacrylamide gel electrophoresis. A schematic diagram of AMD is presented in figure 1.5.

For the detection of mismatched base pairs by chemical cleavage both end-labelled probes, generated during the polymerase chain reaction, or labelled probes resulted from the incorporation of a radioactive substrate in the final PCR product, can be used. The utilisation of both ^{32}P and ^{35}S labelled probes has been reported (Montandon et al., 1989; Saleeba and Cotton, 1991). The length of the DNA fragment screened can be up to 2 kb (Cotton and Malcolm, 1991).

The ability to position one or more mutations in a particular region of the fragment examined and to provide information about the type of mismatch are particular advantages of the method. The most unpleasant disadvantage of the method is the use of highly toxic chemicals that require special handling and disposal.

1.13 Denaturing Gradient Gel Electrophoresis (DGGE). Principle and Practice.

Natural DNA can be considered as a composite copolymer comprising of four kinds of nucleotides: adenine, guanine, thymine and cytosine. Under ordinary conditions and in an aqueous solution, it adopts a double-stranded structure known as B-form, which is maintained partly by hydrogen bonds between complementary pairs on opposite strands but mainly (3-10 times larger contribution than the previous) by stacking interactions between neighbouring base pairs. The double helical structure of DNA can be disrupted into single stranded random coils by heat, alkali and other denaturing agents. The helix to coil transition of DNA is called thermal denaturation or melting.

The electrophoretic mobility of DNA in a polyacrylamide gel is sensitive to the secondary structure of the molecule with respect to its helicity, partial melting, or complete melting and disassociation of the strands (Lerman et al., 1984). As temperature rises DNA melting proceeds under equilibrium conditions as a series of relatively abrupt transitions of portions of the

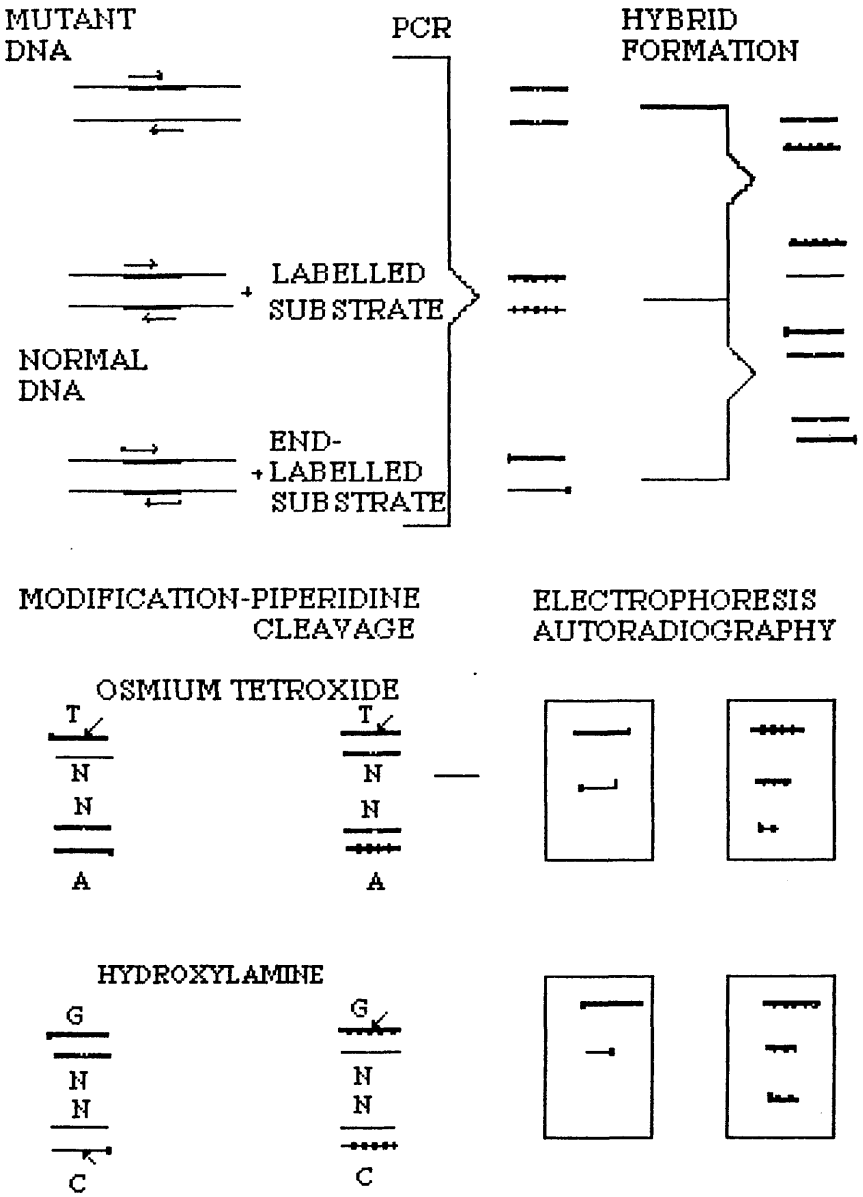


FIGURE 1.5: Schematic diagram showing steps used for the detection of point mutations by means of AMD analysis.

molecule (melting domain) from helix to random chain. The progression from negligible to essentially complete melting of a domain takes place over a narrow temperature interval and the intermediate does not contribute a lot to the result.

If the double helix is regarded as a two-ended polymer, then partial melting changes the molecule into a star polymer with three arms if the melting has proceeded from one end, or into a four armed star in which two pairs of arms are connected by the remaining double helix. The diffusion of branched polymers in a dense relatively immobile matrix is much slower than that of a linear polymer twice as long as any arm since a coincidence in random disposition of the previously paired arms is required. The rate of diffusion declines rapidly as the length of branched polymer increases. DNA molecules differing by a single base will therefore have slightly different melting properties, and they will migrate differently in a polyacrylamide gel containing a linear gradient of DNA denaturants such as urea and formamide. The solvent gradient is equivalent to a shallow, linear temperature ramp . Thus, the physical separations achieved in denaturing gradient gel electrophoresis are determined by the sequence of the molecule rather, than by its length (Lerman et al., 1984).

Denaturing gradient gel electrophoresis, can be applied in two configurations, both parallel and perpendicular. Both require gels of uniform polyacrylamide density maintained at a constant temperature by immersion in a bath near the melting temperature of DNA

Because of the sensitivity of the system, precise temperature control is important. Ohmic heating raises the average temperature of the gel about 1.4^{0}C above the bath under typical conditions (150 V applied, 0044 M total cation, 1.5 mm gel). The environmental variables promoting melting are incorporated in an equivalent temperature T' such that $T' = T_{0} + gC$, where T_{0} is the temperature of the gel determined by the bath and internal heating, C is the concentration of the denaturing solvent, and g is the linear coefficient for the first derivative of melting temperature with respect to solvent concentration (Lerman, 1984). The slope dT_{m}/dC urea = 2.5^{0}C (Klump and Burkart, 1977).

1.13.1 Perpendicular Gradients.

In one configuration, which is termed a perpendicular gradient, a uniform electrical field is applied perpendicular to the direction of the gradient, and each molecule moves along a straight contour of uniform denaturant concentration. The sample is then applied as a continuous line along the cathode edge of the gel either from solution or as a long band cut from a preliminary gel. From the sample, some molecules move through the gel at each level of denaturant concentration. Their migration velocity is determined by the equilibrium structure at that concentration and temperature. The electric field is applied until the fastest - moving molecules

approach the anode edge of the gel. At the edge where the denaturant concentration is very low the fragments are separated according to their length as in conventional gel electrophoresis whereas at the high denaturant edge the apparent mobility is very much smaller. Thus each fragment generates an S-shaped curve with a steep inflection at a characteristic position along the gradient.

The details of the sigmoid curves differ reproducibly from fragment to fragment. Some have a flat baseline before undergoing the sharp change while in others the change is much less sharp, and in others the transition is multiphasic. The changes in mobility across the gradient closely correspond to the extent of loss of helical structure attributable to the denaturing equilibrium.

1.13.2 Parallel Gradients.

In a parallel gradient the migration is in the same direction as the gradient, so that the molecule moves continually into a higher concentration of denaturing solvent. In this system the initial velocity is determined by the length of the molecule as in conventional electrophoresis, but when the molecule reaches a depth in the gradient corresponding to the abrupt decline in mobility seen in perpendicular gradients there is little further travel. In general, further advance effects further reduction in mobility. The gradient patterns become almost time-independent after the molecules that are retarded deepest in the gradient have reached that level, and the focusing provides high resolution.

1.14 Aims of Present Study.

The present study was focused on the molecular pathology of Duchenne and Becker muscular dystrophy. The overall aim of the project was to develop mutational screening strategies for the disease. The specific targets can be summarised as follows:

i) to complete the deletion analysis of all Scottish DMD/BMD cases with the cDNA probe 5b-7; and to screen using cDNA subclones 9, 10 and 11-14, DMD/BMD patients who showed no deletion or duplication after DNA analysis with the cDNA probes 1-2a, 2b-3, 4-5a, 5b-7 and 8.

ii) to develop, perform and evaluate a deletion screening in the DMD/BMD patients by means of the polymerase chain reaction (PCR).

iii) to compare the findings obtained from the multiplex PCR deletion screening with those already available from the deletion screening using the cDNA probes

iv) to develop and apply the techniques of Single-Strand Conformational Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE) and Amplification Mismatch Detection (AMD) analysis for mutational screening on PCR amplified samples of boys affected with Duchenne/Becker muscular dystrophy whose molecular pathology remained unknown despite the application of both Southern analysis and PCR amplification assay.

v) to confirm any mutations detected using the techniques mentioned above, by direct genomic sequence, and

vi) to compare the detection efficiency of SSCP, AMD and DGGE in the detection of sequence alterations.

CHAPTER II

MATERIALS AND METHODS

2.1 Patients.

The patients (110 characterised as DMD and 22 as BMD) in this study were referred to the Duncan Guthrie Institute of Medical Genetics - Glasgow, which is responsible for carrier detection and prenatal diagnosis of X-linked muscular dystrophies as part of the Scottish Molecular Genetics Consortium. Currently in Scotland over 250 pedigrees have been ascerained with at least one member affected by X linked muscular dystrophy. Each case was clinically assessed and investigated in one of the genetic clinics associated with the Scottish consortium. Muscle histology was undertaken in each of the index cases included, and DMD was differentiated from BMD using the age at which the patients became chairbound, as described by Emery (1988). All index cases had calf hypertrophy and markedly raised serum creatine kinase levels. Care was taken to exclude possible cases of autosomal recessive limb-girdle dystrophy. In three cases (GMG 7327, GMG 7484, and GMG 8337), having a BMD phenotype, no pedigree information was able to provide definite evidence of X-linked inheritance.

2.2 Features of cDNA Probes.

Both cDNA probes 5b-7 and 9-14, used in this study are available from the American Type Culture Collection (12301 Parklawn Drive, Rockvill, Maryland 20852, USA) where they were deposited by Dr. Luis Kunkel. The vector used is Bluescribe and the size of the inserts are 2.5 kb and 6.1 kb respectively. They contain no repetitive sequences and they can be released by an EcoR I digest. Simultaneous digestion of the cDNA 9-14 with the restriction endonuclease BamH I gives cDNA probes 9 (1.2 kb), 10 (0.8 kb) and 11-14 (4.1 kb).

Probe 5b-7 detects genomic Hind III fragments: 0.45, 0.5, 1.3, 1.5, 1.8, 4.1, 4.2, 6.0, 6.2, 10.0, 11.0 and 18.0 kb. When Bgl II digested DNA is hybridised with this probe the following fragments are detected: 2.8, 3.1, 3.3, 3.4, 3.5, 6.1, 10.8, 13.2 and 21.0 kb. cDNA probes 9, 10 and 11-14 hybridise to the Hind III and Bgl II fragments as follows. cDNA 9/Hind III: 1.0, 2.3, 6.0, 7.8, 8.3, 8.8 kb; cDNA 9/ Bgl II: 4.1, 6.1, 8.0, 9.5, 16.0 kb; cDNA 10/ Hind III: 2.4, 2.55, 2.8, 3.5, 6.0, 6.6, 12.0 kb; cDNA 10/ Bgl II: 1.2, 1.4, 2.6, 3.3, 4.6, 6.5, 11.0 kb; cDNA 11-14/Hind III: 1.45, 1.5, 1.8, 1.9, 2.1, 5.9, 6.0, 6.8, 7.8, 10.0 kb; cDNA 11-14/Bgl II: 1.4, 4.0, 5.0, 10.0, 18.5 kb (Darras et al., 1988).

A Bgl II RFLP detected by cDNA 5b-7 consists of a 2.3-kb allele and a second allele of

7.0 kb. An additional two allele Bgl II RFLP, 28 and 24 kb, is detected by cDNA 11-14 (Darras et al., 1988).

2.3 Plasmid Preparation.

All the solutions mentioned in the present section as well as in the rest of this chapter are listed in an alphabetical order in Appendix I.

A culture containing 50 µl of glycerol stock from the plasmid of interest in 100 ml of L-broth (100 µg/ml ampicillin) was incubated overnight with shaking at 37^{0}C. The culture was left to stand on ice for 30 minutes before being spun at 5 K for 10 minutes at 4^{0}C (Sorval RC-5B). The bacterial pellet was resuspended in 4 ml of Solution I, containing 5 mg/ml lysozyme (Sigma) freshly prepared, and kept on ice for 30 minutes. Then 8 ml of freshly made Solution II were added and the suspension was further mixed until it became clear. After another 5 minutes on ice 6 ml of Solution III were added, the suspension was mixed and kept on ice for 30 minutes, followed by a centrifugation at 7 k for 5 minutes at 4^{0}C. The supernatant was filtered and the plasmid DNA was precipitated by adding 0.6 volumes of iso-propyl-alcohol, mixed and spun at 8.5 K for 20 minutes at 4^{0}C. 10 ml of Solution IV containing 80 µg/ml boiled RNAase A were added to the pellet, vortexed briefly and incubated at 65^{0}C for 30 minutes. After a phenol and a chloroform extraction, the aqueous phase was precipitated with a tenth volume 3 M sodium acetate (pH=7) and two volumes of 100% ethanol at -70^{0}C for a minimum of 2 hours. Plasmid DNA was pelleted (12 K for 10 minutes), washed twice in 5 ml of 70% ethanol, dried and dissolved in 1 ml of TE buffer.

2.4 Isolation of Inserts.

EcoR I - BamH I digestion of 10 µg plasmid DNA was performed in a total reaction volume of 80 µl, containing 14 µl of BRL "React 3" buffer, 2 µl of 0.1 M spermidine (Sigma), 6 µl of EcoR I (8 U/µl) and 8 µl of BamH I (7U/µl), at 37^{0}C overnight.

The digest was then loaded using 10 µl of loading mix II on a 1% Sea Plaque agarose (FMC) gel, 10 x 13 cm, prepared in 1 x electrophoresis buffer. Gels were subjected to constant current electrophoresis at 100 mA, stained in a 5 µg/ml solution of ethidium bromide in 1 x electrophoresis buffer, and visualised over ultra-violet light.

Inserts were excised using a sterile scapel blade, transferred to a Sarsdedt tube, weighed, and a volume of sterile distilled water equivalent to three times the weight of the insert was added. Then the tube was placed at 100^0C for 10 minutes, and the inserts were either used directly for oligonucleotide labelling or stored at -20^0C.

2.5 Preparation of Radioactively Labelled Probes by Random Oligonucleotide Primer Extension Labelling.

The procedure was carried out essentially as in Feinberg & Vogelstein (1984) using a random primed DNA labelling kit (Boehringer Mannheim). The insert to be labelled was boiled for 5 minutes and left at 37^0C for 10 minutes. A 40 µl reaction was set up with 20 µl of insert, 2 µl of each dATP, dGTP, dTTP, 4 µl of 10 x reaction mixture, 4 µl of double distilled water, 5 µl α-^{32}P dCTP (Amersham International, specific activity 3000 Ci/mmol) and 4 units of Klenow enzyme labelling grade. The reaction was incubated at 37^0C for two hours, and stopped by the addition of 2 µl of 0.5 M EDTA pH=8. Incorporated labell was resolved from unincorporated nucleotides on a pre-packed Nick Column (Pharmacia), containing Sephadex G-50, and eluted in 400 µl 1 x SSC buffer.

2.6 Preparation of Genomic DNA.

DNA was isolated from peripheral blood lymphocytes, and harvested lymphoblastoid cell lines based on the protocol described by Kunkel et al.(1977). Fresh blood samples were mixed with anticoagulant and stored at -20^0C. After thawing, 40 ml of lysis buffer were added to 10 ml of blood, and the contents were mixed thoroughly. This was incubated on ice for 10 minutes, spun at 2.8 K for 10 minutes at 4^0C in a IEC DPR-6000 centrifuge, and the supernatant was discarded. After this step the preparation from the blood was as for the cell lines. The pellet was recuspended in 10 ml of 0.075 M NaCl, 0.024 M EDTA pH=7.5 by vigorous pipetting and 500 µl of 10% SDS and 200 µl of 10 mg/ml proteinase K were added. The tube was mixed gently and incubated at 37^0C overnight. After the incubation, samples were extracted twice with phenol/ chloroform, followed by a chloroform extraction to remove any traces of phenol. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate pH=5.6 and two volumes of absolute ethanol. The DNA fibres were spooled out using a sealed pasteur pipette, rinsed in 70% ethanol, air dried and dissolved in 0.3 - 0.6 ml of TE buffer, pH=7.5. Samples were left to disolve at 4^0C for a minimum of 24 hours before being used.

Alternatively, DNA was extracted from blood samples using the method described by Miller et al., (1988). According to this protocol 5 ml of thawed blood were initially mixed with 40 ml of lysis buffer, mixed gently and centrifuged at 2.8 K for 10 minutes at 4^0C. The pellet was resuspended in 3 ml of nuclei lysis buffer, 200 µl of 10% SDS. 100 µl proteinase K (10mg/ml) were added, and the samples were incubated at 37^0C overnight. 1 ml of 6 M NaCl was then added, and after mixing vigorously for 15 seconds the samples were spun at 2.5 K for 15 minutes at room temperature. The DNA was precipitated by adding 2 volumes of ethanol, washed in 70% ethanol, air dried and left to disolve in 500 µl of TE at 55^0C for about 30 minutes. A phenol/chloroform extraction followed, and the DNA was precipitated from the upper aqueous phase with two volumes of ethanol. The DNA fibres were spooled out as before and resuspended into 500 µl of TE.

The final DNA concentration was estimated by measuring the optical density at 260 nm in a Perkin Elmer 6000 spectrophotometer. For a 10 µl dilution in 1 ml of TE, the DNA concentration in µgr/ml was obtained by multiplying the optical density by five thousand. To estimate the quality of the DNA preparation the OD_{260}/OD_{280} was calculated.

2.7 DNA Restriction Digests, Gel Electrophoresis of DNA and Ethidium Bromide Staining of the Gel.

Digests of genomic DNA were carried out using 7.5 µg of DNA at 37^0C overnight. The 40 µl final volume reaction consisted of DNA, 4 µl of Hind III (NBL, 12 U/µl) or Bgl II (NBL, 8 U/µl) restriction enzyme, 4 µl of React 2 or React 3 (BRL) respectively, 2 µl of 0.1 M spermidine (Sigma), and an approriate volume of sterile distilled water. The enzymes were added to the digest last and the solution spun briefly before incubation.

Gel electrophoresis of the digested DNA was carried out in 0.9% gels SEA KEM GTG agarose (FMC), 20 x 20 cm in size. The gels were made and run usually overnight at 70 mA, in 1 x electrophoresis buffer using the gel electrophoresis Apparatus GNA 200 (Pharmacia).

Samples were spun briefly and one-sixth volume loading mix II was added. DNA was sized using the 1 kb Ladder (BRL) which has molecular weights of 210, 298, 344, 394, 516/506, 1018, 1635, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198 and 12216 kb.

After electrophoresis the gel was stained in a dilute ethidium bromide solution (5 μg/ml in 1 x electrophoresis buffer) for 10 minutes and destained in 1 x electrophoresis buffer for 5 minutes. The gel was then visualised on a U.V. transilluminator (316 nm, Fotodyne) and photographed with Polaroid type 667 (black and white) film in a Polaroid instant print camera fitted with a Kodak 22A Wratten filter.

2.8 Southern Transfer and Hybridisation of Southern Blots.

Southern transfer was performed according to the protocol developed by Southern (1975). Gels were soaked in 0.2 M HCl for 15 minutes to depurinate the high molecular weight DNA and facilitate transfer. The DNA was then denatured in 0.5 M NaOH, 1.5 M NaCl for 20 minutes twice, and neutralised by two 30 minutes washes in 0.5 M Tris-HCl pH=7.4, 3 M NaCl. All washes were carried out with constant agitation. The gel was then transferred to a platform covered in a 3MM paper (Whatman) wick soaked in 10 x SSC buffer which dipped into a reservoir of this buffer beneath the platform. A piece of Hybond-N membrane (Amersham international plc.) labelled and cut to fit the gel exactly was laid gently on the gel. Two sheets of 3MM paper soaked in 2 x SSC and cut to size were placed on top of the membrane. The remaining area of the wick was covered with plastic sheeting and a stack of paper hand towels (Bowater - Scott), a glass plate and a small weight placed on top of the gel.

The transfer was left to proceed for 18 to 24 hours. At the end of the transfer, the filter was removed from the gel and rinsed in 2 x SSC. It was then air-dried and baked at 80^{0}C for 5 hours to fix the DNA. The transfer was confirmed by staining the gel and visualiing on a U.V. transilluminator.

Filters were wetted in 1 x SSC prior to pre-hybridisation. Prehybridisation and hybridisation were performed in the same buffer. Filters were placed in plastic bags and three sides were sealed carefully. Sonicated salmon sperm DNA was boiled for 10 minutes and added to 5 to 6 ml of hybridisation solution, to a final concentration of 10 μl/ml. Prehybridisation was performed at 65^{0}C overnight. The labelled probe was boiled for 10 minutes, cooled rapidly on ice and added directly to the prehybridised filter. Any bubbles were removed before sealing the bag. Hybridisation was performed for 48 hours at 65^{0}C.

After hybridisation the filters were washed in 2 x SSC, 0.1% SDS for 20 minutes at room temperature, followed by 20 minutes washes at either 1 x SSC, 0.5 x SSC or 0.1 x SSC, 0.1% SDS at room temperature or at 65^{0}C, depending on the stringency required. The filters were then air-dried until slightly moist, placed between plastic sheets and autoradiographed at -70^{0}C

with "Dupont lightning plus" intensifying screens and Kodak AR-5 film for 24 hours to one week. Films were developed using Kodak XAR developer and fixative in a Fuji RGII automatic film processor.

After autoradiography the radioactive probe was removed by gentle shaking the filter in 0.4 M NaOH for 45 minutes, followed by a 45 minutes wash in 0.5 M Tris-Cl pH=7.4, 3 M NaCl and one rinse in 1 x SSC, 0.1% SDS. The filter was then ready for re-hybridisation with another probe as detailed above.

2.9 PCR Amplification Primers.

Eighteen separate oligonucleotide primers, designed by Chamberlain and his co-workers (1990), were used in this study for the amplification of DNA from the DMD region. The synthetic oligonucleotide primers were prepared by Oswel DNA Services, Department of Chemistry - University of Edinburgh , including an ion-exchange chromatography step. The purification step proved to be necessary for a successful multiplex PCR amplification since their quality was very much improved. That was easily observed by running 500 ng of each primer before and after the purification step, on a 20% polyacrylamide gel, 16 x 16 cm at 2000 V for 1 hour, figure 2.1.

Their characteristics including: the sequence of all nine primer sets, the exon number or the size of the genomic Hind III fragment that the exon is located on, the size of each exon and the amplified fragment obtained, are described in Table 2.1. The relative location of the nine amplified regions, designated by a letter, relative to a number of commonly used diagnostic probes is shown in figure 2.2. For the remainder of this manuscript, these exons will be referred to as a, b, c, d, e, f, g, h, and i.

All eighteen oligonucleotide primers were between 23-28 bases long to permit a high stringent annealing and therefore greater specificity. The G/C content varied from 40-50%. The priming sites were either overlapping the exon-intron borders or situated in the introns. This ensured that alterations which disrupt RNA splicing leaving the exon intact, could be detectable. Also, use of intronic sequences as priming sites permitted greater flexibility in choosing the size of the region to be amplified, facilitating resolution of the PCR reaction products on an agarose gel. Acrylamide gel electrophoresis would have been necessary if amplification was performed from within exons, as exon sizes were within a narrow range.

2.10 Multiplex Genomic DNA Amplification.

DNA isolated from either peripheral lymphocytes or from Epstein-Barr virus transformed

lymphoblastoid cell lines by the methodology described in paragraph 2.6, was used as a template in the PCR reaction.

For the PCR reaction a 0.5 ml sterilised microfuge tube was used. The reaction mix, which was made up to a total volume of 50 µl with double distilled water , was set up using 5 µl of GeneAmp 10 x PCR reaction buffer (Perkin Elmer Cetus), 16 µl of dNTP's mix containing 1.25 mM of each nucleotide, 2% dimethylsulfoxide (DMSO) and 30 pmol of each oligonucleotide primer. The concentration of each oligonucleotide primer was calculated according to the method described in Appendix II. All reagents were kept at -20^{0}C, vortexed briefly after thawing and spun down before pipetting with positive displacement pipettes (Lab systems). The mix was irradiated for 15 minutes in an Amplirad before the addition of 0.5 - 1 µg of template (Sarkar and Sommer, 1990). Amplirad is a high intensity shortwave cabinet with 4 x G875 germicidal lamps, 1.8 watts each, 254 nm wavelength. The samples were then heated at 95^{0}C for 5 minutes, and 3 units of AmpliTaq DNA Polymerase (Perkin Elmer Cetus 5 U/µl) were added. Each PCR reaction mix was overlayed with 50 - 100 µl of light mineral oil (Sigma), and centrifuged for 5 seconds before it was placed in a Techne PHC 1 automatic thermocycler.

For each set of PCR reactions a positive and a negative control was prepared simultaneously. In the positive control the template added was genomic DNA from an individual with no abnormality in the area of the DMD gene. In the negative control no DNA was added.

The PCR temperature cycling protocol was as follows. Initial denaturation was for 1.5 minutes at 94^{0}C. Subsequent denaturation was for 0.5 minutes at 94^{0}C. Annealing and extension were carried out at 53^{0}C for 0.5 minutes and 64^{0}C for 4.2 minutes respectively, with a final extension time of 7 minutes at 64^{0}C. The number of cycles performed was 31. All samples were kept at 4^{0}C until analysis.

2.11 Amplification of Single Exons by PCR.

For the amplification of single exons slight modifications to the protocol described above were necessary to optimise the yield of the PCR reaction specifically for each different primer set. The final concentration of the reagents in the PCR reaction mix, 50 µl total volume, were kept the same with the exception of dNTPs whose amount was reduced by 50%. The reactions were also exposed to UV light (254 nm) for 15 min before the addition of DNA (Sarkar, 1990).

a)

b)

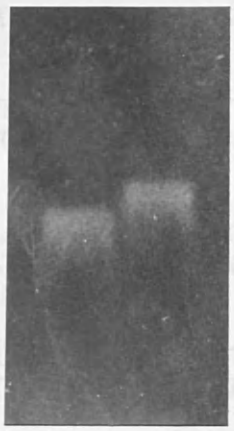


FIGURE 2.1: Electrophoresis of synthetic oligonucleotide primer in a polyarylamide gel a) before and b) after the purification step using ion exchange chromatography. The primers shown are those flanking region b of the dystrophin gene.

EXON and SIZE	PRIMER SEQUENCE	SIZE OF AMPLIFIED PRODUCT (bp)
i. Exon 4 / 8.5 kb Hind III 78bp; (probe 1-2a)	F-TTGTCGGTCTCTCTGCTGGTCAGTG R-CAAAGCCCTCACTCAAACATGAAGC	196
a. Exon 8 / 7.5 kb Hind III 182bp;(probe 1-2a)	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360
g. Exon 12 / 4 kb Hind III 151bp; (probe 2b-3)	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCCAACATAAGATACACCT	331
b. Exon 17 / 1.7 kb Hind III 178bp;(probe 2b-3)	F-GACTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTTCC	416
c. Exon 19 / 3.5 kb Hind III 88bp; (probe 2b-3)	F-TTCTACCACATCCCATTTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459
d. Exon 44 / 4.1Kb HindIII 148bp; (probe 5b-7)	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCTTCAGAACCTGATTCT	268
e.Exon 45 / 0.5Kb HindIII 176bp; (probe 5b-7)	F-AAACATGGAACATCCTTGTGGGGAC R-CATTCCTATTAGATCTGTCGCCCTAC	547
f. Exon 48 / 1.2/3.8Kb HindIII 186bp; (probe 8)	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506
h. Exon 51 / 3.1Kb HindIII 233bp; (probe 8)	F-GAAATTGGCTCTTTAGCTTGTGTTTC R-GGAGAGTAAAGTGATTGGTGGAAAATC	388

TABLE 2.1: Features of the primer sets used for the amplification of regions of the dystrophin gene (Chamberlain et al., 1990). Letters a-h designate each amplified region of the dystrophin gene. For each region the number of the exon included is listed and the size of the genomic Hind III fragment that the exon is located on after hybridisation to a cDNA probe. The sequence of the primers are shown in 5'-3' orientation, where F: forward primer, hybridises 5' of the exon; and R: reverse primer, hybridises 3' of the exon.

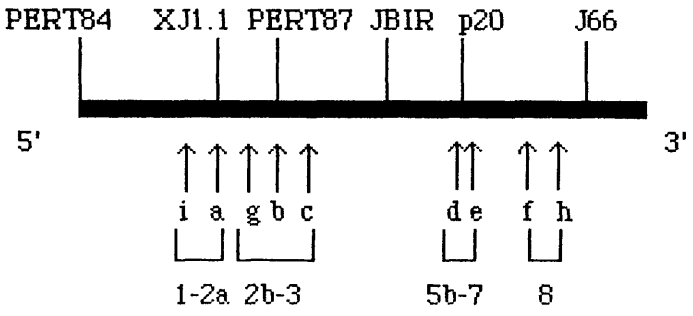


FIGURE 2.2: Relative position of the DMD amplified fragments. The arrows, a-i, indicate the position of the fragments amplified by the primer sets described at table 2.1 relative to several genomic probes. The cDNA probes hybridise to the amplified exons are indicated underneath.

