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Detection of point mutations in the dystrophin gene

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Detection of Point Mutations in the Dystrophin Gene

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"I certify that this thesis does not incorporate without acknowledgement any material submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text."

ABSTRACT

The dystrophin gene has been localised to Xp 21.1. Mutations of this gene can lead to the clinical manifestations of Duchenne and Becker muscular dystrophies (DMD/BMD). In the majority of DMD and BMD patients the disease-causing mutation is a deletion detectable by southern analysis or multiplex PCR, however in 30% of patients no deletion is observed using these conventional tests. Using PCR amplification of cDNA it was possible to detect a deletion in the product of the dystrophin gene of one such individual affected with BMD. It was then necessary to characterise the mutation in order to determine whether this was the disease-causing mutation. The mutation was found to be a point mutation by genomic sequencing at the donor splice site consensus sequence of intron 19. This resulted in alternate splicing of exon 19, two mRNA transcripts being produced one with exon 19 and the other without. This was determined sufficient to cause BMD in the family. It was then necessary to provide an easy and efficient diagnostic test in order to determine the carrier status of females and also to provide those females determined to be carriers with an accurate prenatal diagnostic test. A new technique, single strand conformation polymorphism, performed on genomic DNA provided evidence of conformational difference between the affected allele and the normal allele. Allele specific primers were also designed and optimised. These primers, 19FA and 19FC, only varied at one base, one being the "normal" allele primer and the other the "affected" allele primer. The amplified product using primers for the normal and the affected allele were then run on agarose gels and

iii

stained with ethidium bromide providing a quick and easy method of determining the carrier, affected or normal status of all family members.

The localisation and characterisation of this splice site mutation involved many molecular techniques including, RNA extraction, cDNA synthesis, nested amplification, sequencing, single strand conformation polymorphism and the amplification refractory mutation system. All of these techniques can be used to isolate and characterise point or minor mutations in the 30% of Western Australian dystrophic families, who currently lack an accurate diagnostic test for their dystrophic condition.

CONTENTS

Chapter 1: INTRODUCTION

1.1 General introduction

Mutations in the dystrophin gene can result not only in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) but also familial X-linked myalgia and cramps, which is a non progressive myopathy associated with a deletion in the dystrophin gene (Gospe et al, 1989; Hodgson et al, 1989; Prior et al, 1990). DMD and BMD are progressively degenerative diseases which show a great deal of variation in both genetic and protein structure. These variations along with other as yet unknown factors, determine the speed at which the disease progresses (Koenig et al, 1989).

1.2 Duchenne and Becker muscular dystrophy

Duchenne muscular dystrophy and Becker muscular dystrophy are allelic disorders caused by mutations of the dystrophin gene (Koenig et al, 1989). DMD, the more severe of these dystrophinopathies, occurs in approximately 1 in 3500 male births (Hodgson et al, 1989). BMD, the milder allelic disorder, has a much lower incidence of approximately 1 in 30 000 males (Koenig et al, 1989; Prior et al, 1990). The incidence of BMD may prove to be much higher now that complementary deoxyribonucleic acid (cDNA) probes have been used to reclassify many limb-girdle muscular dystrophy and spinal muscular atrophy patients as having BMD (Norman et al, 1989; Patel et al, 1989).

The phenotypes of affected boys have been sub-classified into 3 groups based on the age at which ambulation was lost. Group 1 boys have DMD and are wheelchair bound by age 13. Group 2 boys form

1

an intermediate group and are wheelchair bound between the ages of 13 and 16. Group 3 boys have BMD and become wheelchair bound after the age of 16 or may never lose ambulation (Hodgson et al, 1989; Prior et al, 1990).

Duchenne and Becker muscular dystrophies are X-linked recessive disorders. X-linked recessive disorders generally only affect males and are passed through carrier women. Carrier women are usually unaffected since the mutated gene is compensated for by the normal gene on their other X chromosome. All daughters of affected men are carriers, and the disease is never