2.5 units of AmpliTaq polymerase (Perkin Elmer Cetus) were used per reaction, which was overlayed with 50 µl of mineral oil (Sigma).

All samples were heated at 95^{0}C for 5 minutes before the addition of the enzyme, and then with the enzyme at 94^{0}C for 90 seconds before one of the following reaction cycles was applied 28 times using a Techne PHC 1 automatic thermocycler. With primer sets i and d: 94^{0}C for 30 seconds, 55^{0}C for 30 seconds and 73^{0}C for 50 seconds. Final extension time 7 minutes at 73^{0}C. With primer sets a, b, g and h: 94^{0}C for 60 seconds, 55^{0}C for 60 seconds and 73^{0}C for 120 seconds. Final extension time 7 minutes at 73^{0}C. With primer set c: 94^{0}C for 30 seconds, 55^{0}C for 30 seconds and 73^{0}C for 55 seconds. Final extension time 7 minutes at 73^{0}C. With primer set e: 94^{0}C for 60 seconds, 55^{0}C for 80 seconds and 73^{0}C for 180 seconds. Final extension time 8 minutes at 73^{0}C. With primer set f: 94^{0}C for 30 seconds, 53^{0}C for 30 seconds and 65^{0}C for 55 seconds. Final extension time 7 minutes at 65^{0}C.

2.12 PCR Amplification of Degraded Genomic DNA.

An increased amount, 2 µg, of degraded genomic DNA was added to a 0.5 ml sterilised microfuge tube containing : 2% DMSO, 200 µM of each dNTP, 1 x GeneAmp PCR reaction buffer (Perkin Elmer Cetus), and 30 pmol of each of the four oligonucleotide primers necessary for the amplification of regions g and h. The samples were irradiated for 15 min with 254-nm ultraviolet bulbs in an Amplirad before the addition of the template (Sarkar and Sommer, 1990), and heated at 95^{0}C for 5 minutes, before 2.5 units of AmpliTaq DNA Polymerase (Perkin Elmer Cetus 5U/ml) were added. For the amplification of the degraded genomic DNA 45 cycles were performed on a Techne PHC 1 automatic thermocycler; each cycle 94^{0}C for 1 minutes, 55^{0}C for 1 minutes,72^{0}C for 2 minutes. The final extension time was 7 minutes at 72^{0}C.

2.13 PCR Amplification of Genomic DNA Extracted from Haematoxylin and Eosin Stained Sections.

Haematoxylin and eosin (H&E) stained sections, prepared from blocks of muscle tissue, and processed for histological examination were kindly provided by Professor A. Emery (University of Edinburgh). The sections were 4 to 5 mm thick, cut from the paraffin-embedded tissue block, de-waxed, stained, and then mounted on a slide in a resin under a glass coverslip. The slides had been stored at room temperature and they were between 1 - 15 years old.

The slides were soaked in histoclear solution (National Diagnostics) for a period of four days to one week until the coverslip could be removed gently. They were rinsed with 100% ethanol and left to dry before DNA was extracted using one of the following procedures. The tissue section was scraped from the glass slide using a sterilised razor blade and transfered to a sterilised 0.5 ml microfuge tube. 50 µl of nuclei lysis mix and 5 µl of proteinase K (10 mg/ml) were added, and the samples were incubated for 3 hours at 50^0C. Alternatively the slides were treated directly with proteinase K (10 mg/ml) in 40 µl of nuclei lysis mix, under a sterile coverslip sealed with rubber cement, at 50^0C for 3 hours (Jonveaux, 1991). The coverslip was gently removed and the slide rinsed with 100 µl of nuclei lysis mix. The DNA was used as a template for a PCR reaction either without any purification, or after being purified using the GeneClean II kit (BIO 101 Inc., Stratech Scientific). According to the protocol provided by the manufacture, 150 µl of NaI and 5 µl of glass milk were added mixed vigorously and left on ice for 10 minutes. The pellet was washed three times with 400 µl of diluted ice-cold New solution, and resuspended in 20 µl of T.E. buffer. After incubation at 50^0C for 3 minutes the glass milk was spun down, and 4 µl of the supernatant were used per PCR reaction. In the case of unpurified DNA the whole sample was boiled for 10 minutes, and 10 µl were transferred to the PCR reaction. Amplification of single exons was performed as it is described in paragraph 2.11.

2.14 Analysis of PCR Amplification Products.

On completion of the reaction, the samples were spun down for 5 seconds and the mineral oil was removed with a pipette. 8 µl of each sample was electrophoresed on either a 1.4% SEAKEM agarose (FMC Bioproducts) or a 3% NuSieve agarose gel (FMC Bioproducts), containing 0.5 µg/ml ethidium bromide, at 4 mA/cm in 1 x TBE buffer.

The results were visualised by placing the gel on a 254-nm Fotodyne 1000 transilluminator, and photographed using a Polaroid instant print camera fitted with a Kodak 22A Wratten filter. The type of film used was a Polaroid type 667 (black and white) film.

2.15 Phosphorylation of the 5' Ends of the Oligonucleotide Primers with T_4 Polynucleotide Kinase.

The synthetic oligonucleotides were prepared without a phosphate group at their 5' termini, and could therefore be labelled by transfer of the γ-^{32}P from [γ-^{32}P]ATP using the enzyme T_4 polynucleotide kinase (Maniatis, 1982)

30 pmol of each oligonucleotide primer were labelled with 2.5 µl [γ^{32}P]ATP (50 pmol, 3000 Ci/mmol, Amersham), using 1 µl of T_4 polynucleotide kinase (10 U/µl, NBL) in 10 µl of 0.05 M Tris (pH 7.6), 0.01 M $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine (Sigma), 0.1 mM EDTA (pH 8.0). The reaction mix was incubated for 45-60 minutes at 37^{0}C. The T_4 polynucleotide kinase was inactivated by heating at 68^{0}C for 10 minutes.

The efficiency of transfer of γ^{32}P to the primer was monitored by absorption to DE81 filter paper (Whatman) . 0.5 µl of the reaction mixture were applied to two separate filter paper discs . after incubation at 37^{0}C. The oligonucleotide binds tightly to the positevely charged filters whereas unicorporated radiolabell is removed by repeated washings with 0.5 M Na_2HPO_4. The percentage of radiolabell transferred from [γ^{32}P] ATP to the oligonucleotide was calculated based on the results of a 1215 rackbet liquid scincillation counter (LKB Walla). Under the conditions described above, approximately 90% of the radioactivity was transferred to the oligonucleotide.

2.16 PCR-SSCP Analysis.

The final concentration of the reagents in the PCR reaction mix, which was made up to a total volume of 25 µl with double distilled water were 1 x PCR reaction buffer (Perkin Elmer Cetus), 200 mM of each dNTP, 15 pmoles of each of the labelled primers, and 0.5-1 µg of genomic DNA. Kination products were added without purification. The PCR amplification was performed according to the protocols described in paragraph 2.11.

The amplified product was diluted 1:25 with 0.1% SDS, 10 mM EDTA, followed by a 1:2 dilution in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. Two samples were prepared for each individual. One sample was heated at 80^{0}C for 10 minutes to denaturate the DNA. Both samples were loaded on to a 6.5% non-denaturing polyacrylamide gel (20 cm x 40 cm x 0.4 cm) containing 10% glycerol. The extent of cross-linking (%C) was 3.3. Electrophoresis was carried out at 25-30 W for 6-9 hours, with a fan heater, set on cold, directed at the gel as a cooling device. The gels were dried on a gel dryer, model 583 (Biorad). After drying they were placed on a sheet of Kodak XAR-5 film in an autoradiographic cassette with "Dupont lightning plus" intensifying screens and exposed for 3-16 hours at -70^{0}C.

2.17 Sample Preparation for Amplification Mismatch Detection Analysis.

The method used for the amplification mismatch detection analysis was a modification of the protocols described by Cotton et al. (1988), Montandon et al. (1989) and by Boyd and Glasgow (personal communications).

The region e of the dystrophin gene from the individuals to be examined and from a normal individual, were amplified by PCR according to the method described in paragraph 2.11. The oligonucleotide primers used in the PCR of the control samples were radioactive end-labelled according to the protocol provided in section 2.15, and the radioactive amplification product was the "probe" sequence necessary for the application of the amplification mismatch detection analysis.

After PCR amplification a sample of the unlabelled reaction products was run on a 1% SEA-KEM GTG agarose gel (FMC) to ensure efficient amplification and the remainder was purified using Geneclean (Bio 101) and eluted in a final volume of 20 µl. Detailed description of the purification method is given in section 2.13.

For the radioactive PCR product gel purification was performed in an apparatus for micro-electroelution developed by Sandhu and Kline (1989). This technique entailed i) cutting out a small block containing the band of DNA from an ethidium bromide stained agarose gel (1.2% SEA-KEM GTG agarose (FMC) in the currently described case), ii) placing the gel piece in a 1.5 ml microfuge tube transfixed on a 25-gauge hypodermic needle, and iii) performed the microelution as is shown in figure 2.3 for 2 hours, using 450 µl of 1 x TBE buffer. The gel block was pulled out and viewed under ultraviolet light to confirm the completion of electro-elution. DNA was extracted by adding 0.1 volume of 3 M sodium acetate and two volumes of ethanol to the buffer. Precipitation was for 15 min at -70^{0}C, and DNA was recovered by centrifigation for 10 min at 12000 rev/min. DNA pellet was resuspended in TE buffer at 28000 cpm/µl.

2.18 Chemical Cleavage of PCR Products.

A heteroduplex was formed by mixing the labelled probe with a 5 to 10- fold molar excess of amplified target DNA in 125 µl of 2 x hybrid buffer , made up to 250 µl total volume with double distilled water. The mixture was heated 5 minutes at 100^{0}C and annealed for 1 hour at 42^{0}C. The heteroduplex was precipitated with two volumes of 5 M sodium acetate, 2.5 volumes of iced-cold ethanol and 4 µl of glycogen (Boehinger Manheim), at -70^{0}C for 1 hour. The pellet was washed once with 70% ethanol and resuspended in 6 µl of double distilled water, approximately 1000 cpm/µl.

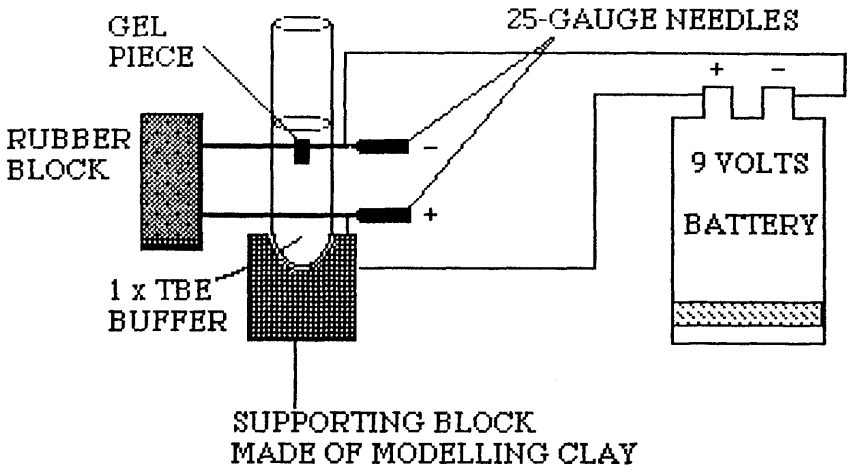


FIGURE 2.3: Diagrammatic representation of the microelution apparatus (Sandhu and Kline, 1989), used for the purification of the probe in the amplification mismatch detection (AMD) analysis.

Hybrid DNA was treated with 20 µl of a 4 M solution of hydroxylamine hydrochloride ph=6 (hydroxylamine modification) at 37^{0}C for 90 minutes. The reaction was stopped by transferring the mixture to ice and adding 200 µl of stop solution. DNA was precipitated with 750 µl of ice-cold ethanol. The DNA pellet was resuspended in 200 µl of 0.3 M sodium acetate pH=5.2. After a further ethanol precipitation, the DNA pellet was washed once with 70% (v/v) ethanol and dried.

Chemical cleavage of the C bases reacted with hydroxylamine was achieved by incubating the heteroduplexes with piperidine. 50 µl of freshly prepared 10% piperidine were added to the dry DNA pellet and incubated at 90^{0}C for 30 minutes. The reaction was stopped by placing the samples on ice for 2 minutes. DNA was precipitated with 50 µl 0.6M sodium acetate pH=5.2 and 300 µl of 100% ethanol. The pellet was washed once with 70% ethanol, and dried.

2.19 Radioactive Labelling of 1 kb Ladder (BRL).

The DNA fragments of the 1 kb ladder (BRL) were labelled by filling in of the 3' recessed ends with Klenow enzyme using the dATP, dGTP and dTTP nucleotides included in the Random Primed DNA labelling Kit (Boehringer Mannheim). 1 µg of 1 kb ladder was mixed with 1 µl of each dNTP, 2 µl React 3 buffer (BRL), 2 µl of α-^{32}P dCTP (Amersham international, specific activity 3000 Ci/mmol), 2 units of Klenow enzyme and made up to a final volume of 20 µl with double distilled water. After incubation at 37^{0}C for 30 minutes the volume of the reaction was increased to 100 µl and the labelled fragments were separated from other radioactive material on a pre-packed Nick Column (Pharmacia), containing Sephadex G-50, and eluted in 400 µl 1 x SSC buffer. The radioactively labelled 1 kb ladder was combined with loading mix II before being applied on a gel.

2.20 Electrophoresis of Chemical Cleavage Products.

Samples were incubated in 7 µl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, at 100^{0}C for 10 minutes before application to a 5% denaturing polyacrylamide gel. During electrophoresis the gel was maintained at 50^{0}C. Electrophoresis was performed until the xylene cyanol dye was at a distance of approximately 30 cm from the loading point. The gel was fixed by immersion for 15 minutes in a solution containing 10% glacial acetic acid and 10% methanol, dried and autoradiographed with "Dupont lighnting plus" intensifying screen using Kodak XAR-5 film at -70^{0}C for 72 hours.

2.21 Direct DNA Sequencing of an Asymmetric PCR product.

The PCR technique provides a simple and fast way of producing DNA templates for sequencing. Direct sequencing of the PCR products without an additional cloning step in addition to the benefit of simplicity, reduces potential errors due to imperfect fidelity of Taq polymerase Any random misincorporations in an individual template molecule will not be detectable against the much greater signal of the original sequence.

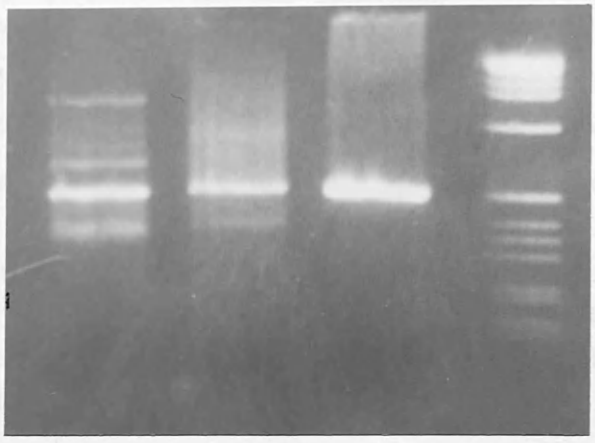
A double-stranded PCR amplified fragment used as a template in a sequencing reaction can cause many problems due to the rapid reassociation of the short linear template strands. Thus, a modified type of PCR, asymmetric PCR, has been developed which utilizes unequal concentration of the two amplification primers to produce single-stranded DNA of a chosen strand (Gyllensten, 1988). During the initial cycles most of the product is double-stranded, but as the low concentration primer becomes depleted an excess of one of the two strands, depending on which of the primers is limited , is generated.

The asymmetric PCR reaction was performed according to the protocol developed by Mgone (1991). An unequal ratio of 100:1 was used for the generation of single stranded DNA. 5 µl of a 10 µM and a 0.1 µM dilution of each primer were used in a PCR reaction in a total volume of 100 µl. The number of cycles was increased to 41 without any further modification in the concentration of the reagents, or in the cycle-profile used for the generation of double stranded DNA.

Reactions were monitored for the production of single-stranded DNA on a 1.2% TBE agarose gel, and visualised by staining with ethidium bromide (10 µg/ml). An example is shown in figure 2.4. Single-stranded DNA (ssDNA) runs slower than the double strand (dsDNA), and fluoresence is much reduced relative to an equivalent amount of double-stranded DNA.

Single-stranded DNA was purified from residual dNTPs and primers prior to the sequencing reaction. The mineral oil was removed and the DNA was precipitated by adding an equal volume of 4 M ammonium acetate and a double volume of isopropanol. After incubation at room temperature for 10 minutes, mixing occasionally, samples were centrifuged for 15 minutes at high speed using a IEC centra-4X microfuge. The pellet was washed once with 70% ethanol, dried and resuspended in 5-10 µl of double distilled water.

The sequencing reaction was performed using a Sequenase 2.0 kit (USB), according to the protocol provided. The primer limited in the asymmetric PCR was added to the annealing mixture in a final concentration of 0.1 µM. The annealing mixture containing 5 µl of single-stranded DNA (about 50% of the total product generated by the asymmetric PCR), 2µl of 5 x



1 2 3 4

FIGURE 2.4: Agarose gel analysis of single-stranded DNA generated by asymmetric PCR. 1 and 2) single-stranded DNA using unequal ratio 100:1; 3) double stranded DNA, product of a standard PCR; 4) 1 kb ladder (BRL).

annealing buffer was heated at 65^{0}C for 2 minutes and then cooled below 35^{0}C within 30 minutes. The labelling reaction consisting of 1 µl of 0.1 M Dithiothreitol (DTT), 2 µl labelling nucleotide mix (1.5 mM each of dGTP, dCTP and dTTP), diluted 1:5, 1000 mCi/mMol of a-^{35}S dATP and 3 units of Sequenase T7 DNA Polymerase, was incubated at room temperature for 5 minutes. 3.5 µl of the labelling reaction was transfered to 2.5 µl of each dideoxy termination mixture prewarmed at 37^{0}C. Each termination nucleotide mixture contains 80 µM of each dGTP, dATP, dTTP and dCTP, and 50 mM NaCl. In addition, the "G" mixture contains 8 µM dideoxy-dGTP; the "A" mix, 8 µM ddATP; the "T", 8 µM ddTTP; and the "C" 8 µM ddCTP. After 3 min incubation at 37^{0}C the termination reactions were stopped by adding 4 µl of stop solution, and stored at -20^{0}C until their analysis on a sequencing gel.

A sequencing gel can resolve DNA strands of n and n+1 nucleotides. Polyacrylamide gels 5% acrylamide (5%C), 7M Urea, were prepared for the analysis of the products generated by the sequencing reaction. Their size was 200 x 400 x 4 mm and the BRL model S1 sequencing system was used for running the gels using 1 x TBE buffer.

Samples were heated at 75^{0}C for 3 minutes before loading. Gels were run at 50^{0}C+ 5^{0}C for 2 to 6 hours. After electrophoresis the gels were soaked in 10% methanol, 10% acetic acid for 15 minutes and dried using a gel dryer model 583 (Biorad). The gels were autoradiographed with "Dupont lightning plus" intesinfying screen using Kodak XAR-5 film at -70^{0}C overnight.

2.22 Preparation of Samples Used in the Denaturing Gradient Gel Electrophoresis.

The samples analysed using the method of denaturing gradient gel electrophoresis were the same as those used in the PCR-SSCP analysis. The preparation of these samples is described in section 2.16. In those cases were a further DGGE assay was necessary, further amplification of genomic DNA for a particular area of the DMD/BMD locus from the individual to be tested or from normal controls was performed according to the protocols described in 2.11.

Either the amplification products of four different DMD/BMD samples (20 µl each) were combined with 40 µl of DNA amplified from the same region of a control sample and run on a perpendicular gel or, in later stages 20 µl of a single DMD/BMD sample were combined with 20 µl of amplified DNA from a normal individual.

2.23 Electrophoresis of Genomic DNA on Perpendicular Denaturing Gradient Gels.

Perpendicular denaturing gradient gels contain a fixed acrylamide concentration and a linearly increasing gradient of the denaturants (formamide and urea) perpendicular to the direction of the electrophoresis. For the preparation of perpendicular denaturing gradient gels spacers 0.75 mm thick, lightly greased, were placed on the sides and along the top edge of the glass plates. A spacer slightly shorter than the spacer used as a comb was inserted in the fourth side. The glass plates were clamped together with bulldog clamps. The frame was rotated 90^0 so that the side adjacent to the gap was now uppermost. The gel was poured through the gap which was then sealed with modelling clay.

For the preparation of the gradient a gradient former, model 230 (Biorad) was used. An equal volume (6 ml) of the stock solutions of denaturants and acrylamide (80% denaturant - 7.5% acrylamide and 0% denaturant - 7.5% acrylamide) with 1/200th volume 20% ammonium persulfate and 1/2000th volume of TEMED (N',N',N',N'-tetramethylmethylenediamine) in each, whose total volume would just fill the plates, was used. Before the pouring of the gel some of the solution of higher denaturant concentration was allowed to pass into the empty chamber of the gradient maker to ensure that no air bubbles block the passageway between the two chambers. Then the excess was transfered back to the other chamber with a pasteur pipette. The gel was poured using a Varioperpex II peristaltic pump (LKB), and at the same time the solution of higher denaturant concentration was stirred by a magnetic stirrer so that no air bubbles would be introduced into the monomer solution. After polymerisation the top spacer, used as a comb, was removed to reveal one, large, flat well.

For the electrophoresis of DNA a Protean II vertical electrophoresis apparetus (Bio-Rad) was used. A Varioperpex II peristaltic pump (LKB) circulated the buffer, 1 x TAE, from the water bath into the top (cathode) chamber. This was necessary to avoid significant increase in pH during electrophoresis. The gels were prerun for about 30 minutes to ensure that the temperature at the part of the gel containing no urea or formamide, was raised to 60^0C. A temperature indicator (Bio-Rad) was also applied to this region of the gel. The samples were loaded into a single large well at the top of a perpendicular denaturing gradient gel with 40 µl of loading mix II. 150V were applied for a sufficient time to allow the fragments to reach the edge of the gel were no denaturants were present. The gels were analysed as described in section 2.7.

2.24 A Computer-Derived Simulation of Melting Behavior of Known Sequence DNA Fragments.

The theoretical pattern of thermal stability of DNA molecules and the expected changes in

the electrophoretic mobility in a denaturing gradient gel were calculated using the computer programmes MELT 87 and MUTRAV respectively developed by Lerman and Silverstein (1987).

The melting programme MELT 87 calculates the theoretical melting map (the temperature at which each base will be at equilibrium with equal probability of helix or random chain configuration), of a known sequence of DNA using the Fixman-Friere (1977) modification of Poland's algorithm (1974) for DNA melting (PFF) (Lerman, 1984). The theory assumes that : a) the status of every base is described either helical or melted, b) the strength of the cooperativity is independent of the sequence, and c) the probability of melting of a sequence bounded by helix at both ends is suppressed according to the inverse 1.5-2 power dependence of the entropy of the loop on its strength. The PFF scheme of calculation depends on recursion relations between adjacent base pairs or base pair doublets as it proceeds along the sequence; partition functions and statistical weights are implicit in the probability relations. There are no a priori assumptions as to where the disordering will begin, nor are any assumptions made with respect to the limits of regions that melt at different temperatures; these are all consequences of the calculation. The intrinsic stability of each base pair is influenced by its nearest neighbours but not by interactions over a longer distance. Each base pair or each local group can be characterised by an enthalpy and entropy of melting near the temperature of 1:1 equilibrium. The entropy change is assumed to be uniform for all pairs. Also the gel matrix has no significant effect on the melting equilibrium. The values used for the intrinsic stabilities of the ten types of nearest neighbour base pair doublets, are those calculated by Gotoh and Tagashira (1981) for an aqueous enviroment containing about 0.02 M of sodium ion which is slightly different from the gel medium that contains in addition Tris ion as well as urea and formamide. Cooperativity and the improbability of forming melted loops, link the base pairs into regions, termed domains, of closely similar melting probability.

The length of the entire sequence, the first and the last bases in a selected fragment, the starting temperature of the calculation and the temperature increment between iterations are the data required by MELT 87 (Lerman,1987). Five of the output files contain temperature contours base by base, at which there is a particular, fixed equilibrium ratio between the melted and helical state. The probability levels are 5, 25, 50, 75 and 95%. In addition the programme returns files containing an estimate of the number of nonhelical bases at each temperature (pbr), the change in the fraction of nonhelical bases between temperature iterations (dtht), the log of the dissociation constant for complete separation of the strands at each temperature (dsklg), and the coherence (coop) which estimates the extent along the sequence of close coupling in the melting transition (in base pairs).

In the mobility programme MUTRAV melted strands are assumed to behave as independent branches joined at the boundary of the helical portion (Lerman and Silverstein, 1987). The mobility declines exponentially with the length of the melted portion of the molecule. The ratio

of the electrophoretic mobility of each partially melted molecule relative to that of the intact double helix is calculated by the relation $m/m_{()} = \exp(-Lm/Lr)$, where Lm is the total number of non-helical base pairs inferred from the melting algorithms, and Lr is the length of the flexible unit in the melted strand. The length of this unit appears in the programme as RETL and a value of 75 bases has been suggested appropriate for a number of medium-length molecules (Myers, 1987). A plot of MUTRAV versus T corresponds fairly closely to the curve given by a perpendicular gradient gel. It shows the temperature required for each drop in mobility, the steepness of the velocity transition, the number of domains that can be resolved, and an indication of the size of the domains.

2.25 Comparing Gels and Calculations.

An approximate relation between denaturant concentration and calculated equivalent temperature is DENCONC = 3.2 X (EQTEMP -57) where DENCONC is the concentration of the standard stock solution of urea and formamide in percent (v/v), EQTEMP is a temperature derived from the melting calculation assuming that the bath temperature is $60^{()}$C (Lerman, 1987). Ohmic heating raises the average temperature of the gel about $1.4^{()}$C above the bath under typical conditions: 150 V applied, 0.044 M total cation, 1.5 mm gel, (Lerman et al., 1984). The relative electrophoretic mobility as a function of equivalent temperature calculated by the computer programme MUTRAV neglects certain experimental aspects as the gradient of solvent viscocity that accompanies the gradient of denaturant concentration and the possible effects of denaturant on DNA charge density and porosity of the gel. Since the viscocity increment is effectively linear in urea-formamide concentration, the calculated curve by the MUTRAV computer programme can be corrected by dividing the calculated mobility at each point in the gradient by the relative viscocity, which has been found to be :

VREL = 1 + (4.3 X DENCONC X 10^{-3}), where DENCONC is the concentration of stock denaturant (v/v) in percent (Lerman and Silverstein, 1987).

CHAPTER III

RESULTS

3.1 Deletion Screening of DMD/BMD Patients Using cDNA Probe 5b-7.

Seventeen DMD/BMD patients were studied with cDNA probe 5b-7 using Bgl II-digested DNA, and an example of hybridisation patterns is presented in figure 3.1. One individual, GMG 4180, was deleted for the 3.1, 3.4, 6.1 and 10.8 kb fragments normally detected by cDNA probe 5b-7. Southern analysis of Hind III-digested DNA from the same individual, had previously shown a deletion of the following fragments: 18.0, 11.0, 6.2, 6.0, 4.2, 4.1, 1.8 and 1.5 kb (Cooke, personal communication). In the rest of the DMD/BMD cases screened by cDNA 5b-7, no deletion was observed in the region covered by this probe.

A correspondence of the Bgl II and Hind III fragments detected by cDNA 5b-7 was established based on the described deletion of individual GMG 4180. Since the 4.1, 0.5, 1.5 and 10 kb Hind III fragments were precisely mapped respectively to 3.4, 2.8, 3.3 and 3.5 kb Bgl II fragments (Darras et al., 1988) and Bgl II fragments in this region can be related to the Hind III exon-containing fragments as shown in figure 3.2.

3.2 Deletion Analysis of DMD/BMD Patients at the 3' end of the dystrophin gene.

The panel used for deletion screening at the telomeric end of the DMD/BMD locus consisted of those individuals showing no deletion or duplication after DNA analysis with the cDNA probes 1-2a, 2b-3, 4-5a, 5b-7 and 8 (Koenig et al., 1987). Two individuals, GMG 7589 and GMG 7641, were also included since their deletion extended to the most distal exon-containing Hind III fragment detected by cDNA 8.

Hind III and / or Bgl II digests were prepared and screened with the cDNA probes 9, 10 and 11-14, whose relative position is shown in figure 1.2. The number of patients tested with each probe and the number of deletions detected are shown in table 3.1.

Probe cDNA 9 reveals six Hind III fragments : 8.8, 8.3, 7.8, 6.0, 2.3 and 1.0 kb, with the last 1.0 kb band being a doublet. The same probe hybridises to five Bgl II fragments : 16.0, 9.5, 8.0, 6.1 and 4.1 kb in size (Darras et al., 1988). For the deletion screening using this probe both Bgl II and Hind III restriction enzyme digests were used. Seven deletions were detected varied in size from a single fragment, GMG 2790, GMG 4629 (figure 3.3), GMG 7589, GMG 8396 and GMG 9011 to the whole length of the DMD gene detected by this probe, GMG 7641 (figure 3.4). DMD patient GMG 7643 was deleted for the Hind III fragments 8.3, 7.8 and 2.3 kb, (figure 3.5). The analytical results of the thirty-six individuals tested with

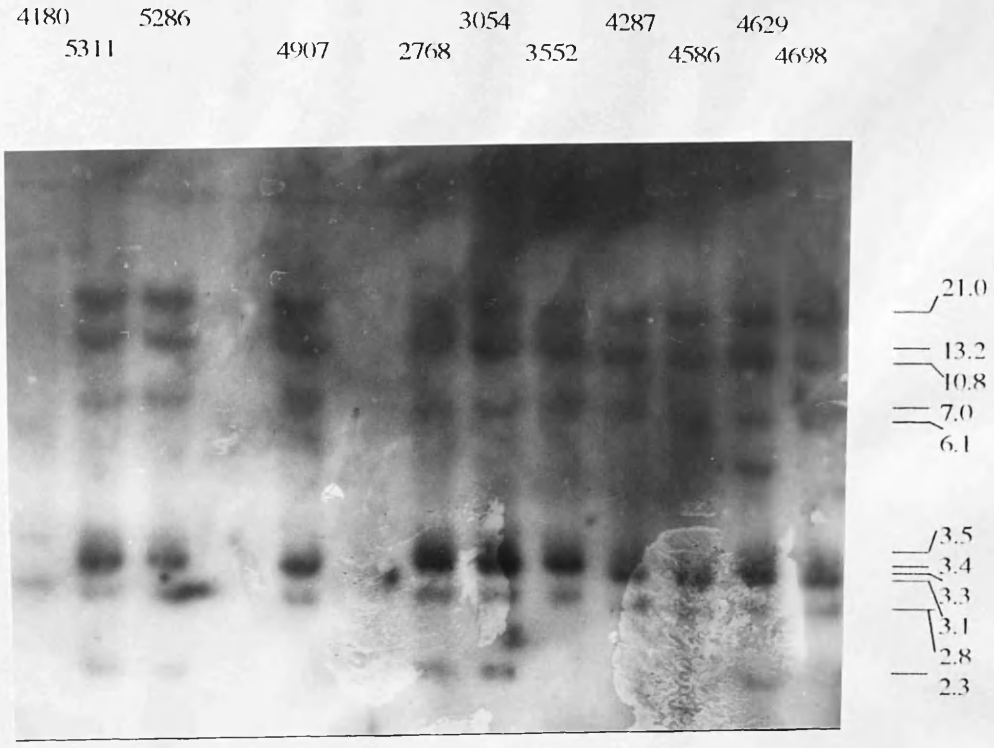


FIGURE 3.1: Panel of Bgl II-digested DNA from DMD/BMD patients hybridised with cDNA probe 5b-7. The size of the fragments is shown in kb.

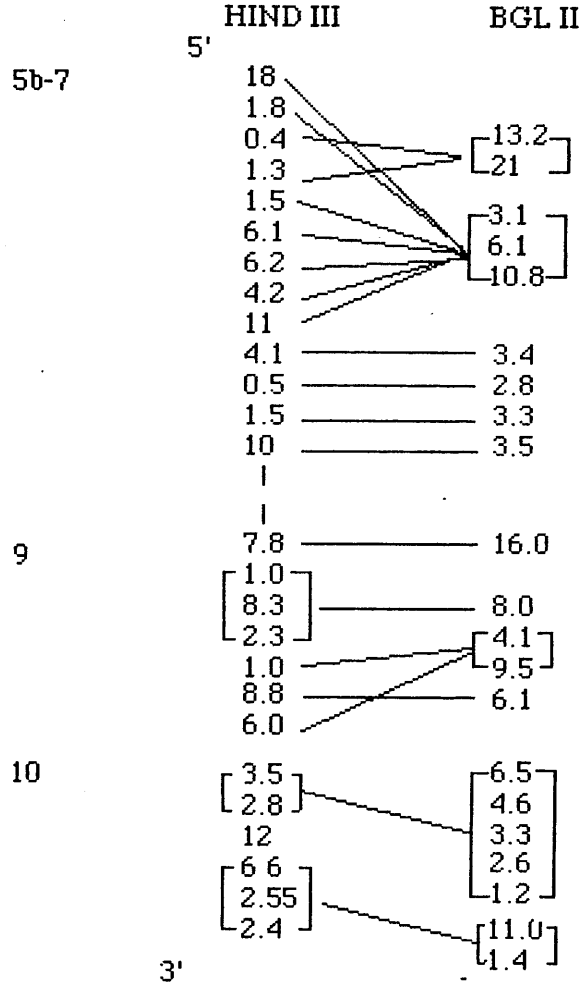


Figure 3.2: Mapping of Bgl II genomic fragments. Corresponding Hind III and Bgl II fragments, according to the results obtained in the present study and from Darras et al. (1988) are connected by lines. The orientation of the Hind III fragments is according to Koenig et al., 1989.

cDNA PROBE	NUMBER OF DNA SAMPLES TESTED	DELETIONS DETECTED
9	35	7
10	36	1
11-14	28	0

TABLE 3.1: Southern analysis of DMD/BMD patients using cDNA subclones 9, 10 and 11-14 (Koenig et al., 1988).

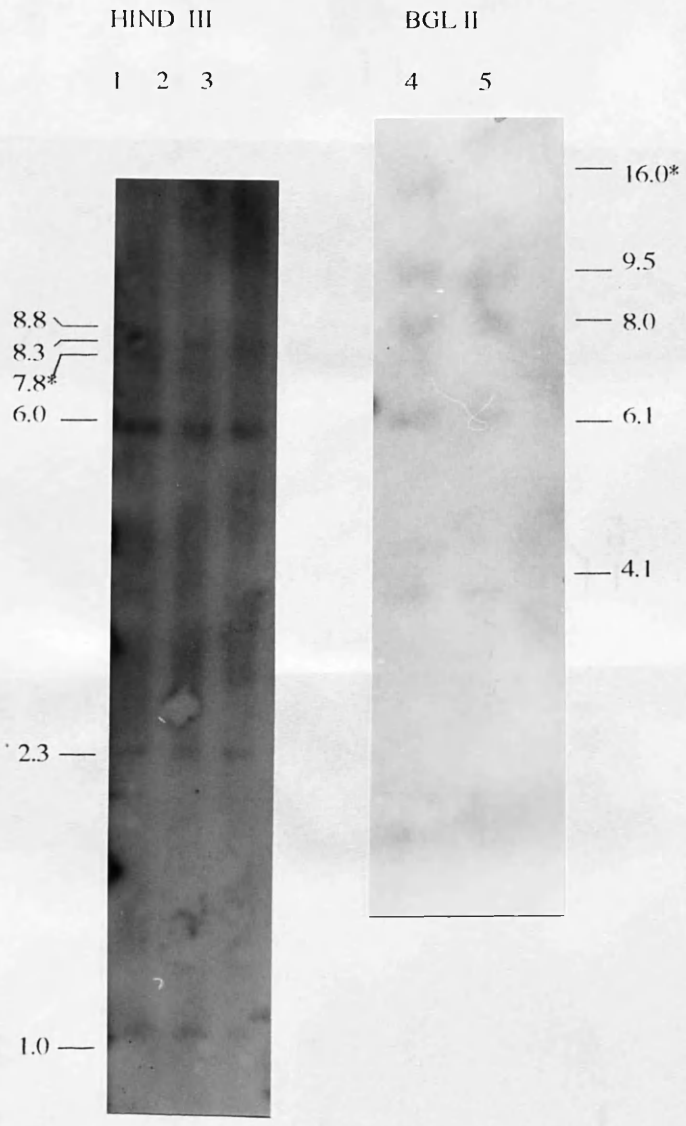


FIGURE 3.3: Autoradiographs of Southern blot analysis performed on Hind III and Bgl II digested DNA from individual GMG 4629 using cDNA probe 9. The cDNA fragment sizes are indicated in kb. Deleted fragments are marked with an asterisk. Lanes 1, 2 and 4 are control individuals.

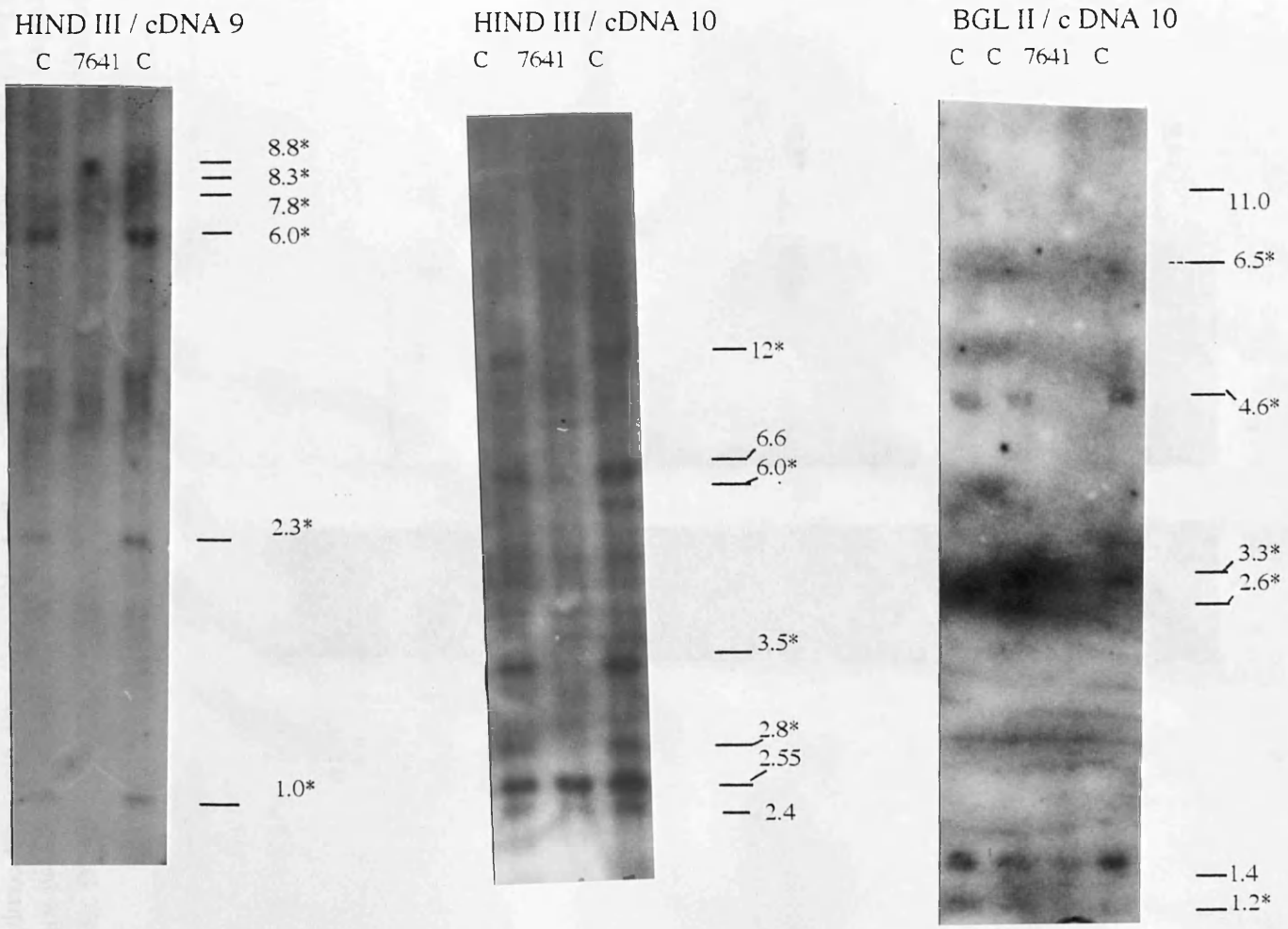


FIGURE 3.4: Southern blot analysis of DNA from GMG 7641. DNA was digested with Hind III and Bgl II (as indicated at the top), and hybridised to cDNA probe 9 and cDNA probe 10. The complete deletion involves cDNA 8 sequences also. The normal pattern of fragments detected by the probe is shown on the right of each autoradiograph. Deleted fragments are marked with an asterisk. Lanes C are shown the complete pattern of hybridisation. Sizes are given in kilobases.

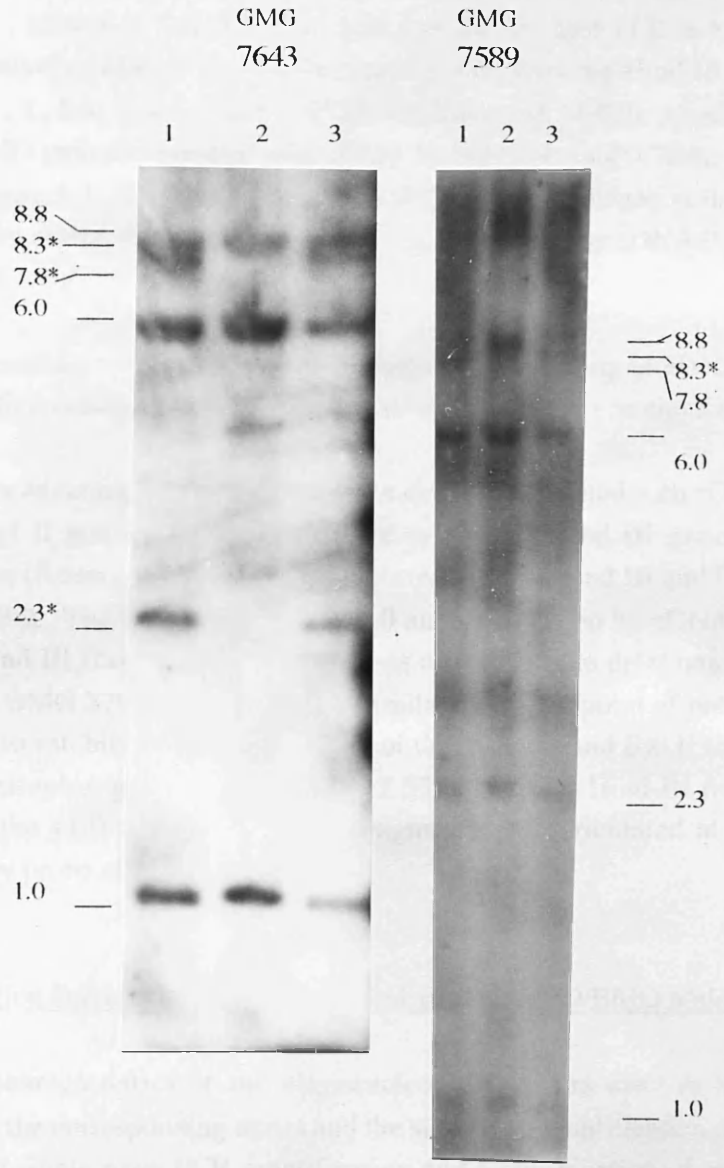


FIGURE 3.5: Hind III digested DNA from individuals GMG 7643 and GMG 7589 blotted and probed with cDNA 9. The fragments deleted for each individual are marked with an asterisk. The cDNA fragment sizes are indicated in kb. Lanes 1 and 3 are shown the complete pattern of hybridisation.

c-DNA 9 are presented in appendix III. The 7.8 kb Hind III fragment was deleted in three of the seven patients deleted with probe cDNA 9 (deletion frequency of 43%).

Probe cDNA 10 detects seven fragments in both Hind III- and Bgl II-digested DNA. Their sizes are : 12.0, 6.6, 6.0, 3.5, 2.8, 2.55 and 2.4 kb, and 11.0, 6.5, 4.6, 3.3, 2.6, 1.4 and 1.2 kb respectively. cDNA 11-14 hybridises to the following Hind III fragments : 10.0, 7.8, 6.8, 6.0, 5.9, 2.1, 1.9, 1.8, 1.5 and 1.45 kb (Darras et al., 1988). Among the group of thirty-six DMD/BMD patients screened with cDNA 10 only one, GMG 7641, was deleted for part of this probe, figure 3.4. This individual had a deletion which began at the 7.0 kb Hind III fragment detected by cDNA 8, spanned the whole region covered by cDNA 9 and extended in the area of cDNA 10.

No deletions were detected in any patients with the use of cDNA probe 11-14. Analytical hybridisation results with cDNA probes 10 and 11-14 are presented in appendix III.

Taking advantage of the different size deletions detected with cDNA probes 9 and 10, some of the Bgl II genomic fragments were related to Hind III genomic fragments of known orientation (Koenig et al., 1989). The corresponding Hind III and Bgl II fragments are shown in figure 3.2. The Bgl II fragments 16.0 and 6.1 kb seen by cDNA 9 were related to 7.8 and 8.8 kb Hind III fragments respectively, as a result of the deletions detected in patients GMG 4629 and GMG 2790 (appendix III). Similarly, the endpoint of patient's GMG 7641 deletion was used to establish a correspondence of the Hind III and Bgl II fragment groups in this area of the dystrophin gene. Thus the 6.6, 2.55 and 2.4 kb Hind III fragments were related as a group to the 11.0 and 1.4 kb Bgl II fragments, and orientated at the distal end of the area covered by probe cDNA 10.

3.3 Deletion Screening of the Dystrophin Locus in DMD/BMD Males by PCR Amplification.

The characteristics of the oligonucleotide primers used in this project including the sequence, the corresponding exons and the size of the amplification products are shown in table 2.1. Both single exon PCR amplification and a combination of multiple primer sets in one reaction, depending on availability, were employed to detect deletions in the dystrophin gene. Addition of each extra set frequently required modification of primer annealing temperatures, time of annealing, primer extension time and temperature. The conditions for the multiplex amplification of all nine primers and for individual exon amplification using a single primer set are described in materials and methods, in sections 2.10 and 2.11. In all cases the primer sets used were HPLC purified as attempts at amplification with unpurified primers early in the project were not successful. Occasionally difficulties presenting themselves during the application of multiplex PCR on genomic DNA samples extracted by the addition of 6M NaCl,

were easily overcomed by a simple ethanol precipitation in 3 M sodium acetate.

Genomic DNA from 118 boys with BMD/DMD were screened with all nine sets of oligonucleotide primers and the results are presented in appendix IV. Examples of multiplex PCR amplification of DMD samples are shown in figure 3.6. Deletions were detected in fifty-seven of the 118 samples tested, thus identifying the mutation in 48.3% of the patients. The deletions varied in size from a single region to as many as six. None of the patients was deleted for all nine regions. Primer sets f, e and h detected thirty-nine of the fifty-seven mutations detected, (68.5%).

3.4 Evaluation of the Oligonucleotide Primers Used in the Multiplex PCR Assay.

The deletion frequency for the product of each primer set is given in figure 3.7. Primer set f revealed deletions in twenty-two individuals tested while primer set d identified deletions in five cases. The initial PCR based assay published by Chamberlain et al., (1988) which screens for deletions in six regions of the dystrophin gene amplified by primer sets a, b, c, d, e and f would have detected the forty-five of the fifty-seven mutations found. The other twelve mutations were detected by the multiplex assay including the additional primer sets g, h and i. The oligonucleotide primer set g did not find any deletion among the individuals screened which could not be detected by the other primer sets.

3.5 Comparative Analysis of Deletions Detected in the DMD Gene with PCR and Southern Analysis.

The cDNA probes as they were developed by Koenig et al, (1987) were used for the characterisation of the molecular pathology of boys with Duchenne or Becker muscular dystrophy, even before the beginning of this project. Screening of the 116 samples used in the PCR assay with most of the cDNA probes revealed sixty-four deletions. The multiplex PCR assay which scans for deletions at nine separate regions of the dystrophin gene enabled the direct detection of approximately 86% of all DMD/BMD mutations (fifty-five of the sixty-four deletions).

The PCR method initially used for screening of deletions in the DMD gene, showed inconsistencies were compared with Southern analysis. The frequency of false positives was higher than that of false negatives. In seventeen cases of inconsistencies fourteen showed false positive results while in three cases false negative results were obtained. A repeat assay employing the set of primers flanking the disputed region yielded results which confirmed those predicted by conventional analysis. The number of false positives dropped to zero when certain

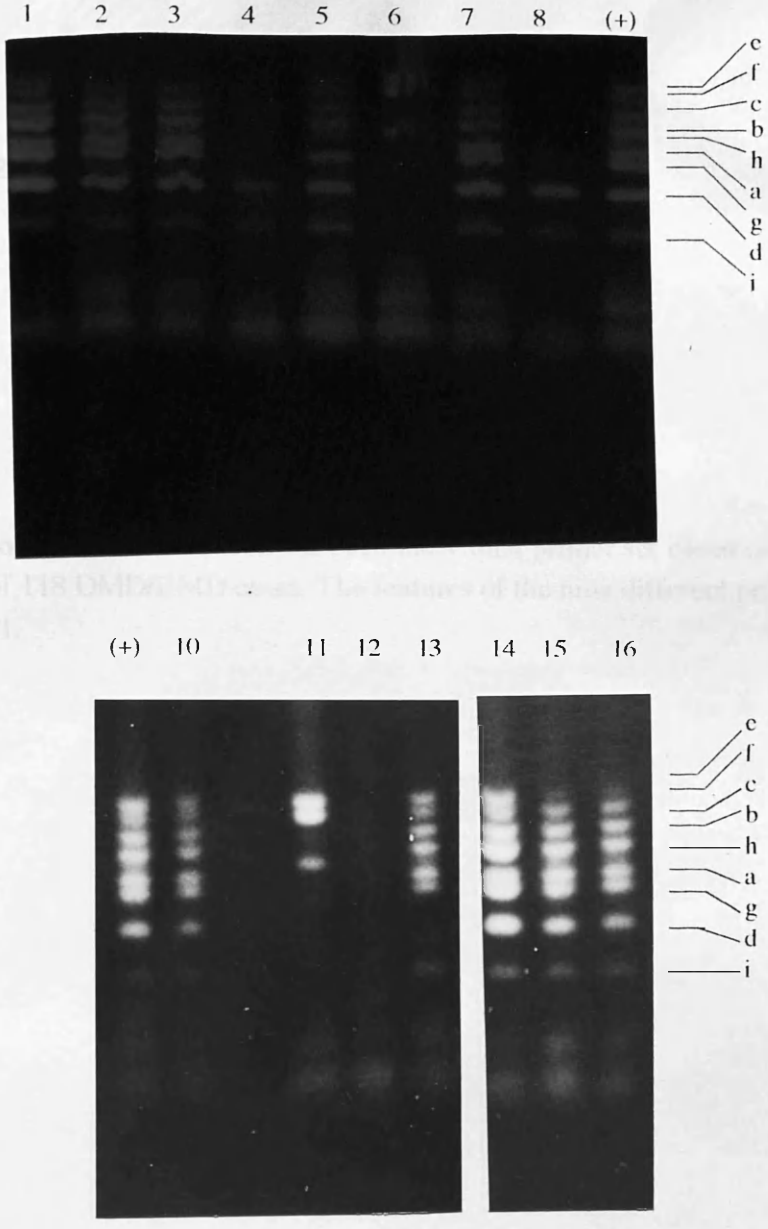


FIGURE 3.6: Products of amplification from the multiplex PCR assay. Example of normal control (+) and several deleted samples analysed by agarose gel elctrophoresis and stained with ethidium bromide. The corresponding regions of the dystrophin gene are shown on the right. The amplification patterns of GMG 1596, deleted for exon 45 (primer set e), and of GMG 5099, deleted for exons 4, 8, 12, 17, 19 and 44, are presented in lanes 3 and 6 respectively. Lane 12 is a negative control.

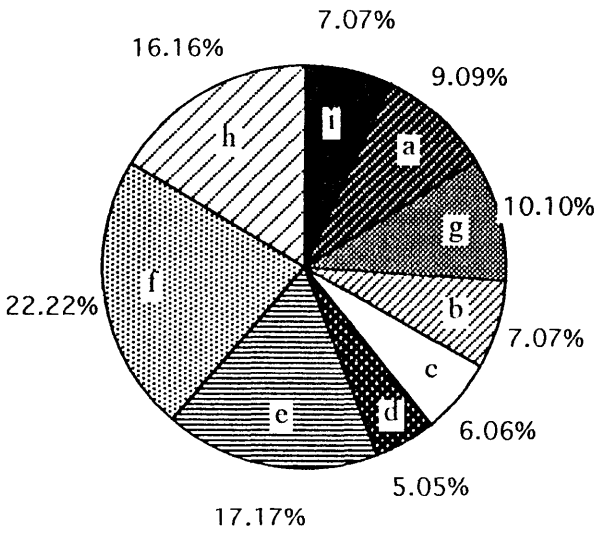


FIGURE 3.7: Deletion detection frequency of each individual primer set based on a deletion detection screening of 118 DMD/BMD cases. The features of the nine different primer set are described on Table 2.1.

measures were taken to reduce the risk of carry-over of amplified DNA from sample to sample or from sample to reagent stock; e.g: aliquoting of the reagents to minimise repeated sampling; use of positive displacement pipettes; minimum sample handling. A major contribution to the preparation of false-positive free PCR amplification was the ultraviolet light treatment of the samples before the addition of template DNA (Sarkar, 1990). The false negative cases most probably were due to lack of experience of the method as they were also eliminated at the later stages of this project. Thus the results from the samples tested with the PCR reaction were in agreement with the deletions determined by Southern cDNA analysis.

In two cases, GMG 5113 and GMG 5165, where a shift of the 8.5 Hind III fragment containing exon 4 was detected by Southern analysis, a positive signal was observed in the multiplex PCR.

3.6 Refining the Results from the Southern cDNA Analysis.

In 122 DMD/BMD cases screened for deletions with cDNA probe 5b-7 using Southern blot analysis the 0.5 kb Hind III fragment detected by cDNA 7 weakly hybridised in forty-one cases. This problem was circumvented using the oligonucleotide primer set e in thirty-five cases, appendix IV (in italic), allowing the accurate mapping of the deletion end-points in two of them, GMG 5099 and GMG 5534. GMG 5099 deletion extends from the first exon of the dystrophin gene up to the 10 kb Hind III fragment with cDNA 7, exon 44 (figure 3.6), while for the individual GMG 5534 the deletion starts from the 0.5 kb Hind III fragment, exon 45. Also in the case of individual GMG 4089 who was scored positive for the region amplified by primer f, his 3' deletion end point was defined to exon 47.

Also a new deletion was detected via the multiplex PCR amplification assay for individual GMG 1596 in the region flanked by primer set e, figure 3.6. This sample was not screened with cDNA 5b-7 through lack of genomic DNA for a Southern analysis.

3.7 Postnatal-detection Screening.

Since the PCR based assay traced successfully most of the deletions in the DMD gene of male patients, two DNA samples GMG 8602 and GMG 6272, which had not been previously characterised by Southern analysis,were analysed. In each case a deletion was detected within 48 hours including the extraction of DNA. Individual GMG 6272 was deleted for exon 4 while individual GMG 8602 was deleted for exons eight and twelve figure 3.8. Southern analysis confirmed both results.

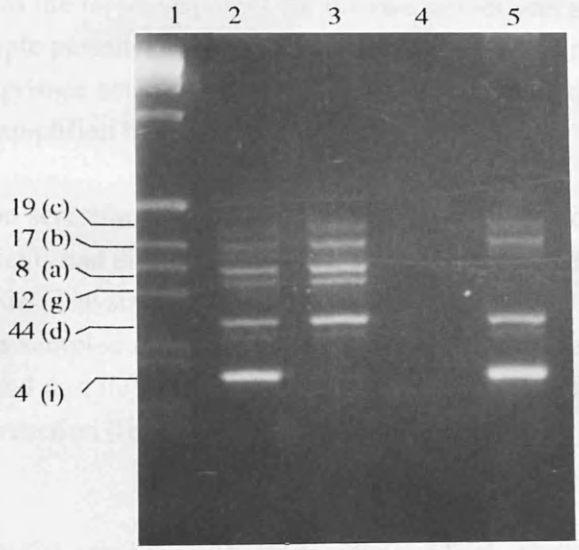


FIGURE 3.8: PCR amplification for postnatal diagnosis of DMD/BMD on samples GMG 6272 (lane 3) and GMG 8602 (lane 5). Lane 2 is a positive control (amplification of DNA from a normal individual). Lane 4 is a negative control (no DNA). The amplified exons are pointed on the left. The corresponding primer sets used for the amplification of each exon are shown in brackets. The marker lane 1 contains DNA from 1kb Ladder (BRL).

3.8 Amplification of a Degraded DNA Sample.

A degraded DNA sample from individual GMG 4027, for whom no other DNA sample was available, was screened for deletions in the area of the DMD locus after slight modification of the PCR reaction (section 2.12). This DNA sample failed to produce a signal on a Southern blot with radioactive cDNA probes. The size of the genomic DNA is presented in figure 3.9a.

This DNA permitted the amplification of products defined by the primer sets a, b, c, g and i, figure 3.9b, but no amplification was observed using primer set h when single exon PCR assays were performed. In order to confirm a deletion of exon 51 (primer set h) the same DNA was used simultaneously as the target sequence for the two primer sets g and h. It can be seen in figure 3.9c that the sample permitted the amplification of the 331 bp product (primer set g), but not the 388 bp one (primer set h). Thus a deletion was revealed in the region of the dystrophin gene normally amplified by the primer set h.

PCR assay for deletion screening was also employed in two other cases, GMG 2758 and GMG 3766, where the patients had died and the available DNA had failed to produce detectable signals by Southern analysis. The size of their DNA is shown in figure 3.10. No amplification was detected in these two samples although the average molecular weight of the templates appeared to be adequate, and despite the fact that 5'-end labelled primers were used to increase the sensitivity of the PCR reaction (figure 3.11).

3.9 Amplification of DNA Extracted from Haematoxylin and Eosin Stained Sections.

DNA was extracted from haematoxylin and eosin stained sections, prepared from blocks of muscle tissue, one to fifteen years old, and used as a template in a PCR amplification reaction using the oligonucleotide primer sets i and f. Specific amplification was obtained in both cases although the PCR product yield for the larger fragment 506 bp, primer set f, was somewhat smaller after 35 cycles of amplification, figure 3.12. Reduced PCR product yield was also observed when the template DNA was extracted from sections not directly treated on the slides but scraped from them and transferred to a microfuge tube, figure 3.13.

3.10 Application of PCR-SSCP Analysis on Regions of the Dystrophin Gene.

Nine regions of the dystrophin gene were screened for sequence changes with PCR-SSCP analysis. These cover 3471 bp of the DMD locus, and are the areas defined by the primer sets i, a, g, b, c, d, f and h which correspond to sequences flanking exons 4, 8, 12, 17, 19, 44, 48 and 51 respectively , table 2.1.

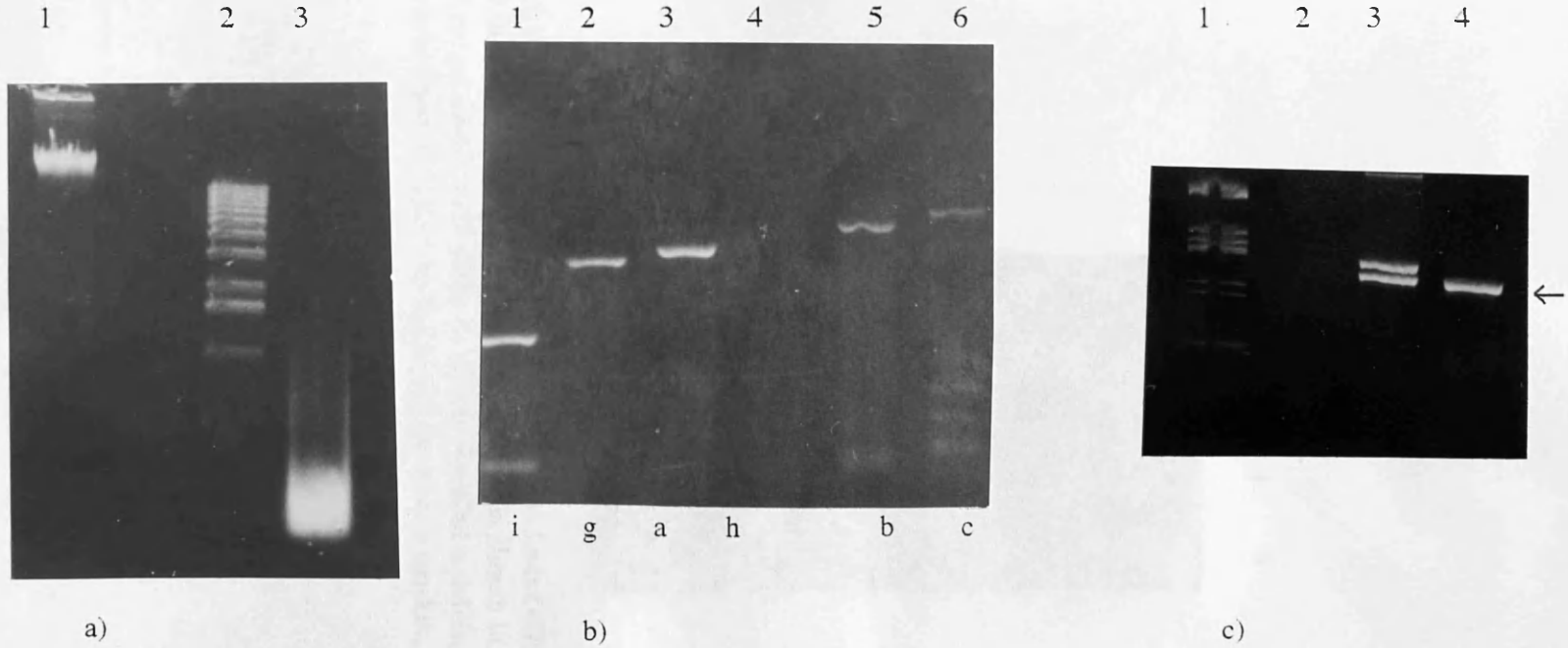


FIGURE 3.9: Amplification of degraded genomic DNA from individual GMG 4027.
a) Size range of genomic DNA from individual GMG 4027. 15 μl of DNA was run on a 1% SEA KEM (FMC) agarose gel and stained with ethidium bromide. A smear is shown below 500 bp (lane 3). Lane 1 is DNA from a non-degraded sample and lane 2 contains DNA from 1 kb ladder (BRL). b) PCR products of sample GMG 4027 obtained by primer sets i, g, a, h, b and c. c) Agarose gel electrophoretic analysis of PCR amplified products from GMG 4027 (lane 4) and a normal individual (lane 3) using primer sets g and h. the deleted fragment is shown with an arrow. The negative control is shown in lane 2. The marker lane 1 contains DNA from Pst/Dde I ladder (IBI). The size of the fragments is 1.652, 540, 465, 426, 409, 288, 252, 166 and 162 kb.

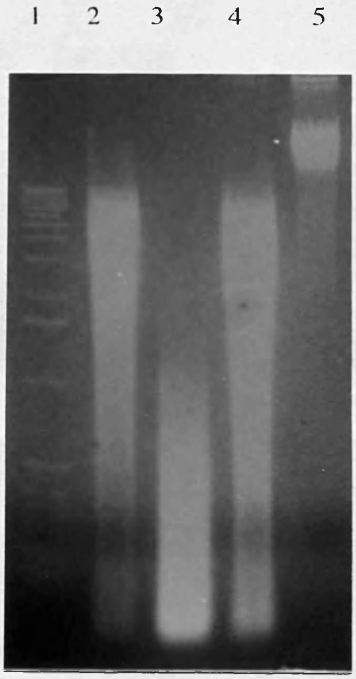
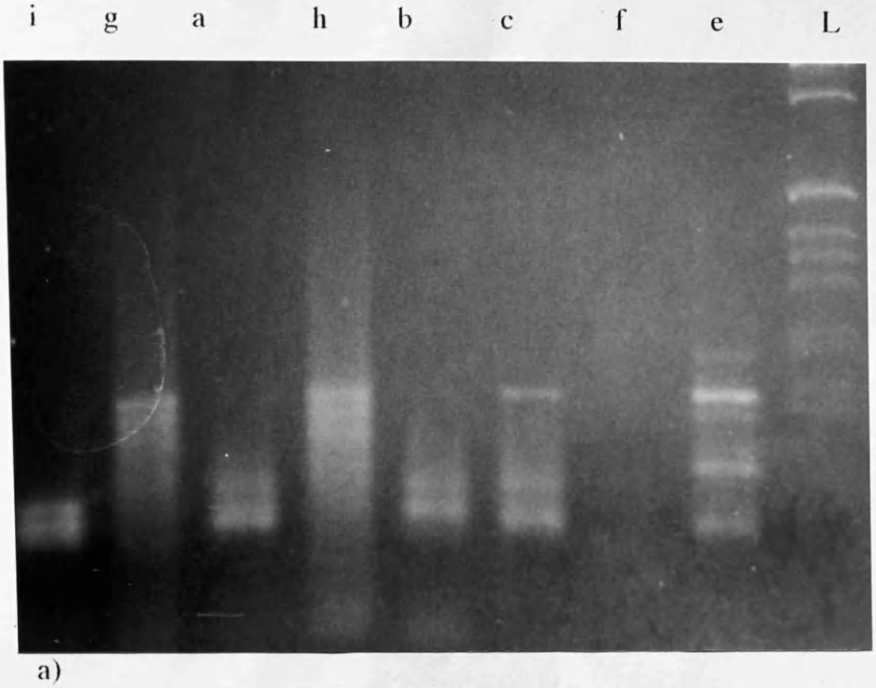
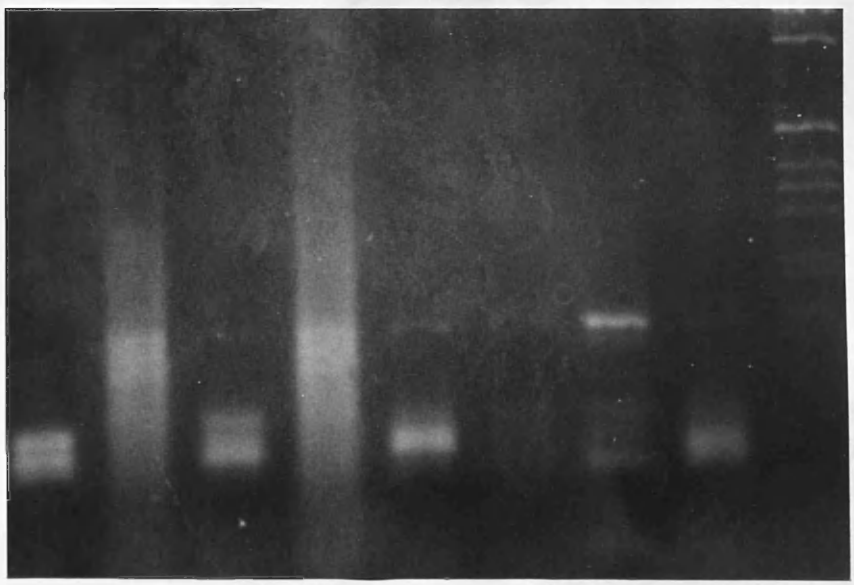


FIGURE 3.10 : Size range of genomic DNA from individuals GMG 2758 and GMG 3766. The size range of their DNAs, lanes 2 and 4 respectively, is shown in comparison to the genomic DNA of individual GMG 4027 (lane 3) which revealed a deletion of exon 51 and to a non-degraded sample (lane 5). The 1 kb ladder was used as a marker, (lane 1).



a)



b)

FIGURE 3.11: PCR assays on genomic DNA of DMD patients GMG 2758 (a) and GMG 3766 (b). Absence of specific amplification was observed with both DNA samples and primer sets i, g, a, h, b, c, f or e.

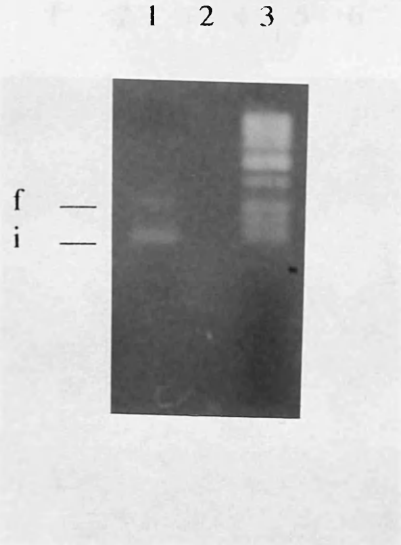


FIGURE 3.12: Amplification of DNA isolated from haematoxylin and eosin stained sections using primer sets f and i, lane 1. Lane 2 is a negative control. Lane 3 contains DNA from a 1 kb ladder (BRL).

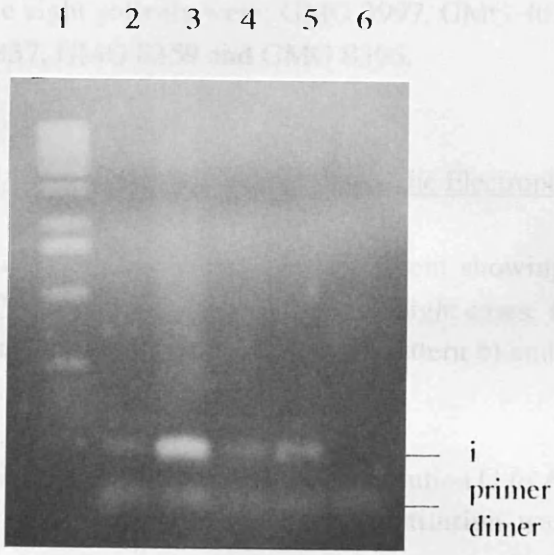


FIGURE 3.13: Effect of the protocol used for the extraction of genomic DNA from haematoxylin and eosin stained sections on its amplification. PCR amplification of DNA using primer set i (196bp). Lane 2: the template (unpurified DNA) was boiled for 10 minutes before added in the PCR mix. Lane 3: positive control (DNA from a normal individual extracted by adding 6M NaCl as described in section 2.6). Lane 4: purified DNA extracted from sections not directly treated on the slides. Lane 5: purified DNA extracted by proteinase K treatment on the slides. Lane 6: negative control (no DNA added). The DNA marker used is the 1 kb ladder (lane 1).

A systematic screening was performed using genomic DNA as template for the PCR reaction from thirty-nine DMD/BMD boys with no deletion or duplication previously detected by either Southern analysis or PCR based multiplex assay. Only one patient, individual GMG 8396, was found later on to be deleted for a part of the cDNA probe 9. Table 3.2 presents the number of cases tested for each primer and comments on the electrophoretic mobility of single-stranded DNA whose shift under non-denaturing conditions, would be an indication for the presence of a sequence variation. Analytical results are presented in appendix V.

The migration pattern of the DMD/BMD patients examined was the same as that of a normal individual for the areas amplified by primer sets i, a, g, b, c, d, h and f. By contrast, primer set e (exon 45) produced two very easily distinguished patterns, figure 3.14. The less frequent (pattern a) was present in eight of the thirty-two DMD/BMD boys examined for this area, including the individual whose molecular pathology had already been defined as a deletion in the area of cDNA probe 9. The eight patients were: GMG 3997, GMG 4026, GMG 4191, GMG 4252, GMG 5105, GMG 8337, GMG 8359 and GMG 8396.

3.11 Sequencing of the DNA Fragment Presented a Shift in the Electrophoretic Mobility.

Asymmetric PCR and direct sequencing of the fragment showing an altered mobility (pattern a) in the PCR-SSCP analysis were performed in all eight cases. The sequence obtained was compared to the sequence of a normal male control (pattern b) and to the published one, (Chamberlain et al., 1988).

The sequence ladder revealed a single nucleotide substitution G to A at position 203 from the 5'end of the sense primer (figure 3.15). This substitution was also confirmed by sequencing of the complementary strand.

The shifted fragment was present in patient GMG 8396 whose molecular pathology had already been defined as a deletion in the area of cDNA 9. The substitution is located in the large intron between exons 44 and 45 of the DMD gene without any affect on the intron-exon border. Therefore, it was predicted to be a harmless polymorphism.

3.12 Mendelian Inheritance of the Polymorphism Detected by PCR-SSCP Analysis.

To confirm that the observed SSCP in the region of intron 44 of the dystrophin gene was due to allelic variants of true Mendelian traits,segregation analysis was performed on the members of six of the DMD/BMD families from which a DNA sample was available. In all cases the genotype of the progenies were consistent with the parental genotypes. The pedigrees

PRIMER SET	SIZE OF AMPLIFIED PRODUCT (bp)	NUMBER TESTED	DIFFERENCES IN MIGRATION PATTERN
I	196	37	NONE
A	360	35	NONE
B	416	34	NONE
C	459	32	NONE
F	506	34	NONE
E	547	32	8
D	268	37	NONE
G	331	34	NONE
H	388	36	NONE

TABLE 3.2: Results of PCR-SSCP analysis in regions of the DMD / BMD gene flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51.

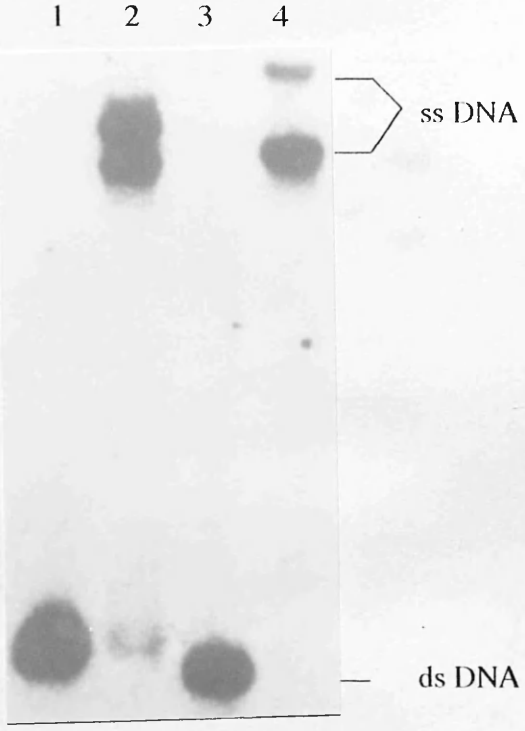


FIGURE 3.14: SSCP analysis of region e of the dystrophin gene on a 6.5% non-denaturing polyacrylamide gel. DNA loaded with no heat denaturation (lanes 1 and 3), or after heat denaturation (lanes 2 and 4). Pattern b is shown in lane 4 while the variant pattern (pattern a) is present in GMG 4252 (lane 2).

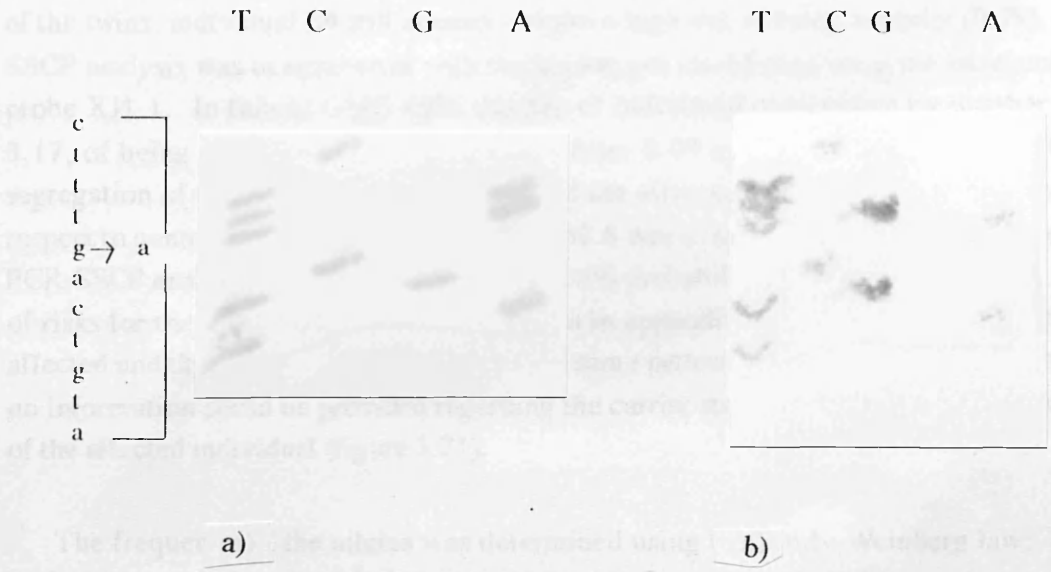


FIGURE 3.15: Direct sequencing of PCR amplified fragments from the region e of the dystrophin gene presenting pattern a (a) and pattern b (b) in PCR-SSCP analysis. The nucleotide sequence is shown on the left. The G to A transition is marked by an arrow.

of the families, haplotype analysis using intragenic probes and SCK levels where available, together with the SSCP analysis are shown in figures 3.16, 3..17, 3.18, 3.19, and 3.20 respectively for the following families GMG 3997, GMG 4026, GMG 4252, GMG 5105, and GMG 8359. For families GMG 8337 and GMG 8396 there were no DNA samples available from all the necessary members to prove a Mendelian mode of inheritance. In family GMG 3997 where all the three sisters of the affected male had low SCK levels PCR-SSCP analysis reduced the risk of individuals II2 and II3 of being carriers from 0.09 to 0.01 while the second of the twins, individual II4 still appears to have a high risk of being a carrier (0.79). The PCR-SSCP analysis was in agreement with the haplotypes established using the intragenic genomic probe XJ1.1. In family GMG 4026 the risk of individual represented by number 9 in figure 3.17, of being a carrier was reduced to 0.1 from 0.09 by SSCP analysis. In GMG 4252 the segregation of haplotype in the two sisters of the affected male, individuals II2 and II3, with respect to genomic probes XJ1.1, 87.15 and 87.8 was in agreement with the results obtained by PCR-SSCP analysis, and both of them had a 95% probability of being carriers. The calculation of risks for the different individuals is shown in appendix VI. In family GMG 4191 both the affected and the unaffected son presented the same pattern in PCR-SSCP analysis and therefore no information could be provided regarding the carrier status of either the mother or the sisters of the affected individual (figure 3.21).

The frequency of the alleles was determined using the Hardy-Weinberg law: a=0.25 and b=0.75 based on the PCR-SSCP results of forty-four unrelated individuals. A sample from the group of individuals demonstrating the altered pattern was also sequenced to confirm the aetiology of the mobility shift.

Investigation of the region of the dystrophin gene amplified by primer set e for restriction enzyme sites, showed that an extra TSpE1 site (recognition sequence AATT) was formed when the G to A substitution was present figure 3.22. Unfortunately, at the present time, the restriction enzyme , TspE I, is not commercially available.

3.13 Detection of the Polymorphic Site in Intron 44 of the Dystrophin Gene by Means of AMD.

Samples GMG 3997, GMG 4026, GMG 4191, GMG 4252, GMG 5105, GMG 8337, GMG 8359 and GMG 8396, showed pattern a when they were analysed by PCR-SSCP analysis, were tested with the method of amplification and mismatch detection using hydroxylamine for the DNA modification. Since the substitution revealed by direct sequencing was a G to A substitution and a C to T change was present in the complementary strand, a mismatch with a normal control should be detected by hydroxylamine and piperidine cleavage.

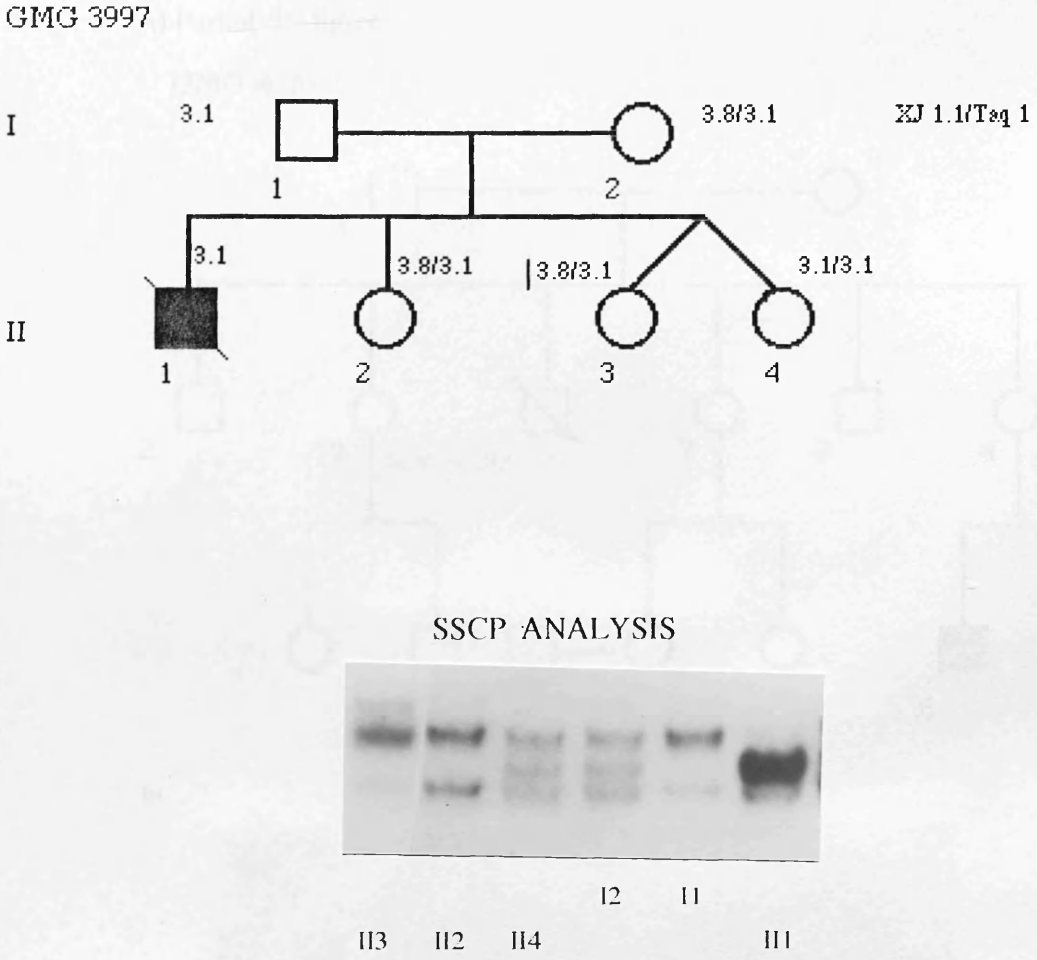
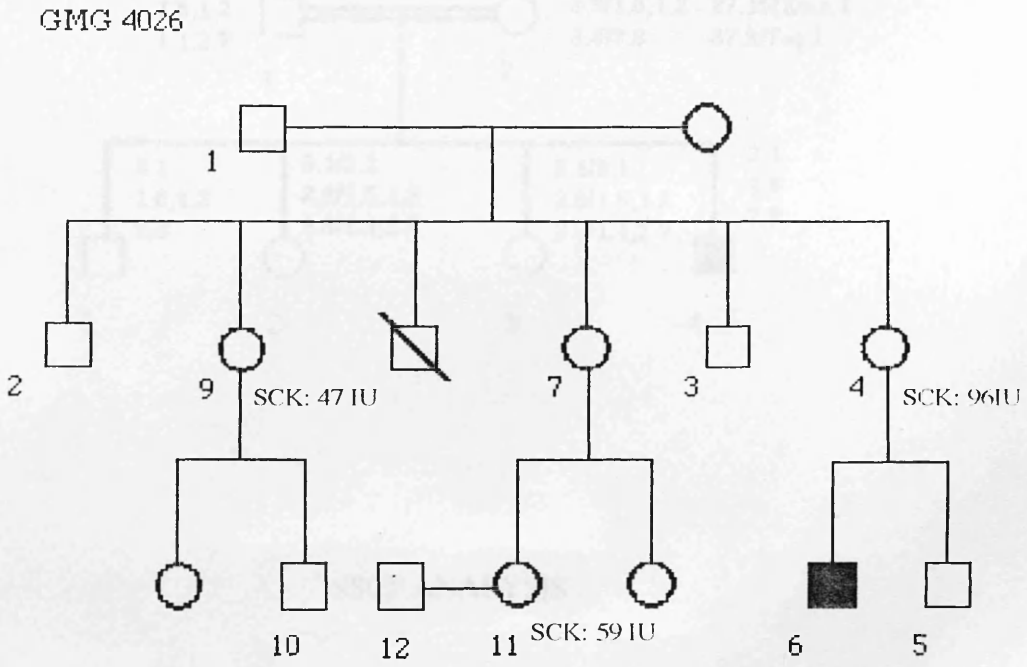


FIGURE 3.16: Study of DMD family GMG 3997. Pedigree and haplotype analysis of DMD family GMG 3997 using the intragenic genomic probe XJ 1.1. The SSCP alleles presented at the bottom panel are segregating in a similar way.

a) Partial Pedigree.



b) SSCP ANALYSIS

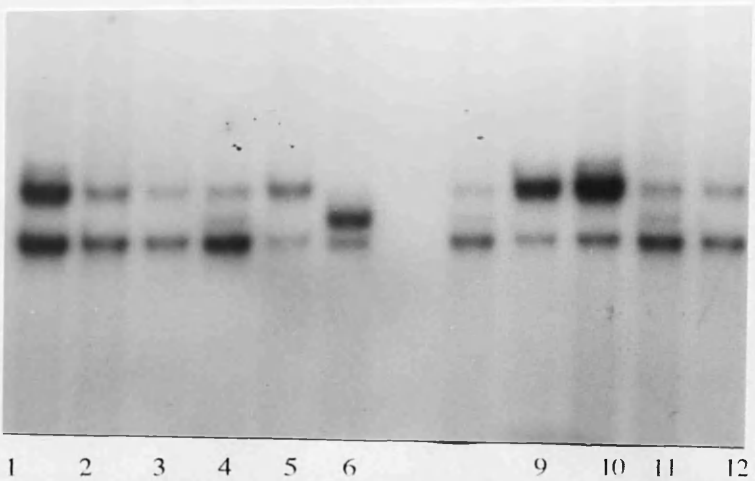
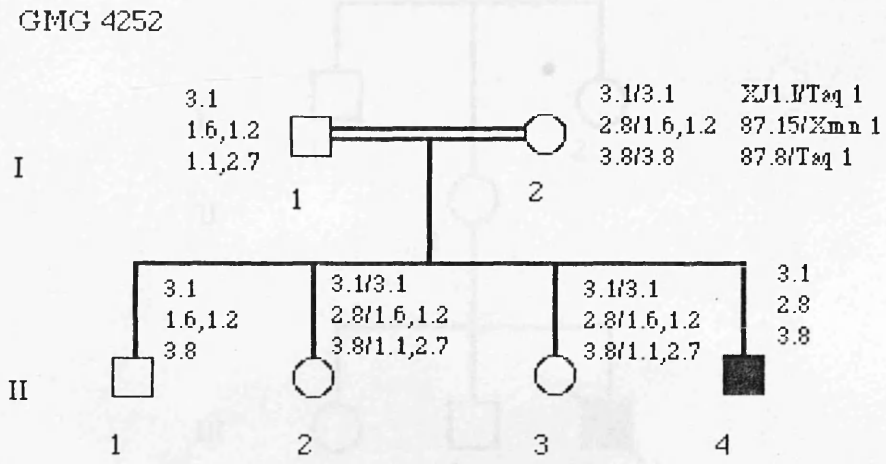


FIGURE 3.17: PCR-SSCP analysis of DMD family GMG 4026.
a) Partial pedigree. b) Pattern of SSCP alleles segregating in a mendelian fashion.
The lanes correspond to the individuals representated by the symbols in (a).



a)

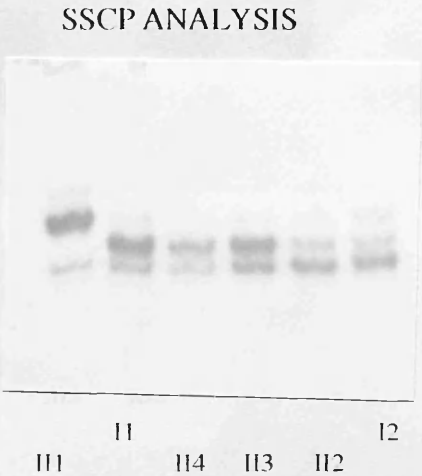


FIGURE 3.18: Study of DMD family GMG 4252.
Pedigree and segragation haplotype in respect to genomic probes XJ1.1, 87.15 and 87.8 (a). The pattern obtained by SSCP analysis is presented in (b). The SSCP alleles demonstrate the same segregation.

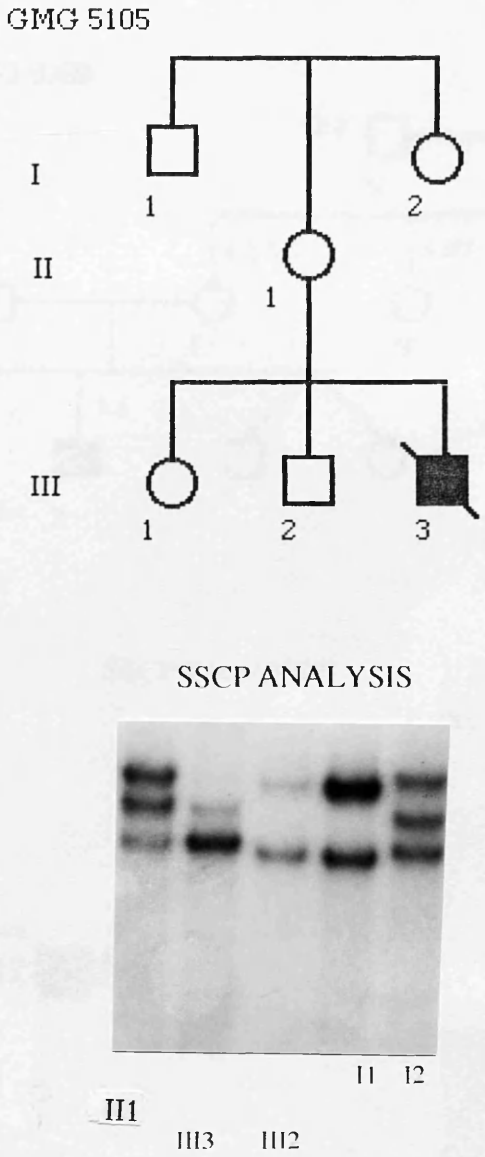
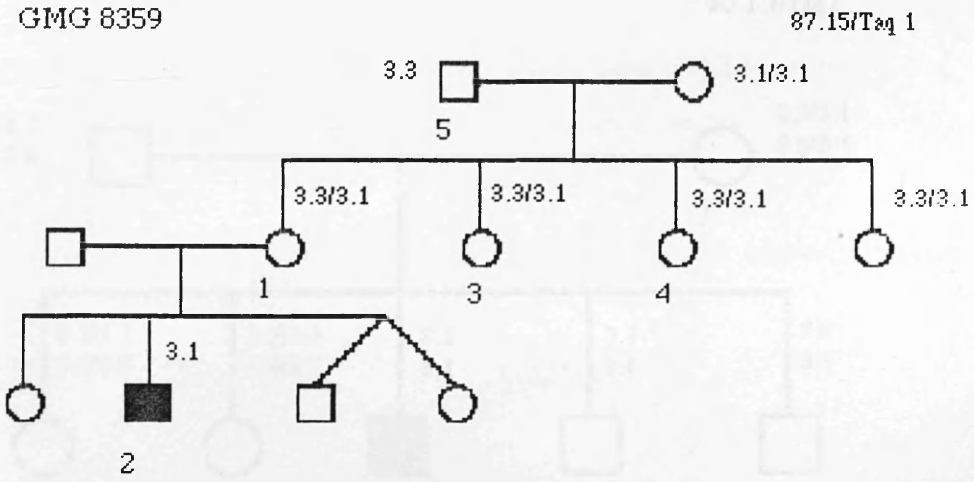


FIGURE 3.19: PCR-SSCP study of family GMG 5105. A partial pedigree of the family GMG 5105 is presented at the top and the results of the SSCP analysis at the bottom.



SSCP ANALYSIS

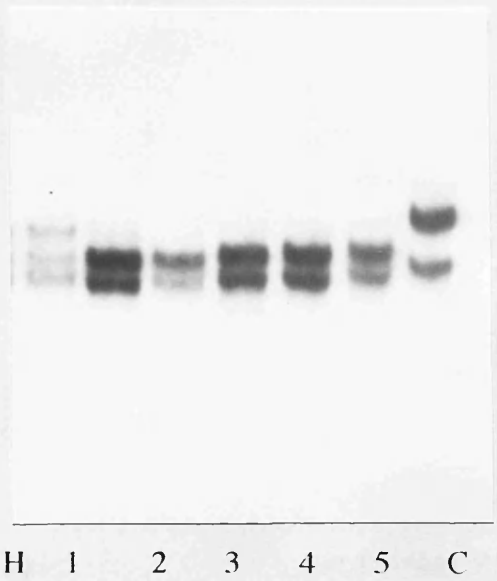
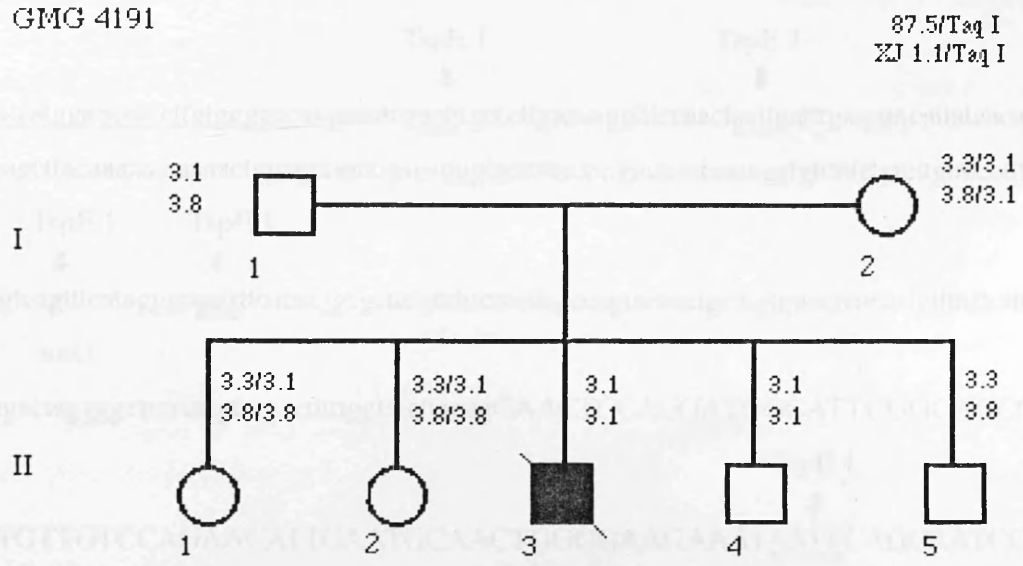
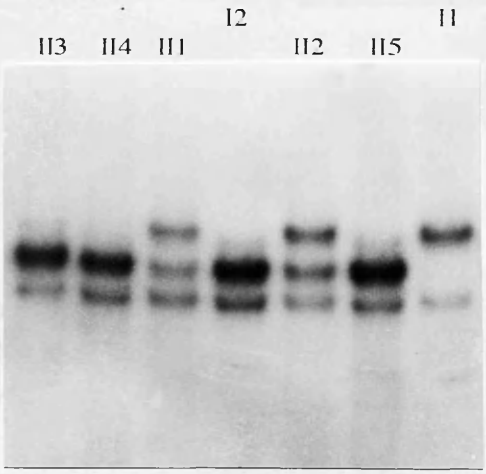


FIGURE 3.20: PCR-SSCP analysis in family GMG 8359. Pedigree and haplotype analysis using the intragenic probe 87.15 (a). The family is uninformative by PCR-SSCP analysis. H : heterozygous female; C :control sample presenting pattern b.



a)



b) SSCP ANALYSIS

FIGURE 3.21: Study of DMD family GMG 4191. a) Pedigree and haplotype analysis of DMD family GMG 4191. b) The SSCP alleles are presented showing a Mendelian mode of inheritance.

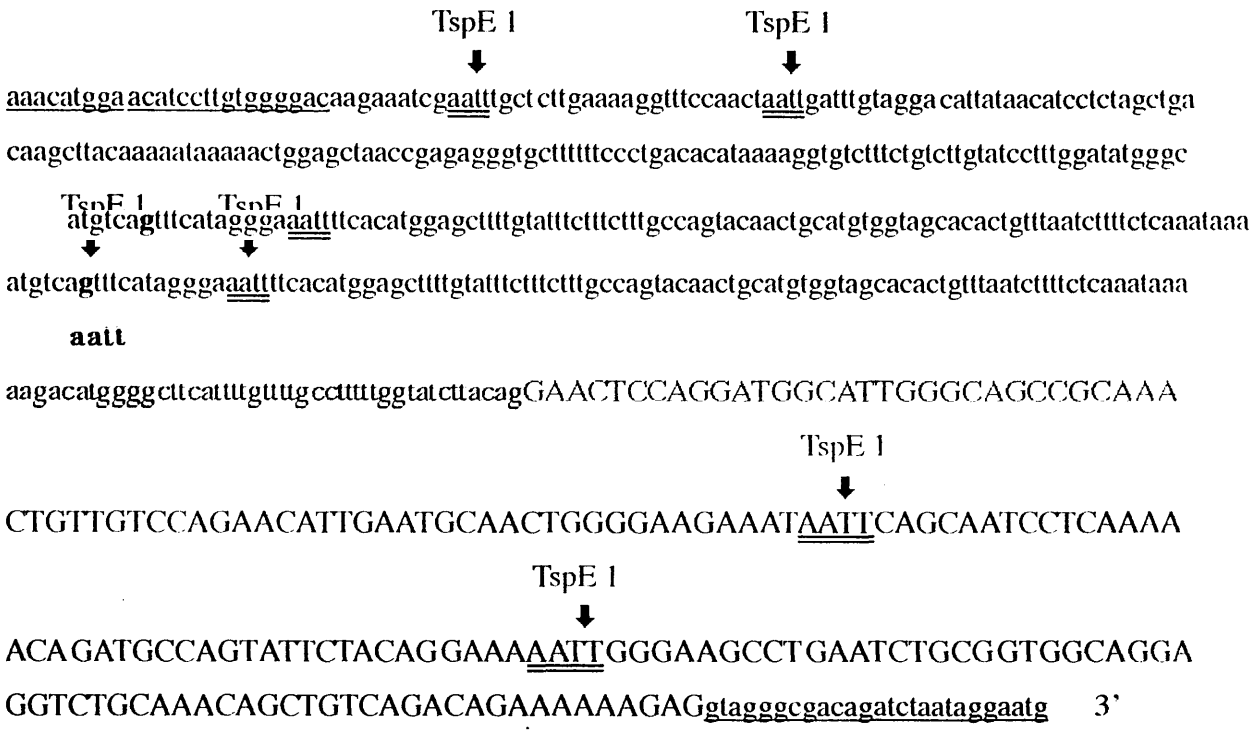


FIGURE 3.22: Sequence of the region amplified by primer set e. Exon sequences are in capital letters and intron sequences are in lower case letters. The sequences of the primers used for the amplification are underlined. The arrows point the TspE I restriction sites. The extra TspE I site, formed when the G to A substitution is present, is shown in bold.

Figure 3.23 shows the products of the chemical cleavage using hydroxylamine - piperidine, in a heteroduplex formed between an unlabelled DNA sample containing the sequence change, (GMG 3997), and a probe labelled at both ends. In the control sample the hybrid was between labelled and unlabelled PCR product of a normal individual. The two bands present, were of the expected size. The 550 bp band, according to the 1 kb ladder, represents the uncleaved strand labelled at the 5'end where a G nucleotide is present in the target position. The band present at the size of approximately 345 bp is the product of chemical cleavage. Thus, the cleavage was at the base predicted by sequence analysis, 344 bp from the 3' end of the antisense primer. When AMD analysis was performed in another seven DNA samples showing a mobility shift (pattern a) by PCR-SSCP analysis, the two bands of 550 and 345 bp were also shown in the autorads. In all cases the control samples presented just the 550 bp band corresponding to the uncleaved labelled strand. Thus, neither false negatives nor false positives were observed from the application of AMD analysis.

3.14 Application of Denaturing Gradient Gel Electrophoresis in Regions of the Dystrophin Gene.

The nine regions amplified by the primers listed on table 1 were analysed for sequence variations by means of denaturing gradient gel electrophoresis (DGGE). The perpendicular gel approach where the gradient of DNA denaturants increase linear from left to right across the gel was adopted. For the perpendicular denaturing gradient gel electrophoresis a single type of gel, 0 to 80% gradient of DNA denaturants, and standardised electrophoresis conditions were used. A similar number of samples (30 for primer set a, 34 for primer set b, 32 for primer sets c and f, 31 for primer sets d, e and h and 29 for primer set g) were tested using the approach of perpendicular gradient gel electrophoresis. To accelerate and economise the screening of the available DNA samples a pooling strategy initially introduced by Gille et al., (1991) for heterozygote screening of the Δf 508 cystic fibrosis mutation was applied. Initially, the amplification products of a certain region of the dystrophin gene from a group of four unrelated DMD/BMD patients and a normal control were mixed and exposed to the whole range of denaturant concentrations. In the presence of a sequence variation in any of the samples a split of the single curve was expected to appear. A repeat assay where each sample was mixed separately with a normal control was performed in such case to provide identification of the mutant fragment. A sequence variation was detected in eight cases in the region amplified by primer set e. The eight individuals where the same with those detected by PCR-SSCP analysis to present pattern a due to the polymorphic site at position 203 from the 5'end of the sense primer flanking the area e of the dystrophin gene. An example of the observed split of the curve at the area of the transition indicating the presence of a sequence variation in region e is shown in figure 3.24. Analytical results of the application of DGGE in the regions of the dystrophin gene flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 of the dystrophin gene are presented in appendix VII.

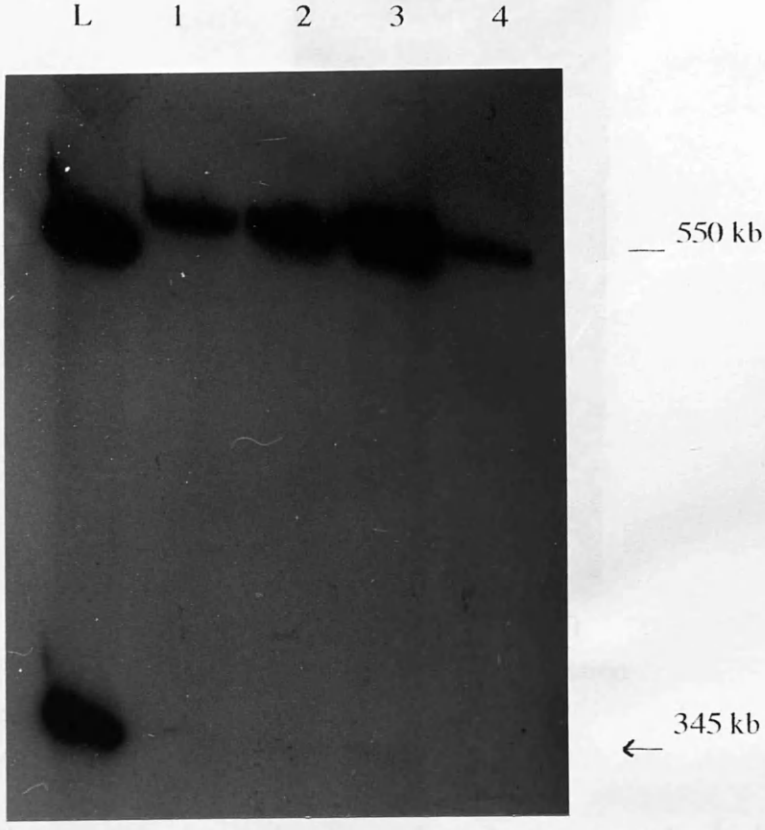


FIGURE 3.23: Autoradiograph of amplification mismatch detection analysis of the polymorphic site in intron 44 of the dystrophin gene. Lane 1: normal control (homoduplex). Lanes 2-4: heteroduplex (GMG 3997). The probe used was end-labeled and the mismatch was detected by hydroxylamine modification. The band at 550 bp represents the uncleaved strand. The arrow indicates the product of chemical cleavage at 345 bp. L contains labeled DNA from 1 kb Ladder (BRL).

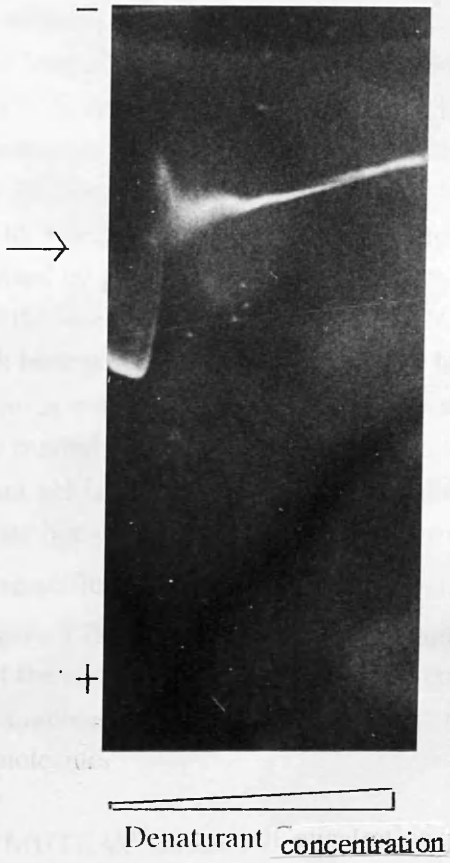


FIGURE 3.24: Identification of a DNA polymorphism by the perpendicular DGGE approach. A mixture of DNA from a control sample and from individual GMG 3997 was electrophoresed in a 6.5% gel in which the denaturant concentration was constant along the path of electrophoretic movement but increased linearly in the perpendicular direction. The sample was applied along the top of the gel. The arrow indicates a split of the curve.

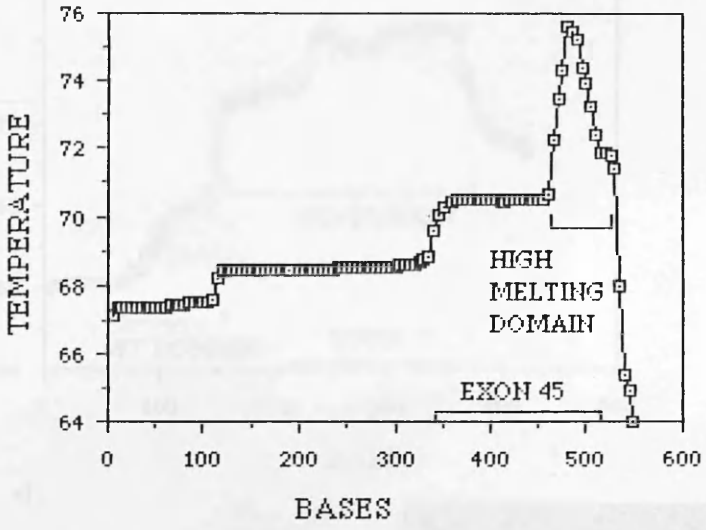
3.15 Melting Calculations for Regions of the Dystrophin Gene Using the Computer Programmes MELT 87 and MUTRAV. (Lerman and Silverstein, 1987).

The melting maps of the regions of the dystrophin gene amplified by primer sets e, b, d and f were plotted using the data derived from the computer programme MELT 87 (Lerman and Silverstein, 1987), listed in appendix VIII and are presented in figure 3.25a, 3.26a, 3.27a and 3.28a respectively. The nucleotide sequence data used in the calculations were those reported by Chamberlain et al. (1988). Only the sequence of exon 17 (primer b) was corrected using experimental data and data reported by Koenig et al. (1988). The nucleotide sequence for the regions of the dystrophin gene amplified by primer sets e, b, d and f are listed in appendix IX. The t_m was plotted as a function of the nucleotide position of the DNA fragment. The line shows the temperatures at which each base pair is in 50:50 equilibrium between the helical and melted configurations. At temperatures above this line, a base pair will be helical, and at temperatures below the line, it will be melted. To show that the DNA to the right of the melting transition in the perpendicular gradient gel is in fact completely melted a mixture, of two PCR amplification products, using primer set b, each one having one of the two strands labelled with ^{32}P was electrophoresed. Each PCR amplification product was also electrophoresed separately. As shown in the autoradiogram of figure 3.29, both labelled DNA strands are moving together indicating a helical DNA molecule at the right half of the gel. On the contrary, the position of one labelled DNA strand does not coincide with that of the other at the left side of the gel indicating a completely melted DNA molecule.

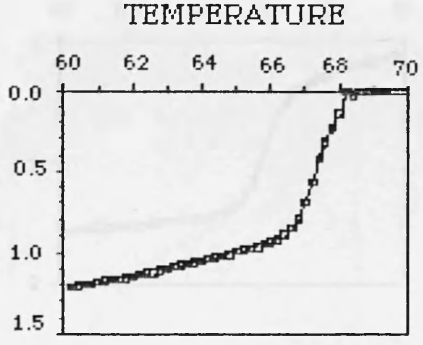
A plot of the data resulting from MUTRAV versus T that is expected to correspond to the curve given by a perpendicular gradient gel is given in figures 3.25b, 3.26b, 3.27b and 3.28b respectively for the regions e, b, d and f. The curve has been corrected for the viscosity gradient present in the gel. The numerical data derived from the computer programme MUTRAV are listed in appendix X.

REGION E. The melting map of the 547 bp region e of the dystrophin gene, shown in figure 3.25a indicates that there is a relative homogeneity in the melting behaviour with the exception of the region between 460 bp and 530 bp melt near the 3' end which melts at an intermediate temperature t_m of 75^0C. On the basis of melting predictions, dystrophin fragments carrying mutations in this domain should not separate by DGGE. The calculated contour falls 0.9^0C between bases 490 and 515, giving the appearance of a steep wall.

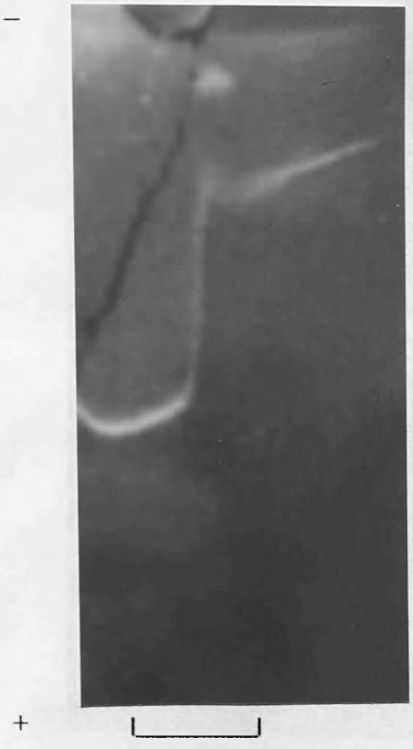
Figure 3.25b is a plot of the relative electrophoretic mobility as a function of equivalent temperature which corresponds to the experimental curve 3.25 c obtained in perpendicular gels for the corresponding areas of the dystrophin gene. As anticipated from the melting map the fragment shows only a single mobility transition at 67^0C. There is a significant similarity



a)



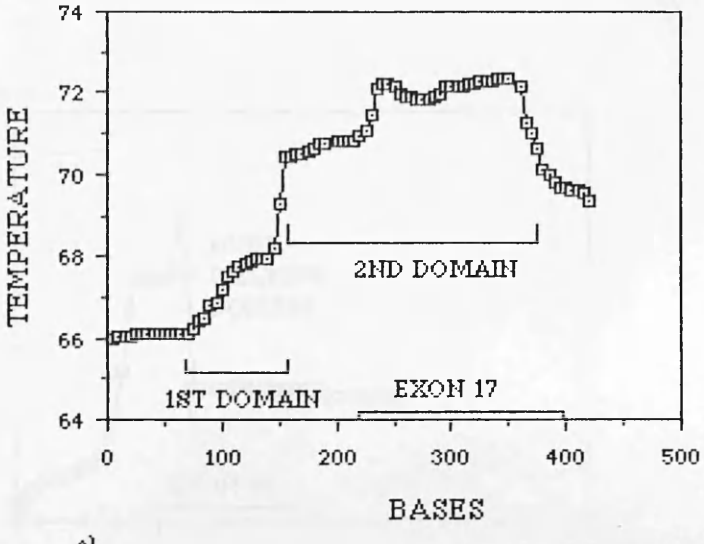
b)



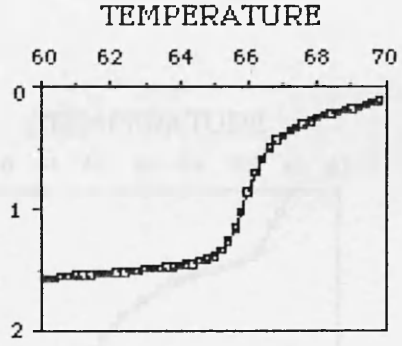
c)

Denaturant Concentration

FIGURE 3.25: Melting behavior of sequence flanking exon 45 (region e) of the dystrophin gene. Melting map of the region as calculated by MELT 87 (a). The relative electrophoretic mobility versus the effective temperature is presented in (b). The experimental pattern obtaining from a perpendicular gel is presented in (c). The area corresponding to the calculated mobility is marked at the bottom.



a)



b)

c)

FIGURE 3.26: Melting behavior of sequence flanking exon 17 (region b) of the dystrophin gene. Calculated melting map for a fully paired helix containing exon 17 of the dystrophin gene (a). The calculated and experimental mobilities gel of the same fragment in a perpendicular denaturing gradient are presented in (b) and (c) respectively. The area corresponding to the calculated mobility is marked at the bottom of the experimental curve.

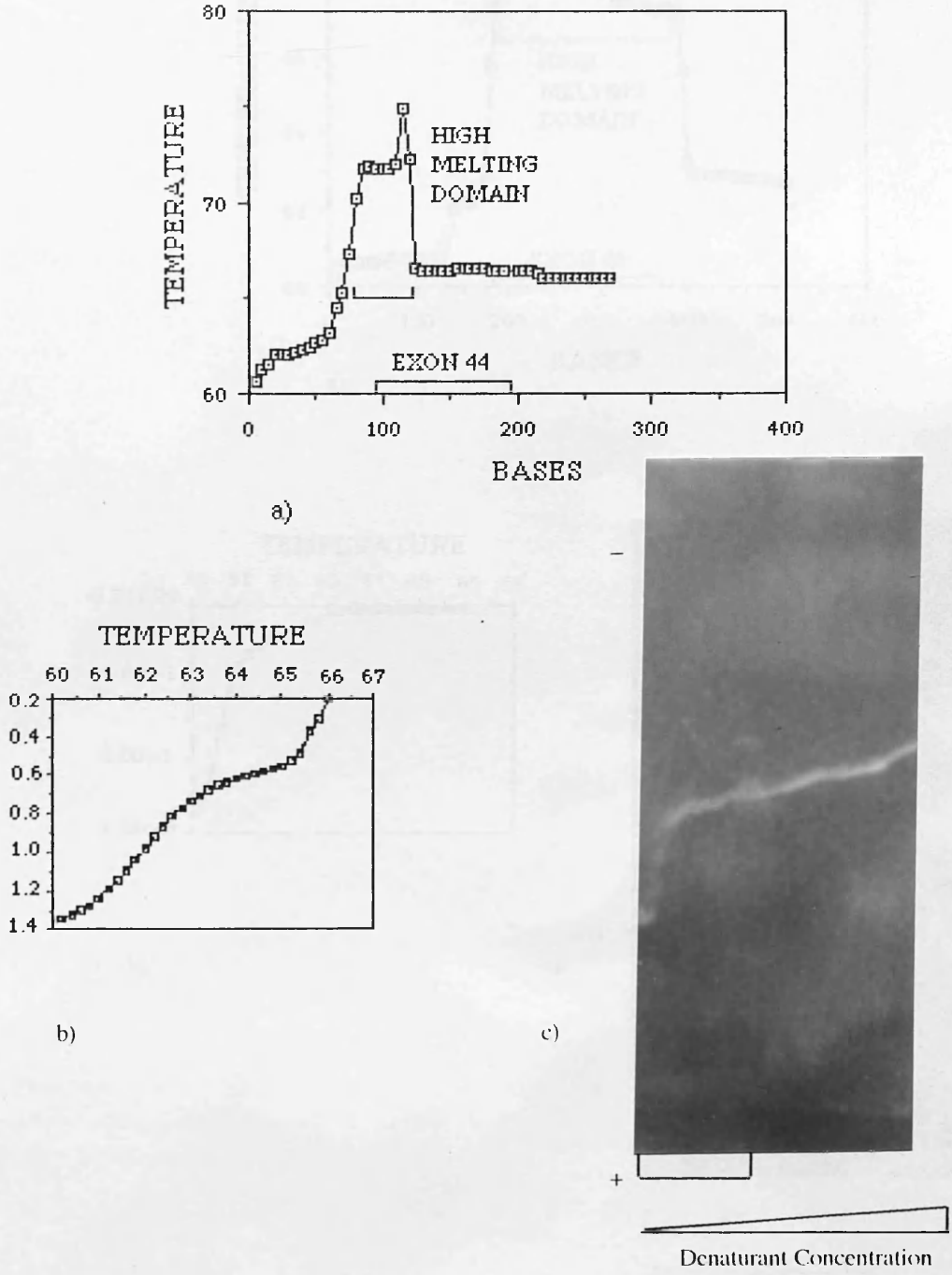
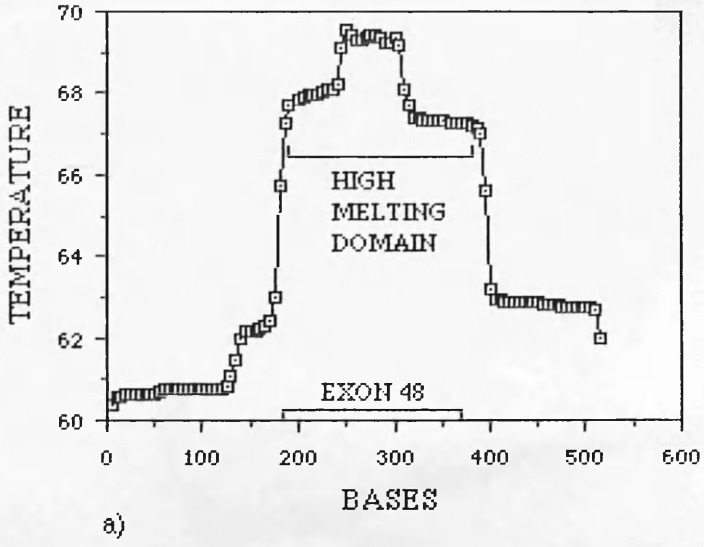
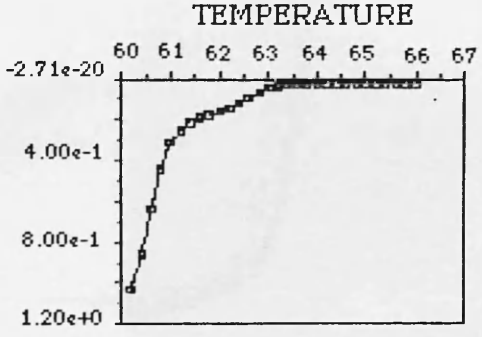


FIGURE 3.27: Melting behavior of sequence flanking exon 44 (region d) of the dystrophin gene. Expected progression of melting of the fragment designated as region D (a). The distribution of each pase pair between the helical and melted states was determined by the MELT 87 computer program. The calculated (b) and experimental pattern (c) of the molecule run perpendicular to a 0-80% denaturant gradient in a 6.5% polyacrylamide gel at 60 °C. The area corresponding to the calculated mobility is marked at the bottom of the experimental curve.



a)



b)

c)

FIGURE 3.28: Melting behavior of sequence flanking exon 48 (region f) of the dystrophin gene. a) Melting map of region f of the dystrophin gene. The plot shows the calculated temperature at which each base pair is distriduted with 50% probability between either the helical or melted states. b) Simulation of perpendicular denaturing gradient gel pattern of the molecule as it derived using the MUTRAV computer program. c) Experimental pattern of electrophoresis of the same fragment in a perpendicular denaturing gradient gel (0-80%) at 60°C.

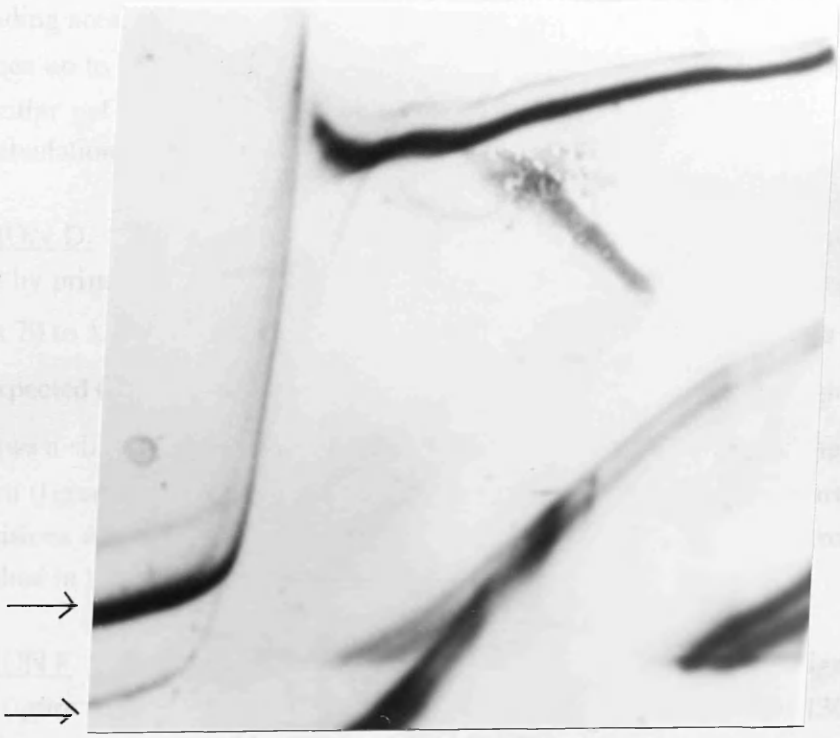


FIGURE 3.29: Denaturing gradient gel electrophoresis of a mixture of two PCR amplification products, using primer set b, each one having one of the two strands labelled with ^{33}P. Each labelled DNA strand moves separately at the left side of the gel, pointed by the arrows, indicating a completely melted DNA molecule.

between the experimental and simulated gel mobility patterns as the temperature at which the DNA ceases to move through the gel as a branched molecule and begins to migrate as denatured single stranded DNA is the same in the two cases.

REGION B. The two panels in figure 3.26 calculated for the fragment amplified by primer set b, illustrate the melting map (a), and the simulated perpendicular denaturing gradient gel pattern (b). A feature derived from the melting map is the absence of a distinguished high temperature melting domain and thus melting and strand dissociation in a large part of the molecule would occur simultaneously preventing the detection of base substitutions in the corresponding area. The mobility transition at 66^0C due to the lower melting domain consisting of the bases up to 160 as predicted by the melting map, can be observed in the experimental perpendicular gel (figure 3.26c) providing further support to the validity of the theoretical melting calculations.

REGION D. The melting calculations for the 268 bp fraction of the dystrophin gene amplified by primer set d are presented in figure 3.27a. The highest melting domain includes base pairs 70 to 120 with a t_m of 71^0C. It can be seen that all of the bases from about 120 to 335 are expected to have a 50% probability of helicity at 65^0C, while the region between 10 and 55 bp shows a slightly lower t_m of 62^0C. The mobility behaviour of the fragment predicted by calculation (figure 3.27b) is consistent with the observed gel pattern (figure 3.27c) since the two transitions at equivalent temperatures corresponding to the two domains can be easily distinguished in the region of 15 to 30% denaturants.

REGION F. The expected melting progression along the molecule designated as region f is shown in figure 3.28a. The first domain to melt includes the bases up to 130. The uniformity of the ordinate value shows that all bases of this region melt as a block. A second domain easily distinguished includes the bases up to 175 with a plateau at 62.5^0C. Additional correspondence between melting theory and the behaviour of the fragment was obtained by comparing the computer-generated gel pattern, figure 3.28b with the experimental data 3.28c. The calculated pattern in perpendicular gradient gel of the molecule shows two inflections corresponding to the two predicted domains. The experimental pattern also shows the two transitions at equivalent temperatures while a third less sharp transition can be observed at a concentration between 30 to 40% denaturants. According to the melting map the bases between 310 to 380 have 50% probability of helicity. For the rest of the molecule there is no temperature at which a partially melted molecule is favoured by the equilibrium, and therefore sensitivity to base substitutions should not be expected.

REGIONS A, C, G, I and H. The observed perpendicular denaturing gradient gel patterns for the regions of the dystrophin gene amplified by primer sets a, c, g, h and i are presented in

figure 3.30. Fragments a, g and i exhibit an abrupt drop in the electrophoretic mobility without showing any sharp transition at a characteristic position along the gradient. An inflection at 30% DNA denaturant concentration can be observed in the perpendicular denaturing gradient gel pattern of region h revealing the existence of a low-melting domain. Domains in the rest of the molecule are indistinguishable. The presence of three domains can be predicted in fragment c based on the pattern of the perpendicular electrophoresis gel. The transitions corresponding to the different melting domains are at 63^0, 66^0 and 69^0C.

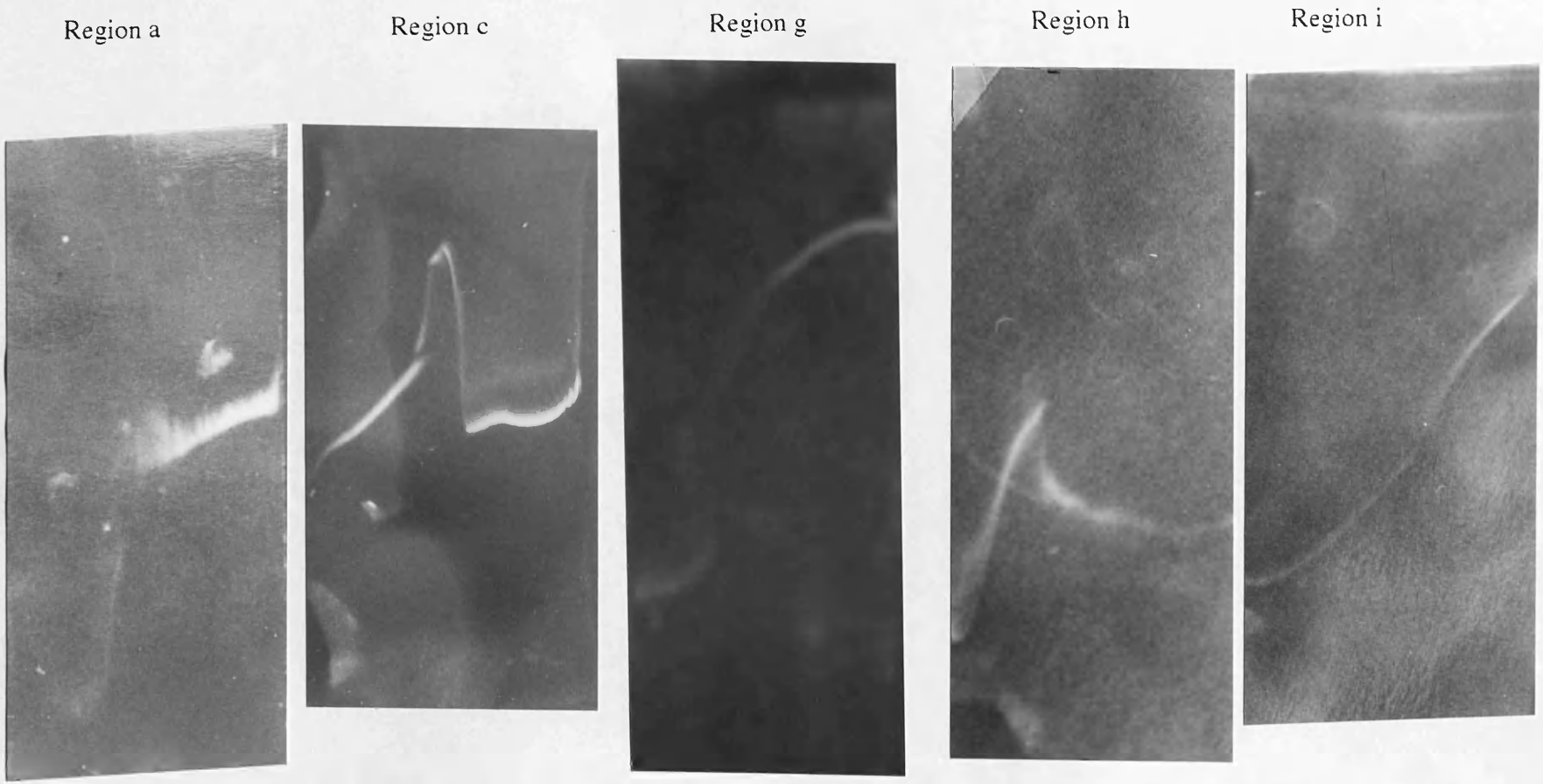


Figure 3.30: Observed perpendicular denaturing gradient gel patterns for the regions of the dystrophin gene amplified by primer sets a, c, g, h and i. Migration was downwards and the denaturant concentration ranges from 0 to 80% (left to right).

CHAPTER IV

DISCUSSION.

4.1 Deletion Screening in the DMD/BMD Gene Using cDNA Probes 5b-7, 9, 10 and 11-14.

In 1989, 254 Scottish pedigrees were known with at least one member affected by X-linked muscular dystrophy. One hundred and thirty-two of these, 110 affected by DMD and 22 affected by BMD, were screened for deletions with the cDNA probes 1-2a, 2b-3, 4-5a, 5b-7 and 8 (Koenig et al., 1987), using Hind III digested DNA. Eighty-five were also studied with cDNA probe 9. The total number of abnormalities detected was eighty-nine deletions and two duplications, 69% (Cooke et al., 1990). The deletions, which ranged from one to thirty-two Hind III fragments in size, where particularly concentrated in the area of probe cDNA 8, while a second smaller concentration was found with probe cDNA 1-2a. cDNA probe 5b-7 detected thirty-seven deletions in a panel of 120 patients tested.

The pattern obtained from the hybridisation of Hind III digested DNA with cDNA probe 5b-7 often presented the problem of weakly hybridising fragments. In order to confirm the presence or absence of such fragments, Bgl II digests were carried out and examined with the cDNA subclone 5b-7 in seventeen patients as described in chapter II. A deletion was confirmed in individual GMG 4190 with the distal endpoint determined to be in intron 44. The proximal endpoint was found to lie in intron 20 (cDNA probe 4-5a). In intron 44 the proximal endpoint of twenty-one cases was found (Cooke et al., 1990). In the rest of the cases confirmation of the presence of the whole range of Hind III fragments was provided.

Cases of DMD/BMD patients showing no deletion or duplication after DNA analysis with the cDNA probes 1-2a, 2b-3, 4-5a, 5b-7 and 8 were further studied using the fragments 9, 10 and 11-14 of the cDNA to detect deletions at the 3' end of the gene. Five cDNA deletions were observed, having both end-points in the distal part of the dystrophin gene, in a sample of thirty-six DMD/BMD affected males showing no deletion or duplication with the rest of the cDNA probes. One deletion (GMG 7589) started from exon 51 had the 3' end-point at the 8.3 kb Hind III fragment, while the one detected by cDNA 10 extended from exon 52 (cDNA 8).

Three of the DMD patients reported here, GMG 8396, GMG 4629 and GMG 7589, have an out-of-frame deletion in the regions of cDNA 8 and 9, figure 4.1. Thus, the correlation between deletion and phenotype fit the reading-frame model introduced by Monaco et al.(1988), to provide a molecular explanation for the phenotypic differences between DMD and BMD patients who bear partial deletions of the Xp21 locus. The suggestion that patients having deletions beginning with exons 54, 55, or 58 could develop a DMD phenotype, due to the synthesis of a dystrophin protein predicted to lack the cysteine-rich and C-terminal domains

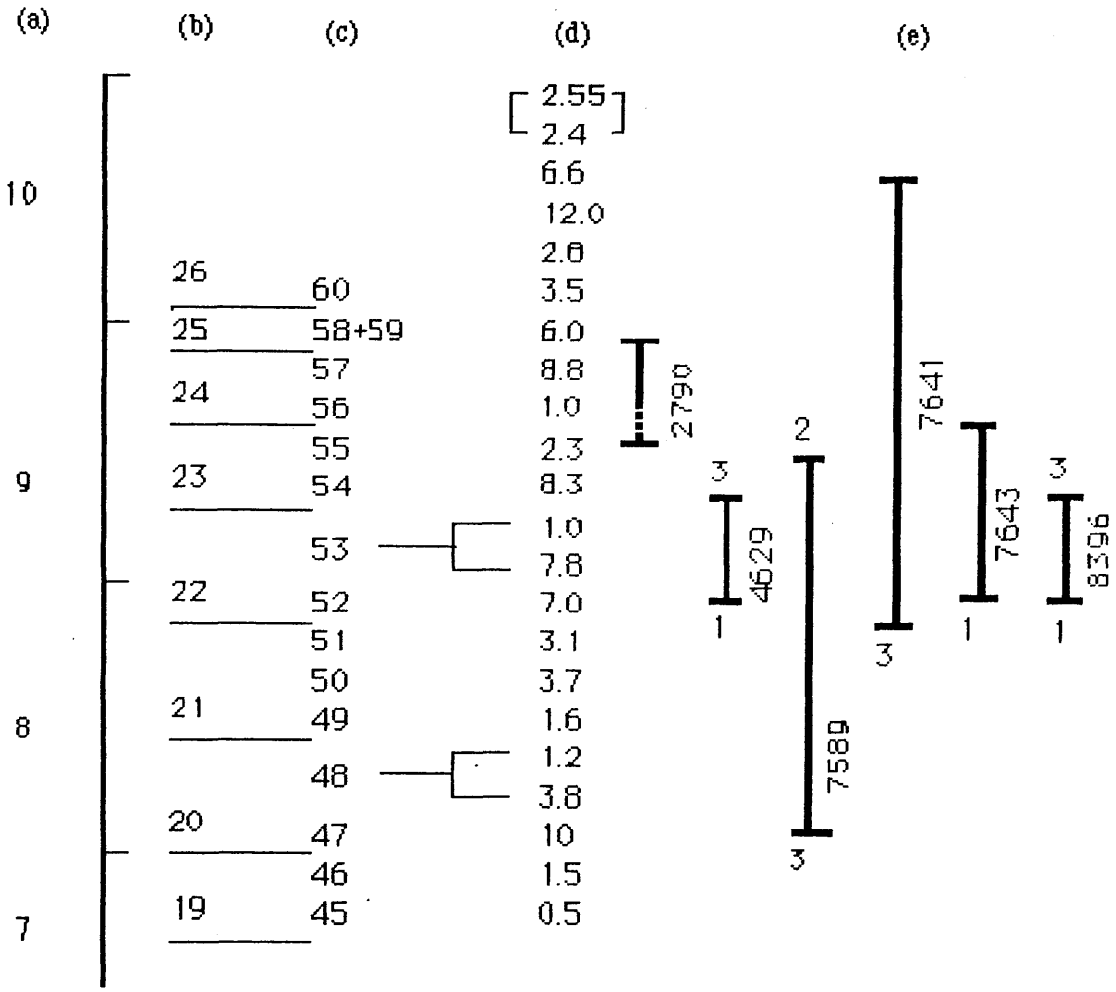


FIGURE 4.1: Extent of six DMD deletions relative to the dystrophin Hind III exon containing map (e). Each deletion is indicated with a line marked by the patient's pedigree number. Bars indicate the beginning and the end of the deletion. a) cDNA probes used for the detection of the deletions; b) distribution of the repeat units (Koenig et al., 1988) relative to exons; d) exon-containing Hind III fragments in kb. The number at the end-points of the deletions represents the exon border type (Koenig et al., 1989).

resulting in an either nonfunctional or unstable molecule (Koenig et al, 1989) could be extended to include exon 53.

The overall frequency of deletions with cDNA 9 was increased to 18.8% in a total of 85 DMD/BMD boys (combination of the results presented here and those reported by Cooke et al., 1990). No deletions were found with cDNA 11-14. Thus, the total number of abnormalities detected with the complete panel of cDNA probes was ninety-four deletions and two duplications in 132 Scottish DMD/BMD boys (73%).

These findings are consistent with those in previous deletion studies on DMD patients. 9% frequency of deletions in the area of cDNA 9-10 is reported by Lindlöf et al. (1989), and in an analysis of forty-two Japanese patients Sugino et al. (1989) describes four deletions extended to the cDNA 9, with only one within that region. No deletions have been confined to the segment covered by probe 11-14 (Koenig et al., 1987; Liechti- Gallati et al., 1989; Lindlöf et al., 1989; Sugino et al.,1989). Deletions involving the 3' portion of the gene have been described in patients with X-linked glycerol kinase deficiency associated with developmental delay and myopathy (Darras and Francke, 1988c). Also, Towbin et al. (1989) reported five patients with glycerol kinase deficiency, adrenal insufficiency and dystrophic myopathy of either Duchenne or Becker type, having large deletions that include the 3' end of the DMD gene. Most of the deletions detected at the distal end of the dystrophin gene are because of the involvement of additional portions of the Xp21 region. Deletions at this part may exist but not produce severe progressive myopathy. DNA analysis of a different patient population using complementary to the 3' end cDNA probes, could provide further evidence supporting this hypothesis.

The deletion in patient GMG 7641 in the region of cDNA 10 rearranges the order of the Hind III fragments suggested by either Koenig et al. (1987) or den Dunnen et al. (1989) to the following : 6.0/3.5/(2.8,12)/(6.6,2.4,2.5); which is in agreement with the one presented by Liechti- Gallati et al.(1989). The 5.9 kb Hind III fragment in the region of probe 11-14 included in the normal Hind III restriction pattern described by Darras and Francke (1988b), could not be distinguished in the experimental work reported here. This fragment is a result of the hybridisation of the subfragment 12b-14 corresponding to the 3' untranslated sequences of the transcript. Application of the cDNA probe 11-14 required the use of more-stringent washing conditions to reduce lane background, resulting from the low-repetitive sequences present and weak bands that do not show X dosage and are presumably of autosomal origin (Darras and Francke, 1988b).

The patterns resulting from the application of different fragments of the cDNA on the DMD/BMD locus, contained in a region of about 2 Mb, are often quite complex and therefore small deletions in affected individuals can be missed because of faintly hybridising bands, unresolvable comigration fragments, or clustered fragments, which do not resolve well with

standard electrophoretic conditions. These pitfalls can be avoided by running longer or higher-percentage gels or by using two restriction enzymes, such as Hind III and Bgl II. The small 0.5 kb Hind III fragment (cDNA 5b-7), corresponding to frequently deleted exon 45, could be easily missed on standard blots while the equivalent 2.8 kb Bgl II fragment can be easily identified offering help in the interpretation of equivocal patterns. Also among the exon-containing Hind III fragments detected by cDNA 9, the 7.8 kb, present in general as a fainter band, and the two co-migrating 1.0 kb fragments can be referred as examples. Thus, availability of both Hind III and Bgl II exon-containing fragment maps facilitate the search of deletions in the DMD/BMD patients and at the same time contributes to the understanding of the genomic organisation of the dystrophin gene. Definition of most deletions detected in this study on both Hind III and Bgl II digested DNA provided more information for the development of a Bgl II exon-containing map.

The investigation of the molecular pathology of the disease with the Southern blot technique, in spite of its advantages over linkage analysis, has its own limitations as exons of this gene are located on at least 65 genomic Hind III restriction fragments (Koenig et al.,1987), which in several cases necessitates hybridisation of Southern blots with eight separate cDNA fragments for diagnosis of genomic alterations. Also use of Southern blot analysis for deletion screening of DMD/BMD patients, especially with the panel of cDNA probes that produce rather complex hybridisation patterns, consist of weakly hybridising bands and clustered or unresolvable fragments, requires a lot of experience for the accurate interpretation of the results. In those cases where a second restriction enzyme has to be used further time (about two weeks) is required to obtain the results. The necessity of radioactive labelling of the probe used in the hybridisation analysis, is another drawback of the Southern approach. Thus, although Southern blot analysis allows a highly reliable prenatal diagnosis in most of the cases, other technologies which are less expensive, tedious and time-consuming but as accurate are demanded for both prenatal diagnosis and carrier detection of the Duchenne and Becker muscular dystrophies.

4.2 Polymerase Chain Reaction : An Alternative Deletion Detection System.

The multiplex PCR assay developed by Chamberlain et al. (1990), was used to perform deletion screening in 118 DMD/BMD samples. An evaluation of sixty-four separate deletions previously found using the cDNA probes, suggests that the multiplex reaction will detect approximately 86% of deletions. The initial PCR assay developed by Chamberlain et al. (1988), would detect 70% of these deletions. The detection of the extra 16% was the result of additional amplifications of exons 4 and 51, primer sets i and h respectively, since amplification with primer set g (exon 12) did not reveal any deletion which could not be detected by the other

primer sets. Discrepancies were not found between the results obtained by the two methods of analysis.

The ability to perform accurate deletion screening by PCR has advantages over traditional Southern blot techniques. Mutations in unrelated families are likely to be of independent origin and a third of all cases arise from new mutations, hence detection of deletions often requires the application of up to seven different subcloned cDNA probes on two different restriction digests of genomic DNA. Eliminating therefore 86% of the cases from the necessity for Southern analysis, clearly illustrates the savings of time and effort which can be achieved with a multiplex amplification protocol. Also the ability of PCR to amplify DNA segments from small amounts of DNA, permitted the development of rapid DNA isolation protocols from different sources including CVS samples making PCR ideal for prenatal diagnosis where time and sample quantity may be at a premium. Additional features of PCR contribute further to their utility. Whereas Southern analysis requires good quality high molecular weight DNA, PCR procedures can use crude and partially degraded samples and therefore allow studies to be done on fixed and embedded specimens such as might be available for deceased patients who have living relatives at-risk for DMD/BMD.

As more sequence data for the DMD/BMD locus are becoming available additional primer sets have been designed to increase the frequency of deletion detection. Similar assays have more recently been developed (Beggs et al.,1990; Abbs et al.,1991) improving the detection of deletions in the dystrophin gene to 98%, tending to make PCR deletion screening the method of choice. Amplification of more coding sequences of the dystrophin gene will withdraw the drawback of the PCR multiplex assay to determine accurately the extent of the deletions which is necessary for the differentiation between Duchenne and Becker muscular dystrophy. Recently direct diagnosis of carriers of Duchenne and Becker muscular dystrophy by amplification of lymphocyte RNA has been reported (Roberts et al, 1990). The entire coding region of the dystrophin mRNA was amplified in ten sections by reverse transcription and nested polymerase chain reaction, and the products were directly visualised on polyacrylamide minigels with ethidium bromide staining. Major structural gene mutations were identified by the appearance of bands different in size of those of the wild type.

4.3 Problems Associated with the Application of PCR.

During the first stages of this project when the amplification conditions for individual primers were being determined some cases gave no detectable amplification. Since the oligonucleotide primers used in this study had already been tested elsewhere, failure of the PCR reaction as a result of their complementarity was excluded. The primer sets used were HPLC

purified as attempts at amplification with unpurified primers proved unsuccessful. Brief mixing of the AmpliTaq polymerase before its addition to the reaction, and complete DNA hybridisation in each cycle by allowing sufficient time at the denaturation plateau temperature, were some of the steps taken to overcome the problem. Also pre-incubation at 95^0C for 5 minutes in the absence of enzyme was applied. Pre-amplification heating may inactivate harmful proteases or nucleases present in the sample and enhance the denaturation of the complex genomic DNA. D'Aquila et al. (1991), speculated that preamplification heating may promote stringent primer annealing and subsequent extension, and minimise the formation of both "primer-dimer" and primer self-annealing. Use of a lower annealing temperature was also helpful as well.

Optimisation of the annealing temperature where priming of a non-target DNA is rare, was important especially since total-genomic DNA which is very complex was used as substrate for the PCR. At sub- and super-optimal annealing temperature values non-specific products can be formed reducing the yield of the amplification product. Occasionally small amounts of non-specific amplification were observed in samples even under optima annealing conditions. These cases were explained by priming of a non-target sequence during the first few cycles of amplification, giving the opportunity for an electrophoretic visible band to accumulate. A variability of the intensities of a specific signal was sometimes observed during the multiplex amplification assay. This was presumed to be the result of the failure of the polymerase to locate and extend all template strands during the first few cycles of the reaction. The presence of fainter than usual bands did not obscure the interpretation of the PCR based multiplex assay results as the "missing exons" did not often form a contiguous deletion. The experiment was repeated if there was any doubt about the result.

Several investigators have observed that inclusion of up to 10% of the cosolvent dimethylsulfoxide (DMSO) facilitates certain PCR assays. In all cases in the present study where a multiplex PCR amplification.was performed, 2% of DMSO was added per reaction. It is not clear which parameters of the reaction the presence of DMSO affects. It may affect the T_m of the primers, the thermal activity profile of Taq DNA polymerase, and/or the degree of product strand separation achieved at a particular "denaturation" temperature (Gelfand and White, 1990). Low concentration of DMSO <1% have no effect on the incorporation activity of Taq polymerase (Gelfand et al., 1989); whereas the presence of 10% DMSO used previously in Klenow-mediated PCR inhibits DNA synthesis by 50% at 70^0C (Scharf et al., 1986).

All PCR reactions performed during this study were overlaid with mineral oil. Perkin Elmer Cetus recommends adding of an oil overlay to the reaction mixture if the standard 0.5 microfuge tubes are to be used, to reduce both cooling and liquid loss due to the evaporation particularly at the denaturation step. Drop of the liquid level in a PCR reaction without oil would change the concentration of the reaction components possibly effecting the yield of the

amplification product, since it is known that PCR is very sensitive to pH and salt concentration present. Presence of more DNA rather than less seemed to be a reason for failure of a PCR reaction. This could be due to the presence of impurities affecting the activity of the enzyme.

The most pernicious problem is contamination of reagents from minute amount of exogenous DNA. This method can amplify sequences which are present only once in 10^5 to 10^6 cells (Saiki et al.,1988), and this necessitates great care to avoid false positives. False positives can result from either sample-to-sample contamination, or from the carry over of DNA from a previous amplification of the same target. Therefore some precautions should be taken during the preparation of the PCR reactions. Positive displacement pipettes, where the units are self-contained, were used to prevent contamination of the samples due to the contamination of the barrels of pipetting devices as a result of aerosolisation. Also aliquoting of the reagents to minimise the number of repeated samplings was found to be helpful, facilitating both the application of PCR on new samples and tracing batches of reagents found to be contaminated. Other precautions included frequent changes of gloves, minimum sample handling and rapid spinning of tubes before opening them. All consumables and solutions were autoclaved and in all cases the DNA template was added last. A major contribution to the preparation of false-positive free PCR amplification was the treatment of the PCR reaction mix with ultraviolet light directly before adding the template (Sarkar and Sommer, 1990). Fifteen minutes of U.V. treatment found to be adequate to sufficiently nick and crosslink any contaminating sequences rendering them unamplifiable without reducing the yield of the PCR reaction. In every set of reactions negative and positive controls were included.

4.4 Amplification of Degraded DNA or DNA Extracted from Haematoxylin and Eosin Stained Sections.

The two cases of degraded samples which failed to amplify, although the average molecular weight appeared to be adequate, could be due either to the presence of contaminants in the samples inhibiting the activity of the Taq polymerase, or to damage to the template DNA. Since dimers of primers were observed indicating that the enzymatic activity of the Taq DNA polymerase was not inhibited, the first possibility was excluded. "Primer-dimers" are produced for two reasons: first, primer complementarity, and second, because Taq polymerase tends to add on a number of non-specific nucleotides to the ends of unused primers which may then overlap and anneal. The two primers are extended along each other by the enzyme to produce a double stranded fragment of DNA, which will appear on the PCR gel. Although increasing amounts of template DNA were added no amplification was detected even when amplification was performed using 5' end labelled primers.

DNA depurination caused by acid fixation does not seem to prevent the amplification of specific DNA sequences. The yield of large fragments during PCR amplification of such DNA appeared to be somewhat smaller. One interpretation is that because of the fragmented nature of the DNA removed from the slides, there are fewer molecules of the appropriate size usable as a template in the PCR reaction. The same interpretation could apply to the reduced PCR product yield observed when the template DNA was extracted from sections not directly treated on the slides but scraped and transferred to a microfuge tube. The use of DNA extracted from sections in PCR amplification has improved our capability to analyse mutations in DMD/BMD families offering accurate genetic counselling in many families where key family members are deceased. Caution should be taken though if the product of such PCR reaction is to be used for sequencing,as DNA damage resulting from fixation may cause replication errors during PCR (Pääbo, 1990).

4.5 Possible Mechanisms of the Mutations Causing DMD/BMD.

The mutation rate in DMD has been estimated to range around 80 to 100 x 10^{-6} mutation per gene per generation , which is considerably higherthan any other X-linked disorder. For haemophilia A and B these values are 32-57 and 2-3 x 10^{-6} respectively (Emery, 1988). The new mutations appear to be equally likely to occur in the formation of a sperm as in an ovum and one third of the cases are considered to be new mutations. Mutations in the formation of ova result in isolated affected males whose mothers are not carriers whilst mutations in sperm result in carrier females who can then have affected sons.

Intragenic deletions appear to be the most common gene defect leading to DMD/ BMD. To a certain extent, the high proportion of deletions detected at the DMD locus is a consequence of its considerable size, although certain regions of the DMD gene appeared to be more deletion-prone than others. The high deletion breakpoint concentration in these regions compared to that of other areas of the dystrophin gene can be attributed to either increased deletion detection level in these regions or to the presence of a structure prone to instability. Some very mild myopathies have been reported to be associated with deletions in the area of the dystrophin gene. England et al., (1990) reported a case where very mild muscular dystrophy was associated with the deletion of almost half the coding sequence. Clustering of breakpoints could be due to either structure-specific (non-homologous) or sequence-specific (homologous) recombination hot spots.

Deletions might occur as a consequence of non-homologous recombination between

widely separated sequences that are physically close as a result of anchorage to the nuclear matrix. One model, involving loss of a complete chromatin loop, predicts deletions of approximately equal size with different end-points. Anand et al. (1988), proposed that deletions might occur by deletion of newly replicated DNA loops, the size of the deletion being dependent on the length of DNA replicated. One-end point of such a deletion is defined by the region immediately adjacent to the nuclear-matrix attachment site, whilst the second is potentially defined by a DNA-polymerase arrest site. Krawczak and Cooper (1991), noted similarities between a consensus sequence that appears to be common to hot spots of deletions found in several different human genes and to the arrest sites for human DNA polymerase α.

Winter and Pembrey (1982) proposed a model to explain the generation of deletions by unequal crossing over between the two X-chromosomes in female meiosis as the result of misalignment of two nonsister chromatids, facilitated possibly by sequence homologous elements. Unequal crossing over, leading to gene deletion or duplication is known to occur in the α-globin gene (Gossens et al., 1980). Some introns of the DMD gene could contain interspersed repetitive DNA such as the Alu family, which have been found capable of mediating unequal chromatid exchange. When 113 breakpoints were mapped to the distal 80 kb of intron 44 which is a recombination hot spot in the dystrophin gene the breakpoints were found to distribute over the entire region, with no significant local variation in frequency which argues against a predominant role of one or a few specific sequences in causing frequent rearrangements (Blonden et al., 1991) In fact, many deletions found in other genetic diseases appear not to result from unequal chromatid exchange but rather to be generated from a single chromatid (Vanin,1983). This type of intrachromatid deletion would not be expected to be a mechanism for generating duplication. Second duplications may not always be stably inherited, in that the duplicated area may undergo spontaneous deletion. Yang et al. (1988), reported a case where the Epstein-Barr-virus-transformed lymphoblasts from a Lesch-Nyhan patient with a partial duplication of the HPRT gene were found to have undergone spontaneous deletion of the duplicated region. It also remains a possibility that some duplications and deletions in the DMD gene may not cause disease phenotype (Nordenskjöld et al.,1990). If so, the true ratio of deletion versus duplication could be different from that observed in patients.

4.6 Screening for Sequence Alterations in Regions of the Dystrophin Gene.

Although that Southern analysis, using cDNA representing exons of the DMD/BMD gene (Cooke et al., 1990) and an assay involving the polymerase chain reaction were employed the molecular pathology of the disease remained unknown in 28% of the patients. The phenotype in the rest of the patients was considered to be the result of point mutations, splicing alterations, or small frameshifting deletions and duplications along the dystrophin gene. Therefore a systematic screening of nine regions of the dystrophin gene from DMD/BMD patients with

unknown molecular pathology was carried out by the single strand conformational polymorphism method.

Using this approach, a polymorphism was detected in the distal part of intron 44. This intron has a size of 170 kb and is already known to be a hot spot for recombination. Blonden et al. (1991), mapped the breakpoints in 113 cases of DMD/BMD patients in the distal 80 kb fragment of intron 44. The apparent high mutation rate in this region where the p20 genomic probe has also been mapped, is considered to reflect a diffused chromosomal instability since the breakpoints were distributed over the entire region. In families with unknown molecular pathology the use of restriction fragment length polymorphisms is the only method for prenatal diagnosis and female carrier risk determination combined with serum creatine phosphokinase measurements and this new polymorphism is a useful additional marker for analysis in such families.

Screening for sequence variations by PCR-SSCP analysis is a simple, fast and efficient approach. All steps including amplification, mutation detection, and sequencing of the fragment containing the alteration were performed using the same pair of primers with standard equipment. The target sequence was amplified and labelled simultaneously by the PCR obviating the need for either restriction enzyme digestion or a hybridisation step. Since in a typical PCR, up to 10% of the substrates (primers and deoxynucleotides) are incorporated into the amplified product, the efficiency of labelling target sequences during the amplification step using labelled primers, was extremely high compared to the efficiency in Southern blotting experiments in which much smaller portions of the labell was attached to the target sequences. Consequently the time of exposure to X ray film was much shorter than in the conventional analysis and the entire procedure including exposure time, could be completed within twenty-four hours. The minimum amount of sample needed due to its high radioactivity, permitted the use of a thin polyacrylamide gel where a steep voltage gradient could be applied without serious Ohmic heating. This reduced the amount of time required for electrophoresis, and increased the resolution.

Currently the effect of sequence change on electrophoretic mobility is unpredictable and it is not known whether nucleotide substitution at any position in a fragment can be detected by SSCP analysis. The PCR-SSCP technique has so far been applied on segments of radiolabelled DNA 100 to 400 bp (Dean, 1990; Labrune, 1991). Thus, the PCR products of primer sets a, b, c, d, g and h of the dystrophin gene were within this limit for analysis by the PCR-SSCP technique. Study of thirty different mutations gave a 97% mutation detection frequency when the method was applied on fragments 100 to 300 base long while this value dropped to 67% for strands of 350-450 bases long (Murakami et al., cited in Hayashi, 1991). Therefore, the possibility of detecting at least one strand shift was high in all fragments with the exception of

those amplified by primer sets e and f. Also, it cannot be excluded that mutations localised close to one end of a fragment would not sufficiently influence the conformation of the single-stranded DNA and would not be therefore, detected by PCR-SSCP analysis. Cases where sequence variations very near the primers used, were detected via PCR-SSCP analysis have not been so far reported. Hence false negatives may occur with the SSCP technique.

No false-positive results were observed. All patients whose patterns of DNA migration were abnormal were consistently found to carry the base substitution in the corresponding area, while in patients whose migration profiles were similar to that of the controls, no base substitutions were present.

PCR-SSCP analysis requires prior information on the representative sequence for the design of the primers, and establishment of amplification conditions where specificity would be the main characteristic. Within this limitation, this method can be applied to a large number of samples without any further complications on the interpretation of the results. The minor faint bands sometimes present in the lanes where denatured samples were loaded but not in the lanes where the samples were electrophoresed without denaturation, were assumed to be different conformers of the same sequence. Usually, intensity of these conformer bands can be different, but the ratio of the intensity between them is constant from sample to sample. Therefore, such sequence-different conformation situations can be easily recognised.

No difference was observed in the electrophoretic pattern of samples electrophoresed immediately or within a week after amplification by the polymerase chain reaction. Labelled PCR samples were kept at 4^0C immediately after PCR amplification.

4.7 Identifying Point Mutations Using the Method of Denaturing Gradient Gel Electrophoresis.

Generally, perpendicular denaturing gradient gels have been used to determine the optimum conditions for the analysis of samples for parallel DGGE (Myers,1987). However, perpendicular denaturing gradient gels can also be used to identify sequence variations in DNA fragments. Similar to parallel DGGE, perpendicular DGGE can identify more than 50% of all base changes within a DNA fragment (Sheffield et al., 1990). Perpendicular DGGE can be efficient and simple, because it utilises single standardised gel and electrophoresis conditions, and the entire DNA sample is exposed to a very broad range of denaturant concentrations. The approach includes amplification of specific DNA fragments by PCR, and electrophoresis on a perpendicular denaturing gradient gel. If an individual is heterozygous for a single base change within the region of the amplified fragment a split in the curve is expected to be seen when the

sample from this individual is electrophoresed on a 0 to 80% perpendicular denaturing gradient gel. Samples amplified from a non-polymorphic individual appear as single DNA curves that do not split into two. In the case of Duchenne muscular dystrophy which shows an X-linked pattern of transmission, a normal chromosome is not present in the DNA of the males to be examined for sequence alterations. To overcome this problem the DNA sample to be examined was combined with DNA from a control sample. A pooling strategy initially introduced by Gille (1991) for identifying heterozygosity of the ΔF508 mutation causing cystic fibrosis, was applied to substantially economise and speed up the screening procedure as only the pool of samples showing a split in the curve would be necessary to be examined individually. This approach was feasible in the case of Duchenne muscular dystrophy as there is a high heterogeneity in the molecular pathology of the disease. All samples were tested for positive amplification by PCR before they were combined and applied on a perpendicular gel for DGGE analysis to prevent false negatives. A sequence variation was detected in eight cases in the region amplified by primer set e. The eight individuals were the same as those detected by PCR-SSCP analysis to bear the polymorphic site at position 203 from the 5'end of the sense primer flanking the area e of the dystrophin gene. Therefore, the results of point mutation analysis in the regions of the dystrophin gene flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 obtained by both the single-strand conformation polymorphism approach and the method of denaturing gradient gel electrophoresis did not present any inconsistencies.

The method of denaturing gradient gel electrophoresis for the identification of mutation sites or polymorphisms can be used to screen up to several hundred base pairs in a single test and as the results can be examined directly by ethidium bromide staining of the gel the use of radioactivity is unnecessary.

The generation of the temperature gradient in the gel requires special equipment. One limitation of DGGE is that aberrant migration will only occur if the point mutation is in certain points of the molecule. This is a consequence of the melting properties of DNA and the relationship between DNA melting and the electrophoretic behaviour of a fragment in the denaturing gradient. As DNA fragments move through polyacrylamide gels containing an ascending gradient of denaturant, small regions (the melting domains) undergo cooperative strand dissociation to produce partially denatured molecules which display a marked decrease in electrophoretic mobility. However, when the most stable domain melts the fragment undergoes complete strand dissociation and the resolving power of the gel is lost. This problem can be circumvented by incorporation of a "GC-clamp" of up to 40 bp on at least one direction during the amplification step (Myers et al., 1985 b,c). DNA melting calculations based on the nucleotide sequence of the fragment to be analysed using the computer programmes available can predict the existence and the location of the low melting domain or domains, providing an indication of the regions within the sequence where base changes are likely to be detected. The reported detection frequency of single nucleotide changes is 50-70% in DNA fragments up to 1

kb in length (Myers et al.,1987). DNA fragments larger than 1 kb are difficult to examine by DGGE because they migrate very slowly in the polyacrylamide gel and because the degree of separation between mutant and wild type decreases due to the melting of multiple melting domains in the larger fragments. Short DNA sequences can undergo a thermal subtransition at a lower temperature if they lie near the end of the molecule, rather than separated from the end by a hundred base pairs or more of a higher melting sequence (Lerman et al., 1984). This could be an explanation for a discontinuous jump in the band observed in some cases.

The interpretation of band patterns of PCR amplified labelled DNA separated by DGGE in a perpendicular configuration was sometimes impeded by the appearance of a false positive split of the sigmoid curve. As the samples used initially for the detection of sequence variations by DGGE were the same as those screened by PCR-SSCP analysis, incomplete kinase labelling reactions yielding mixtures of 5'(^{32}P)-phosphate and 5' hydroxyl primers, whose PCR products contain 5'(^{32}P)-phosphorylated and 5' hydroxyl low melting domains considered to be the reason. This hypothesis was suggested and tested by Pfeiffer and Thilly (1991) who conclude that the presence of a 5' phosphate destabilised the low-melting domain by about 0.013^{0}C.

4.8 Comparison Between Experimental and Theoretical Patterns Obtained by DGGE.

Calculation of the theoretical pattern of thermal stability of DNA molecules of known sequence, together with calculation of the expected changes in electrophoretic mobility in gels under denaturing conditions, is an advantage when searching for sequence variations by means of denaturing gradient gel electrophoresis. The calculations are easy to execute for any sequence on a digital computer and they can provide an indication of the regions within the sequence, where base changes are likely to be detected. Since the separation of DNA fragments differing by a single base substitution is possible only under conditions that generate partially denatured molecules, single base substitutions will not be discerned in DNA fragments containing a single domain.

Nucleotide sequence data were available for the four out of the nine different regions of the dystrophin gene analysed in the present study. Thus, the melting maps and the relative electrophoretic mobility were calculated for the regions e, b, d and f , using the computer programmes MELT 87 and MUTRAV respectively, developed by Lerman and Silverstein (1987). The melting behaviour of DNA molecules predicted by calculation was in agreement with the pattern of electrophoresis on denaturing gradient polyacrylamide gels. The correlation between theory and experimental observations consisted of the effect of sequence context on the

distribution of melting domains, the location and t_m of melting domains and the behaviour of partially denatured molecules during electrophoresis through denaturing gradient gels.

DNA melting calculations using the sequence of the region e predicted the existence of a high melting domain (t_m = 75^{0}C) between 460 and 530 bp near the 3' end allowing the detection of sequence variation in the rest of the fragment. As exon 45, which is flanked by the primer set e, is located between bases 345 and 521 bp sequence changes at the 3' end of the exon were not expected to lead to changes in the electrophoretic behaviour of DNA fragments. The polymorphic site detected by PCR-SSCP analysis could be detected by DGGE analysis as it is located within the low melting domain.

In the region b only polymorphic sites in the 3' end of intron 16 could affect the final gradient penetration of the DNA fragment. According to the calculated melting map only a short region at the 5' end consisting of the bases up to 160 could be considered as a lower melting domain. Due to the absence of a distinguished high temperature melting domain detection of mutations in a large part of the molecule is not possible.

Polymorphic sites at the intronic sequences flanking exon 48 can also be detected in the DNA fragment amplified by primer f. Mutations in the area of the exon should give final gel positions indistinguishable from wild type as they lie within a higher melting domain and the strands would disassociate when the domain is melted.

Two low melting domains with temperature plateaus at 62^0 and 65^{0}C respectively allow identification of sequence variations in the whole of region d with the exception of bases 70 to 120 which are part of the included exon 44.

The relation between electrophoretic mobility and denaturant concentration for the regions of the dystrophin gene amplified by primers a, c, g and h were examined on a perpendicular gradient gel. Although in all cases at least one lower melting domain was present permitting the detection of sequence variations in a part of the molecule the exact position cannot be determined as the melting map of the corresponding DNA fragment could not be plotted due to lack of sequence data.

4.9 Amplification Mismatch Detection System for the Identification of Point Mutations.

The sequence alteration G to A present in intron 44 of eight DMD patients was also identified by the approach of PCR and chemical cleavage of the mismatch (amplification mismatch detection system). While the control samples presented a single band corresponding

to the uncleaved labelled strand individuals showing the migration pattern a by SSCP analysis presented an extra band product of the chemical cleavage. Thus, neither false negatives nor false positives were observed from the application of AMD analysis.

Since the method is based on the chemical reactivity, in a heteroduplex formed from wild-type and mutant DNA, of mismatched T and C bases respectively with osmium tetroxide and hydroxylamine, the type of substitution of one or more mutations in a particular region can be identified. Another advantage of the technique is the ability to provide information regarding the position of the alteration. Dianzani et al., 1991, developed an approach for the discrimination of heterozygotes and homozygotes of a certain point mutation based on the ability of AMD analysis to localise the mutation. When both normal DNA and DNA from a homozygote for the mutation were successively used for the formation of a heteroduplex the pattern of the chemical cleavage obtained permitted the differentiation of the status between a homozygote and a heterozygote for two mutations causing β-thalassaemia. The main disadvantage of the method is the highly toxic reagents, which are dangerous to handle and difficult to dispose of.

4.10 Detection of Point Mutations by Direct Sequencing of PCR Amplified Genomic DNA.

Sequencing of the fragment showing altered electrophoretic pattern was performed in order to detect the sequence alteration causing the mobility shift. The asymmetric PCR approach was chosen, as direct sequencing of double-stranded PCR products is usually more difficult because the short linear template has a strong tendency to reanneal. Since the efficiency of the asymmetric PCR was somewhat lower than the equivalent standard PCR a larger number of cycles was performed. The homogeneity of the asymmetric PCR product was increased by reamplification of a small amount of the product of a standard PCR. The methodology followed has two potential drawbacks. The first is due to the two separated steps involved in making the PCR reaction more succeptable to contamination, and the second is due to the large number of cycles which is a potential source of incorporating errors. Several investigators though estimated that the Thermus aquaticus (Taq) DNA polymerase despite lacking a 3' to 5' proof-reading exonuclease activity can catalyse highly accurate DNA synthesis in vitro, especially if the number of starting templates is large, and that the probability of false diagnosis due to such errors can be less than in one in twenty-five million (Eckert, 1990; Krawczak,1989). As misincorporated bases cannot be proof-read and mismatched bases are inefficiently extended, misincorporation errors that do occur during PCR promote chain termination which restricts the amplification of defective molecules and helps to maintain fidelity. For the Drosophila DNA polymerase α it was shown that enzymatic discrimination against elongating mismatched termini is based mainly on K_m differences. A matched A-T terminus was found to be extended 200 times faster than a G-T mismatch was and 1400 and 2500 times faster than C-T and T-T

mismatches, respectively. As the same is likely to be true for Taq DNA polymerase the concentration of dNTPs in the reaction should have a substantial effect on the fidelity of PCR. Therefore, high annealing temperature (>55^{0}C) and low dNTP concentrations improve the fidelity in the final PCR reaction (Innis and Gelfand, 1990).

No particular technical difficulties were experienced regarding the direct sequence of a PCR amplified single-strand fragment. As long as in both the standard PCR and the asymmetric one a relatively distinct band was observed, the sequencing reaction would normally work. The isotope used for the sequencing reactions was α^{35}S ATP as it was less expensive, had a longer half-life and the bands obtained were uniform through out the gel. On average two-hundred bases were readable per lane in each gel.

4.11 Other Strategies for the Identification of Point Mutations in the DMD/BMD Gene.

Application of both SSCP and DGGE point mutation detection strategies in nine different regions of the dystrophin gene and partial application of AMD analysis, did not reveal any pathogenic sequence variations in a panel of thirty-nine Duchenne and Becker muscular dystrophy patients with no deletion or duplication previously detected by either Southern analysis or PCR based multiplex assay. This is possibly due to the limited size of the region examined (approximately 3.5 kb of genomic sequence, 1500 base pairs of exonic sequence), compared to the genomic size of the locus (2 Mb) which encodes a transcript of about 14 kb. To circumvent this problem the methods of either SSCP, DGGE or AMD can be applied on cDNA corresponding to the entire coding sequence of the dystrophin gene generated by PCR using total RNA from peripheral blood lymphocytes as template for the reverse transcription (Roberts et al., 1991). In this case consideration should be given to a possible transcript-processing differences between muscle and peripheral blood lymphocytes. Currently amplification of small amount of mRNA from peripheral blood lymphocytes can be used for the detection of major structural aberrations of the DMD/BMD gene. Deletion and duplication mutations causing DMD/BMD were identified as bands of different size after electrophoresis of the amplification products on acrylamide minigels (Roberts et al., 1990).

A point mutation, G to T transversion, in the DMD/BMD gene was lately reported by Bulman et al. (1991), at position 3714 that changes a glutamic acid codon to an amber stop codon. The point mutation was identified through Western blot analysis which detected a truncated protein in a Duchenne muscular dystrophy patient. The estimated molecular weight of the protein predicted the approximate location of the mutation in the mRNA and in the gene, facilitating further sequencing of a specific region. This approach of using the estimated protein size to pinpoint the site of mutation could be generally applicable to other patients where the

protein product of the dystrophin gene can be detected to identify additional point mutations, splicing alterations, and small frameshifting deletions and duplications.

4.12 Pursuing the Molecular Pathology of Inherited Diseases.

Genetic disease and congenital malformations occur in approximately two to five per cent of all live births and are the cause of about forty to fifty per cent of deaths occuring under the age of fifteen years. As there is little help available in the way of therapy, rapid and efficient strategies to determine the molecular basis of inherited diseases, and to provide carrier and prenatal diagnosis for genetic counselling are necessary.

By utilising the advances in recombinant DNA technology a significant number of genes of medical interest have been isolated. Currently the human gene map consists of more than 6000 loci of which approximately one third are genes of known function (Human Gene Mapping Workshop). Using the methods of SSCP, DGGE, and AMD analysis the molecular pathology of several inherited disorders have been clarified. Improving the prospectives of carrier and prenatal diagnosis, disease causing mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, in the area of the neurofibromatosis type I locus and in the phenylalanine hydroxylase locus have been identified by SSCP analysis (Dean et al., 1990; Iannuzzi et al., 1991; Cawthon et al., 1990; Labrune et al., 1991). Mutations identified also by the approach of DGGE in the coding sequence of rhodopsin gene were associated with an autosomal form of retinitis pigmentosa, ADRP (Sheffield et al., 1991). The AMD analysis has also been employed for the direct analysis of point mutations on the factor IX gene of haemophilia B patients (Montandon et al., 1989).

Identification of the molecular pathology of genetic diseases eliminates the need for informative segregation of polymorphic markers permitting accurate carrier and prenatal diagnosis. Therefore rapid and efficient strategies such as those provided by SSCP and DGGE are important tools in the study of the molecular pathology of inherited diseases, especially in those cases where the molecular pathology is unknown or highly heterogenic and the size of the gene does not facilitate its entire sequencing.

CHAPTER V

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APPENDIX I

SOLUTIONS

All solutions used were prepared with distilled water. They are listed in alphabetical order.

Acrylamide stock solution (40%) 37.5:1 acrylamide : bisacrylamide) : For 500 ml, 200 g electrophoresis-grade acrylamide (BDH) and 5.35 g bisacrylamide were dissolved in 500 ml double distilled water. The stock solution was kept in a dark bottle at 4^0C.

5 x Annealing buffer (Sequenase 2.0 kit, USB) : 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl. Kept at -20^0C.

Denaturant stock solution (0%) - 7.5% acrylamide in 1 x TAE buffer : For 200 ml : 37.5 ml acrylamide stock solution, 10 ml 20 x TAE and 152.5 ml double distilled water. The stock solution was kept in a dark bottle at 4^0C.

Denaturant stock solution (80%) - 7.5% acrylamide, 5.6M urea and 32% formamide in 1 x TAE buffer : For 200 ml : 37.5 ml acrylamide stock solution, 10 ml 20 x TAE, 67.2 g electrophoresis grade urea (BRL), 64 ml deionised formamide and double distilled water up to 200 ml. The stock solution was kept in a dark bottle at 4^0C.

To deionise formamide 5 g ion exchange resin (Bio-Rad Ag 501-X8 20-50 mesh) was added per 50 ml, stirred with a magnetic stirrer at room temperature for one hour, and filtered twice through Whatman Number one filter paper.

dNTPs mix : A stock solution of dNTPs was made up by mixing 25µl of each of the nucleotides dATP, dCTP, dGTP, dATP (10mM, Perkin Elmer Cetus) and 100µl of double distilled water. Kept at -20^0C.

50 x Electrophoresis buffer (per litre) : 242 gr Tris base, 57.1 ml Glacial Acetic Acid, 100ml 0.5M EDTA pH=8.

10 x Hybrid buffer : 3 M NaCl, 1 M Tris-HCl pH=8. The solution was sterilised in a Denley autoclave equipment.

Hybridisation solution : 4.5 x SSC, 0.1% SDS, 2 x Denhardts, 6% w/v polyethylene glycol. Kept at 4^0C.

L (Luria - Bertani) - Broth (per litre) : 10 g Bacto-tryptone , 5 g Bacto-yeast extract , 10 g NaCl. The solution was sterilised immediately in a Denley autoclave equipment.

Loading mix I : 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water. Kept at 4^0C.

Loading mix II : 0.25% bromophenol blue, 40% (w/v) sucrose in water. Kept at 4^0C.

Lysis buffer : 0.32 M Sucrose, 10 mM Tris-Cl pH=7.4, 5 mM $MgCl_2$, 1% Triton X-100. The solution was autoclaved in a Denley autoclave equipment.

NEW solution : 0.5 ml concentrated New in 10 ml dd$H_2$0 and 11.1 ml 100% ethanol. Kept at -20^0C.

Nuclei lysis buffer : 10 mM Tris-Cl, 0.44 M NaCl, 2 mM EDTA pH=8.2. The solution was autoclaved in a Denley autoclave equipment.

10 x PCR reaction buffer (Perkin Elmer Cetus) : 100 mM Tris-Cl (HCl-neutralised Tris[hydroxymethyl]aminomethane) pH=8.3 (at 25^0C), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin. Kept at -20^0C.

Phenol/ chloroform : 50% Phenol, 50% chloroform, 0.1% 8-hydroxyquinoline equilibrated to pH=7.5 with TE buffer. Kept in a dark bottle.

10% piperidine : 20 µl 99% piperidine in 180 µl of double distilled water.

Solution I : 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH=8. The solution was sterilised in a Denley autoclave equipment , and kept at 4^0C.

Solution II : 0.2 M NaOH, 1% SDS.

Solution III : 3 M KOAc pH=4.8 with glacial acetic acid. The solution was sterilised in a Denley autoclave equipment.

Solution IV : 5 mM mercaptoethanol, 10 mM Tris-Cl pH=7.4.

20 x SSC : 3 M NaCl, 300 mM Na Citrate pH=7.

Stop solution (Sequenase 2.0 kit, USB) : 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. Kept at -20^{0}C.

Stop solution : 0.3 M sodium acetate, 0.1 mM Na_2EDTA containing tRNA (25 μg/ml (baker's yeast, Boehring Mannheim).

20 x TAE electrophoresis buffer : 0.8 M Tris base, 0.4 M sodium acetate, and 0.02 M EDTA pH=7.4 . pH was adjusted to 7.4 with glacial acetic acid.

10 x TBE buffer (for 2 litres) : 54 g Tris base, 22.5 g Boric acid, 20 ml 0.5 M EDTA (pH 8).

T.E. Buffer : 10 mM Tris-Cl (pH 7.4), 1 mM EDTA. The solution was sterilised in a Denley autoclave equipment.

APPENDIX II

CALCULATION OF OLIGONUCLEOTIDE PRIMER CONCENTRATION.

(Invitrogen Corpration, 1991)

The concentration of an oligonucleotide of known sequence can be calculated when the molecular weight of the oligonucleotide and the amount synthesised are known.

The exact molecular weight (M.W.) of an oligonucleotide of known sequence can be calculated using the formula:

$$M.W. = |(A \times 312.2) + (G \times 328.2) + (C \times 288.2) + (T \times 303.2) - 61.0|$$

where the letters A, G, C and T denote the number of respective nucleotides in the oligonucleotide.

The amount of oligonucleotide synthesised is usually described in OD units. One OD corresponds to the amount of oligonucleotide in a 1-ml volume that results in an optical density of 1 in a 1-cm path-length cuvette. This corresponds to approximately 37 μg/ml oligonucleotide.

If M.W. is the molecular weight of an oligonucleotide whose amount is described by N OD units then the concentration (C) in mM of this oligonucleotide can be calculated using the formula :

$$C = (N \times 0.037 / M.W.) \times 10^6$$

APPENDIX III

GMG PEDIGREE NUMBER	CLINICAL CLASIFICATION	cDNA 9 HYBRIDISATION	HIND III	BGL II	cDNA 10 HYBRIDISATION	HIND III	BGL II	cDNA 11-14 HYBRIDISATION
2166	DMD	not-tested			+			+
2768	DMD	+			+			+
2790	DMD	-	8.8	6.1	+			+
3054	DMD	+			+			+
3552	DMD	+			+			+
3699	DMD	+			+			+
3997	DMD	+			+			+
4026	DMD	+			+			+
4030	DMD	+			+			+
4221	DMD	+			+			+
4252	DMD	not-tested			+			+
4287	DMD	+			+			+
4486	BMD	+			+			+
4586	DMD	+			+.			+
4629	DMD	-	7.8	16	+			
4698	DMD	+			+			+
4861	DMD	+			+			not-tested
5100	BMD	+			+			+
5101	DMD	+			+			+
5113	DMD	+			+			+
5117	DMD	+			+			+
5535	DMD	+			+			+
5539	DMD	+			not-tested			not-tested
5658	DMD	+			+			+
7327	**BMD**	+			+			+
7499	DMD	+			+			+
7579	DMD	+			+			+
7589	DMD	-	8.3		+			not-tested
7641	DMD	-	8.8/8.3/7.8/ 6.0/2.3/1.0		-	12.0/6.0 3.5/2.8	6.5/4.6/3.3/ 2.6/1.2	not-tested
7643	DMD	-	8.3/7.8/2.3		+			not-tested
7799	DMD	+			+			not-tested

GMG PEDIGREE NUMBER	CLINICAL CLASIFICATION	cDNA 9 HYBRIDISATION	HIND III	BGL II	cDNA 10 HYBRIDISATION	HIND III	BGL II	cDNA 11-14 HYBRIDISATION
8337	**BMD**	+			+			+
8359	DMD	+			+			+
8381	DMD	+			not-tested			not-tested
8396	DMD	-		16/8.0	+			+
9011	DMD	-		9.5	+			not-tested
9436	DMD	+			+			+
9772	BMD	not-tested			+			not-tested

APPENDIX III: Southern analysis of DMD/BMD patients using cDNA probes 9, 10 and 11-14. (+): indicates a positive hybridisation signal of all fragments detected by the cDNA probe. (-): indicates a deletion of at least one of the fragments detected by the cDNA probe using Hind III and/or Bgl II digests. In the cases where a deletion was observed the fragments deleted are presented according to size. **BMD** are possible sporadic cases.

APPENDIX IV

GMG PEDIGREE NUMBER	MEDICAL DIAGNOSIS	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
107	DMD	+	+	+	+	+	+	+	+	deleted
120	***DMD***	+	+	+	+	+	+	+	+	***not-tested***
198	DMD	+	+	+	+	+	+	+	+	+
948	BMD	+	+	+	+	+	+	+	+	+
1406	***DMD***	+	+	+	+	+	+	+	+	+
1596	DMD	+	+	+	+	+	+	deleted	+	+
1987	BMD	+	+	+	+	+	+	+	deleted	deleted
2161	BMD	+	+	+	+	+	+	deleted	deleted	+
2166	DMD	+	+	+	+	+	+	+	+	+
2672	DMD	+	+	+	+	+	+	deleted	deleted	deleted
2768	DMD	+	+	+	+	+	+	+	+	+
2790	DMD	+	+	+	+	+	+	+	+	+
3054	DMD	+	not-tested	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
3328	DMD	+	+	+	+	+	+	+	+	deleted
3468	BMD	+	+	+	+	+	+	deleted	+	+
3485	DMD	deleted	deleted	deleted	deleted	+	+	+	+	+
3486	DMD	not-tested	not-tested	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
3552	DMD	+	+	+	+	+	+	+	+	+
3623	DMD	+	+	+	+	+	+	deleted	deleted	deleted
3699	DMD	+	+	+	+	+	+	+	+	+
3816	BMD	+	+	+	+	+	+	+	deleted	+
3896	DMD	+	+	+	+	+	+	deleted	+	+
3997	DMD	+	+	+	+	+	+	+	+	+
4002	***BMD***	+	+	+	+	+	+	+	+	+
4026	DMD	+	+	+	+	+	+	+	+	+
4027	DMD	+	+	not-tested	+	+	not-tested	+	+	deleted
4028	***DMD***	+	+	+	+	+	+	+	+	+
4030	DMD	+	+	+	+	+	+	+	+	+
4031	***DMD***	+	+	+	+	+	+	+	+	+
4033	DMD	+	+	+	+	+	+	+	+	+
4034	DMD	+	+	+	+	+	+	deleted	+	+
4045	DMD	+	+	+	+	+	+	+	deleted	deleted

GMG PEDIGREE NUMBER	MEDICAL DIAGNOSIS	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
4089	DMD	+	+	+	+	+	+	+	+	
4191	DMD	+	+	+	+	+	+	+	+	+
4221	*DMD*	+	+	+	+	+	+	+	+	+
4252	DMD	+	+	+	+	+	+	+	+	+
4287	DMD	+	+	+	+	+	+	+	+	+
4477	BMD	+	+	+	+	+	+	deleted	deleted	+
4486	BMD	+	+	+	not-tested	+	+	not-tested	+	+
4586	DMD	+	+	+	+	+	+	+	+	+
4698	*DMD*	+	+	+	+	+	+	+	+	+
4786	*DMD*	*not-tested*	+	*not-tested*	+	+		+	+	
4907	*DMD*	+	+	***deleted***	***deleted***	***deleted***	+	+	+	+
4947	DMD	+	+	+	+	+	+	deleted	deleted	+
5097	*BMD*	+	***deleted***	+	+	+	+	+	+	+
5098	*DMD*	+	+	+	+	+	+	+	*deleted*	+
5099	*DMD*	*deleted*	*deleted*	*deleted*	*deleted*	*deleted*	*deleted*	+	+	+
5100	BMD	+	+	+	+	+	+	+	+	+
5101	*DMD*	+	+	+	+	+	+	+	+	+
5102	DMD	+	+	+	+	+	+	deleted	+	+
5104	DMD	+	+	+	+	+	+	+	+	+
5105	*DMD*	+	+	+	+	+	+	+	+	+
5110	*DMD*	+	+	+	+	+	+	+	+	+
5113	DMD	+	+	+	+	+	+	+	+	+
5114	*DMD*	+	+	+	+	+	+	+	+	+
5116	*DMD*	*deleted*	*deleted*	*deleted*	*deleted*	*deleted*	+	+	+	+
5117	DMD	+	+	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
5120	*DMD*	+	+	+	+	+	+	+	+	+
5165	BMD	+	deleted	+	+	+	+	+	+	+
5190	BMD	+	+	+	+	+	+	deleted	+	+
5219	DMD	+	+	+	+	+	not-tested	+	+	+
5265	*DMD*	*not-tested*	+	*not-tested*	+	*not-tested*	*not-tested*	+	+	
5286	DMD	+	+	+	+	deleted	+	+	+	+
5294	*DMD*	+	+	+	+	+	+	+	*deleted*	+

GMG PEDIGREE NUMBER	MEDICAL DIAGNOSIS	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
5311	DMD	+	+	deleted	deleted	deleted	+	+	+	+
5313	*DMD*	+	+	*deleted*	*deleted*	*deleted*	*not-tested*	+	+	+
5530	DMD	+	+	+	+	+	deleted	+	+	+
5534	*DMD*	+	+	+	+	+	+	*deleted*	*deleted*	*deleted*
5535	DMD	+	+	+	+	+	+	+	+	+
5536	DMD	+	+	+	+	+	+	+	+	+
5538	DMD	not-tested	+	not-tested	+	+	+	+	deleted	not-tested
5539	DMD	+	+	not-tested	+	+		+	+	+
5540	*DMD*	+	+	+	+	+	+	+	+	*deleted*
5551	BMD	+	+	+	+	+	+	+	deleted	+
5552	DMD	+	+	not-tested	+	+	not-tested	+	+	+
5561	DMD	+	+	+	+	+	+	+	deleted	+
5635	DMD	+	+	+	+	+	+	+	+	not-tested
5637	DMD	+	+	+	+	+	+	+	deleted	+
5657	***DMD***	+	+	+	+	+	***deleted***	+	+	+
5658	***DMD***	+	+	+	+	+	+	+	+	+
5972	***DMD***	+	+	+	+	+	***deleted***	+	+	+
6272	BMD	deleted	+	+	+	+	+	+	+	+
7484	***BMD***	+	+	+	+	+	+	*not-tested*	+	*not-tested*
7498	DMD	+	+	+	+	+	+	+	+	+
7499	DMD	+	+	+	+	+	+	+		+
7500	DMD	+	+	+	+	+	+	+	+	
7509	BMD	deleted	deleted	deleted	+	+	+	+	+	+
7579	***DMD***	+	+	+	+	+	+	+	+	+
7589	DMD	+	+	+	+	+	+	not-tested	deleted	deleted
7643	*DMD*	+	+	+	+	+	+	+	+	+
7651	DMD	deleted	deleted	deleted	deleted	deleted	deleted	+	+	+
7799	DMD	+	+	+	+	+	+	+	+	+
7805	DMD	+	+	+	+	+	+	deleted	+	+
7833	BMD	+	+		+	+	+	deleted	+	not-tested
7866	DMD		+	+	+	+	not-tested	+	+	+
7868	***DMD***	+	***deleted***	***deleted***	+	+	+	+	+	+

GMG PEDIGREE NUMBER	MEDICAL DIAGNOSIS	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
8037		+	+	+	+	+	+	+	+	+
8068	*DMD*	+	+	*not-tested*	+	+	*not-tested*	+	+	*deleted*
8285	DMD	+	+	+	+	+	not-tested	+	deleted	+
8281	*DMD*	+	+	*not-tested*	+	+	*not-tested*	+	+	*deleted*
8322	DMD	+	+	not-tested	+	+	+	+	deleted	+
8337	***BMD***	+	+	+	+	+	+	+	+	+
8359	*DMD*	+	+	+	+	+	+	+	+	+
8381	*DMD*	+	+	+	+	*not-tested*	*not-tested*	*not-tested*	*not-tested*	+
8394	*DMD*	+	+	*not-tested*	+	+	+	+	+	*not-tested*
8395	DMD	+	+	+	+	+	+	+	+	+
8396	DMD	+	+	+	+	+	+	+	+	+
8477	BMD	not-tested	+	not-tested	+	+	not-tested	not-tested	deleted	deleted
8602	DMD	+	deleted	deleted	+	+	+	+	+	+
8652	BMD	+	+	+	+	+	+	+		+
8832	DMD	+	+	+	+	not-tested	+	+	+	deleted
9028	DMD	+	+	+	+	+	+	deleted	deleted	deleted
9289	DMD	deleted	+	+	+	+	+	+	+	+
9371	DMD	+	+	+	+	+	+	+	+	deleted
9421	BMD	+	+	not-tested	+	+	+	deleted	+	+
9436	DMD	+	+	+	+	+	+	+	+	+
9614	DMD	+	+	not-tested	+	+	+	+	deleted	+
9772	BMD	+	+	not-tested	+	+	+	deleted	deleted	+

APPENDIX IV: Analytical results of deletion screening of 118 DMD/BMD patients by means of the polymerase chain reaction. The patients with ambiguous results for the 0.5 kb Hind III fragment (cDNA 7) by Southern analysis are presented in italic. **BMD** are possible sporadic cases.

APPENDIX V

GMG PEDIGREE NUMBER	CLINICAL CLASSIFICATION	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
2166	DMD	N	N	N	N	N	N	N	N	N
2768	DMD	N	N	N	N	N	N	N	N	N
2790	DMD	N	N	N	N	N	N	N	N	N
3054	DMD	N	not-tested	not-tested	N	not-tested	not-tested	not-tested	not-tested	not-tested
3486	DMD	not-tested	not-tested	not-tested	N	not-tested	not-tested	not-tested	not-tested	not-tested
3552	DMD	N	N	N	N	N	N	N	N	N
3699	DMD	N	N	N	N	N	N	N	N	N
3997	DMD	N	N	N	N	N	N	**A**	N	N
4026	DMD	N	N	N	N	N	N	**A**	N	N
4030	DMD	N	N	N	N	N	N	N	N	N
4191	DMD	N	N	N	N	N	N	**A**	N	N
4221	DMD	N	N	N	N	N	N	N	N	N
4252	DMD	N	N	N	N	N	N	**A**	N	N
4287	DMD	N	N	N	N	N	N	not-tested	N	N
4486	BMD	N	N	N	not-tested	N	N	not-tested	N	N
4586	DMD	N	N	N	N	N	N	not-tested	N	N
4698	DMD	N	N	N	N	N	N	N	N	N
5100	BMD	N	N	N	N	N	N	N	N	N
5101	DMD	N	N	N	N	N	N	N	N	N
5104	DMD	N	N	N	N	N	N	N	N	N
5105	DMD	N	N	N	N	N	N	**A**	N	N
5110	DMD	N	N	N	N	N	N	N	N	N
5113	DMD	N	N	N	N	N	N	N	N	N
5117	DMD	N	N	not-tested	N	not-tested	not-tested	not-tested	not-tested	not-tested
5219	DMD	N	N	N	not-tested	not-tested	not-tested	not-tested	not-tested	N
5535	DMD	N	N	N	N	N	N	not-tested	N	N
5536	DMD	N	N	N	N	N	N	N	N	N
5539	DMD	N	N	N	N	N	N	N	not-tested	N
7484	**BMD**	N	N	N	N	N	N	not-tested	N	not-tested
7499	DMD	N	N	N	N	N	N	N	N	not-tested
7579	DMD	N	N	N	N	N	N	N	N	N
7799	DMD	N	N	N	N	N	N	N	N	N
8337	BMD	N	N	N	N	N	N	**A**	N	N
8359	DMD	N	N	N	N	N	N	**A**	N	N
8381	DMD	N	N	N	N	not-tested	not-tested	not-tested	N	N
8396	DMD	N	N	N	N	N	N	**A**	N	N

GMG PEDIGREE NUMBER	CLINICAL CLASSIFICATION	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
8652	BMD	N	N	N	N	N	N	N	N	N
8832	DMD	N	N	N	N	not-tested	N	N	N	not-tested
9436	DMD	N	N	N	N	N	N	N	N	N

APPENDIX V: Results of PCR-SSCP analysis in nine regions of the dystrophin gene flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51. N: indicates an SSCP pattern identical to a normal individual. A: indicate the presence of aberrant alleles.

APPENDIX VI

CALCULATIONS OF CARRIER RISKS

FAMILY GMG 3997.

Individual II 2.

Probability	Carrier	Not a carrier
Prior risk	0.5	0.5
Conditional		
SCK 47	0.2	1
DNA	0.05	0.95
Joint	0.005	0.475

Posterior (of being a carrier) : 0.005/0.005+0.475 = 0.01.

Individual II 3.

Probability	Carrier	Not a carrier
Prior risk	0.5	0.5
Conditional		
SCK 49	0.2	1
DNA	0.05	0.95
Joint	0.005	0.475

Posterior (of being a carrier) : 0.005 / 0.005+0.475 = 0.01.

Individual II 4

Probability	Carrier	Not a carrier
Prior risk	0.5	0.5
Conditional		
SCK 53	0.2	1
DNA	0.95	0.05
Joint	0.095	0.025

Posterior (of being a carrier) : 0.095 / 0.095 + 0.025 = 0.79

FAMILY GMG 4026.

Individual 9

Probability	Carrier	Not a carrier
Prior risk	0.5	0.5
Conditional		
SCK 47	0.2	1
DNA	0.05	0.95
Joint	0.005	0.475

Posterior (of being a carrier) : $0.005 / 0.005 + 0.475 = 0.01$

FAMILY GMG 4252

Individual II2 and II3.

Probability	Carrier	Not a carrier
Prior risk	0.5	0.5
Conditional		
DNA	0.95	0.05
Joint	0.475	0.025

Posterior (of being a carrier) : $0.475 / 0.475 + 0.025 = 0.95$

APPENDIX VII

GMG PEDIGREE NUMBER	CLINICAL CLASSIFICATION	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
2166	DMD	+	+	+	+	+	+	+	+	+
2768	DMD	+	not-tested	+	+	+	+	+	+	+
2790	DMD	+	not-tested	not-tested	+	+	+	not-tested	+	not-tested
3054	DMD	+	not-tested	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
3486	DMD	+	not-tested	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
3552	DMD	+	+	+	+	+	+	not-tested	+	+
3699	DMD	+	+	+	+	+	+	+	+	+
3997	DMD	+	+	+	+	+	+	*	+	+
4026	DMD	+	+	+	+	+	+	*	+	+
4030	DMD	+	+	+	+	+	+	not-tested	+	not-tested
4191	DMD	+	+	+	+	+	+	*	+	+
4221	DMD	+	+	+	+	+	+	+	+	+
4252	DMD	+	+	+	+	+	+	*	not-tested	+
4287	DMD	+	+	not-tested	+	+	not-tested	+	+	+
4486	BMD	+	+	not-tested	+	+	+	+	+	+
4586	DMD	+	+	+	+	+	+	+	not-tested	+
4698	DMD	+	+	+	+	+	+	+	+	+
4861	BMD	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested	not-tested	not-tested
5100	DMD	+	+	+	+	+	+	+	+	+
5101	DMD	+	+	+	not-tested	+	+	+	not-tested	+
5104	DMD	+	not-tested	+	+	+	+	+	+	+
5105	DMD	+	+	not-tested	+	+	+	*	+	+
5110	DMD	+	not-tested	+	+	+	+	+	+	+
5113	DMD	+	+	+	+	+	+	+	+	+
5117	DMD	+	+	not-tested	+	not-tested	not-tested	not-tested	not-tested	not-tested
5219	DMD	+	+	not-tested	+	not-tested	not-tested	+		+
5535	DMD	+	+	+	+	+	+	+	+	+
5536	DMD	+	+	+	+	+	+	+	+	+
5539	BMD	+	+	+	+	+	+	+	not-tested	+
7484	DMD	+	+	+	+	+	+	not-tested	+	not-tested
7499	DMD	+	+	+	+	+	+	+	+	+

GMG PEDIGREE NUMBER	CLINICAL CLASSIFICATION	I EXON 4	A EXON 8	G EXON12	B EXON 17	C EXON 19	D c-DNA 7 4.1 Kb Hind III	E c-DNA 7 0.5 Kb Hind III	F c-DNA 8 1.2/3.8 Kb Hind III	H c-DNA 8 3.1 Kb Hind III
7579	DMD	+	+	not-tested	+	+	+	+	+	+
7799	BMD	not-tested	not-tested	+	+	+	+	+	+	+
8337	DMD	+	+	+	+	+	not-tested	*	+	+
8359	DMD	not-tested	+	+	+	+	+	*	+	+
8381	DMD	not-tested	not-tested	+	+	not-tested	not-tested	not-tested	+	not-tested
8396	DMD	+	+	not-tested	+	+	+	*	+	+
8652	BMD	+	+	+	+	+	+	+	+	+
8832	DMD	+	+	+	+	not-tested	+	+	+	+
9436	DMD	+	not-tested	+	+	+	+	+	+	+

APPENDIX VII: Screening of DMD/BMD samples for sequence variations in regions i, a, g, b, c, d, e, f and h of the dystrophin gene flanking exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 by means of denaturing gradient gel electrophoresis (DGGE). The samples tested for each region are indicated by (+). An asterisc marks the samples caused a split of the sigmoid-curve during perpendicular DGGE.

APPENDIX VIII

BASES	REGION B	REGION D	REGION E	REGION F
5	65.951	60.642	67.117	60.398
10	66.036	61.303	67.353	60.561
15	66.052	61.5	67.38	60.605
20	66.061	62.007	67.396	60.62
25	66.073	62.037	67.398	60.623
30	66.076	62.076	67.398	60.622
35	66.078	62.201	67.399	60.622
40	66.081	62.282	67.4	60.626
45	66.082	62.382	67.399	60.635
50	66.081	62.654	67.4	60.647
55	66.082	62.75	67.398	60.716
60	66.081	63.179	67.399	60.751
65	66.084	64.455	67.402	60.753
70	66.092	65.226	67.411	60.761
75	66.227	67.32	67.413	60.763
80	66.396	70.2	67.455	60.763
85	66.483	71.669	67.494	60.764
90	66.803	71.825	67.533	60.763
95	66.866	71.731	67.542	60.763
100	67.215	71.719	67.545	60.763
105	67.479	71.762	67.548	60.764
110	67.627	71.915	67.623	60.767
115	67.749	74.886	68.188	60.769
120	67.821	72.276	68.407	60.772
125	67.889	66.559	68.448	60.801
130	67.919	66.397	68.456	61.06
135	67.939	66.376	68.457	61.472
140	67.954	66.376	68.457	61.981
145	68.216	66.382	68.458	62.176
150	69.286	66.423	68.458	62.189
155	70.407	66.442	68.458	62.196
160	70.452	66.444	68.46	62.247
165	70.499	66.445	68.46	62.268
170	70.513	66.442	68.46	62.451
175	70.578	66.437	68.461	62.975
180	70.648	66.429	68.461	65.713
185	70.731	66.422	68.462	67.286
190	70.757	66.394	68.464	67.732
200	70.786	66.382	68.464	67.852
205	70.794	66.36	68.464	67.889
210	70.801	66.344	68.464	67.933
215	70.845	66.268	68.466	67.958

BASES	REGION B	REGION D	REGION E	REGION F
220	70.951	66.038	68.467	67.986
225	71.073	65.997	68.469	68
230	71.437	65.984	68.469	68.068
235	72.092	65.968	68.471	68.098
240	72.187	65.963	68.477	68.207
245	72.203	65.959	68.493	69.11
250	72.141	65.952	68.495	69.583
255	71.986	65.949	68.496	69.455
260	71.897	65.947	68.497	69.271
265	71.878	65.946	68.497	69.32
270	71.855	65.945	68.495	69.382
275	71.835	65.945	68.486	69.429
280	71.852	65.948	68.484	69.397
285	71.877	65.949	68.484	69.367
290	71.952	65.949	68.484	69.209
295	72.135	65.946	68.495	69.256
300	72.167	65.94	68.534	69.35
305	72.18	65.938	68.567	69.144
310	72.184	65.938	68.568	68.082
315	72.191	65.936	68.578	67.712
320	72.19	65.937	68.614	67.411
325	72.254	65.936	68.645	67.385
330	72.286	65.932	68.707	67.354
335	72.307	65.854	68.782	67.308
340	72.336		69.542	67.293
345	72.336		70.038	67.295
350	72.324		70.269	67.293
360	72.155		70.41	67.289
365	71.233		70.463	67.286
370	71.017		70.472	67.261
375	70.622		70.47	67.248
380	70.092		70.469	67.181
385	69.97		70.467	67.145
390	69.788		70.464	66.994
395	69.659		70.464	65.627
400	69.639		70.463	63.173
405	69.632		70.462	62.921
410	69.626		70.457	62.907
415	69.565		70.456	62.892
420	69.369		70.463	62.88
425			70.464	62.879
430			70.464	62.878

BASES	REGION B	REGION D	REGION E	REGION F
435			70.471	62.868
440			70.481	62.862
445			70.482	62.847
450			70.494	62.837
455			70.513	62.821
460			70.622	62.799
465			72.233	62.775
470			73.488	62.773
475			74.319	62.77
480			75.591	62.768
485			75.462	62.769
490			75.251	62.769
495			74.401	62.769
500			73.938	62.769
505			73.267	62.757
510			72.381	62.671
515			71.899	62..607
520			71.854	
525			71.821	
530			71.437	
535			67.966	
540			65.382	
545			64.901	
547			64.022	
550				

APPENDIX VIII: Numerical data derived from the computer program MELT 87 (Lerman, 1988), used for compiling the melting maps of regions b, d, e and f of the dystrophin gene.

APPENDIX IX

REGION B

5'tgactttcgatgttgagattactttcccttgctatttcagtgaaccaaacttaagtcagataaaacaattttatttggcttcaatatggtgctattttgatc
tgaaggtc aatctaccaacaagcaagaacagtttctcatta ttttcctttgccactccaagcagtctttactgaa gtctttcgagcaatgtctgacctct
gtttcaatacttctc acagATTTCACAGGCTGTCACCACCACTCAGCCATCACTAACACAGACAAC
TGTAATGGAAACAGTAACTACGGTGACCACAAGGGAACAGATCCTGGTAAAGCATGC
TCAAGAGGAACTTCCACCACCACCTCCCCAAAAGAAGAGGCAGATTACTGTGGATTC
TGAAATTAGGAAAAGgtgagagcatctcaagctt 3'

REGION D

5'tgtccaaaatagt tgactttctttctttaatcaataaatatattactttaaagggaaaaattgcaaccttccatttaaaa tcagctttatattgagtatttttt
taaaatgttgtgtgtacatcgtaggtgtgtatattaatttttatttgttacttgaaactaaactctgcaaatgcaggaaactatcagagtgatatctttgtca
gata accaaaaaaatatacgct atatctctataatctgt tttacataatccatct attttttcttgatccata tgcttttacctgcagGCGATTTGAC
AGATCTGTTGAGAAATGGCGGCGTTTTCATTATGATATAAAGATATTTAATCAGTGGCT
AACAGAAGCTGAACAGTTTCTCAGAAAGACACAAATTCCTGAGAATTGGGAACATGC
TAAATACAAATGGTATCTTAAGgtaagtct ttgatttgttttttcgaaattgtatttatcttcagcacatctggactctttaacttcttaa
agatcaggttctgaagggtgatggaaattactttgactgttgttgtcatcattatattactagaaagaaaa 3'

REGION E

5'tacaacatttcatagactattaaacatg gaacatccttgtggggacaagaaatcgaatttgctcttgaaaaggtttccaactaattgatttgtagga
cattataa catcctctagctgacaagcttacaaaaataaaa actggagctaaccgagagggtgctttttttccctg acacataaaaggtgtctttctgt
cttgtatcctttggatatgggcatgtcagtttcatagggaaattttcacatggagcttttgtatttctttctttgccagtacaactgcatgtggtagcacac
tgtttaatcttttct caaataaaaagacatggggcttcatttttgttttgccttttttggtatcttacagGAACTCCAGGATGGCATTGG
GCAGCGGCAAACTGTTGTCAGAACATTTGAATGCAACTGGGGAAGAAATAATTCAGC
AATCCTCAAAA ACAGATGCC AGTATTCTA CAAGGAAAA ATTGGGAAG CCTGAATCTG
CGGTGGCAGGAGGTCTGCC AAACAGCTGTCAGA CAGAAAAAAGAGgta gggcgacagatct
aataggaatgaaaacattttagcagacttttaagctt 3'

REGION F

5'ttttgtagacggttaatgaataattgaatacattggtta aatcccaacatgtaatatatgtaaataatcaata ttatgctgctaaaataacacaaatca
gtaagatt ctgtaatatttcatgataaataactttgaaaa tatattttaaacattttgcttatgccttgagaat tatttacctttttaaaatgtattttccttttcag
GTTTCCAGAGCTTTACCTGAGAAACAAGGA GAAATTGAAGCTC AAATAAAAGACCTT
GGGCAGCTTGAAAAA AAGCTTG AAGACCTTGAAGA GCAGTTA AATCATCTGCTGC T
GTGGTTATCTCCTATTAGGAATCAGTTGGA AATTTATAACCAA CCAAACCAAGAAGGA
CCATTTGACGTTAAGgtaggggaacttttgctttaatatttttgtctttttaa gaaaaatggcaatatcactgaatttctcatttggtatc
attattaaagacaaaatattacttgttaaa gtgtggtaaggaagactttattcaggataaccacaataggcacagggaccactgcaatggagtatta
caggaggttggatagagagagattgggctcaactctaaatacagcacagtggaagtaggaatttatagc 3'

APPENDIX IX: Sequences flanking: exon 17 (region b), exon 44 (region d), exon 45 (region e) and exon 48 (region f) of the dystrophin gene. The sequences of the oligonucleotide primers used for DNA amplification are underlined. Exon sequences are in capital letters and intron sequences are in lower case letters.

APPENDIX X

TEMPERATURE	DENCON%	REGION B	REGION D	REGION E	REGION F	VREL	REGION B(REL)	REGION D(REL)	REGION E(REL)	REGION F(REL)
60.2	10.24	1.6353	1.489	1.2624	1.08	1.044	1.566	1.348	1.211	1.034
60.4	10.88	1.6347	1.4693	1.2595	0.9022	1.046	1.562	1.327	1.2	0.862
60.6	11.52	1.634	1.4452	1.2565	0.6735	1.049	1.557	1.301	1.197	0.642
60.8	12.16	1.6334	1.416	1.2532	0.4664	1.0522	1.552	1.276	1.191	0.443
61	12.8	1.6327	1.3806	1.2497	0.3336	1.055	1.547	1.237	1.183	0.316
61.2	13.44	1.6321	1.3356	1.246	0.2641	1.0577	1.544	1.194	1.178	0.249
61.4	14.08	1.6312	1.2891	1.2419	0.2284	1.06	1.538	1.149	1.17	0.215
61.6	14.72	1.6303	1.233	1.2376	0.207	1.063	1.533	1.097	1.164	0.194
61.8	15.36	1.6294	1.1718	1.2328	0.1905	1.066	1.528	1.039	1.155	0.178
62	16	1.6286	1.1075	1.2279	0.174	1.068	1.524	0.98	1.142	0.162
62.2	16.64	1.6277	1.0434	1.2225	0.1545	1.071	1.519	0.918	1.14	0.143
62.4	17.28	1.6266	0.9825	1.2166	0.1297	1.074	1.513	0.865	1.132	0.12
62.6	17.92	1.6255	0.9272	1.2106	0.1007	1.077	1.508	0.815	1.123	0.092
62.8	19.2	1.6242	0.8787	1.204	0.0729	1.082	1.5	0.77	1.112	0.067
63	19.84	1.6229	0.8376	1.1971	0.0526	1.085	1.494	0.732	1.103	0.048
63.2	20.48	1.6214	0.8035	1.19	0.0408	1.088	1.488	0.701	1.09	0.037
63.4	21.12	1.6197	0.7754	1.1824	0.0345	1.09	1.485	0.675	1.08	0.031
63.6	21.76	1.6178	0.7522	1.1744	0.0312	1.093	1.479	0.6535	1.07	0.028
63.8	22.4	1.615	0.733	1.1661	0.0294	1.096	1.473	0.635	1.058	0.026
64	23.04	1.6115	0.7168	1.1574	0.0283	1.099	1.465	0.62	1.052	0.025
64.2	23.68	1.6066	0.7026	1.1485	0.0275	1.101	1.458	0.606	1.043	0.025
64.4	24.32	1.5991	0.6896	1.1395	0.027	1.104	1.448	0.592	1.031	0.024
64.6	24.96	1.5876	0.6769	1.1301	0.0265	1.107	1.433	0.58	1.02	0.023
64.8	25.6	1.5691	0.6627	1.1207	0.0262	1.11	1.413	0.567	1.009	0.023
65	26.24	1.5387	0.6443	1.1109	0.0258	1.112	1.383	0.55	0.99	0.023
65.2	26.88	1.489	0.6158	1.1011	0.0255	1.115	1.335	0.524	0.987	0.022
65.4	27.52	1.4105	0.5666	1.091	0.0251	1.118	1.261	0.48	0.974	0.022
65.6	28.16	1.2941	0.4821	1.0803	0.0247	1.121	1.154	0.37	0.963	0.022
65.8	28.8	1.1396	0.3579	1.0689	0.0242	1.123	1.014	0.31	0.95	0.021
66	29.44	0.9636	0.2265	1.0554	0.0235	1.126	0.855	0.2	0.936	0.02
66.2	30.08	0.7962		1.038		1.129	0.705		0.919	
66.4	30.72	0.6613		1.0127		1.132	0.583		0.89	
66.6	31.36	0.5642		0.9718		1.134	0.497		0.85	
66.8	32	0.4965		0.9032		1.137	0.436		0.794	

TEMPERATURE	DENCON%	REGION B	REGION D	REGION E	REGION F	VREL	REGION B(REL)	REGION D(REL)	REGION E(REL)	REGION F(REL)
67	32.64	0.4475		0.7942		1.14	0.392		0.69	
67.2	33.28	0.4087		0.6453		1.143	0.356		0.564	
67.4	33.92	0.3754		0.4872		1.145	0.327		0.425	
67.6	34.56	0.3452		0.3585		1.148	0.3		0.312	
67.8	35.2	0.3178		0.2634		1.151	0.275		0.23	
68	35.84	0.293		0.1719		1.154	0.253		0.147	
68.2	36.48	0.2709		0.0763		1.156	0.233		0.0066	
68.4	37.12	0.2514		0.0278		1.159	0.216		0.0239	
68.6	37.76	0.2338		0.0154		1.162	0.2		0.013	
68.8	38.4	0.2177		0.0123		1.165	0.186		0.01	
69	39.04	0.2024		0.0112		1.167	0.173		0.0095	
69.2	39.68	0.1872		0.0106		1.17	0.16		0.009	
69.4	40.32	0.172		0.0102		1.173	0.146		0.008	
69.6	40.96	0.1565		0.0096		1.176	0.132		0.008	
69.8	41.6	0.1408		0.0089		1.178	0.118		0.007	
70		0.125		0.0078						

APPENDIX X: Numerical data derived from the computer program MUTRAV (Lerman, 1988) used for plotting the simulated perpendicular denaturing gradient gel pattern. The DENCON (concentration of stock solution of urea and formamide in percent v/v), was calculated using the formula DENCON = 3.2 x (EQTEMP - 57), where EQTEMP is the temperature derived from the melting calculation when the bath temperature assumed to be 60^{0}C. The VREL (relative viscocity) was calculated subsequently using the equation: VREL=1+(4.3 x DENCON x 10^{-3}).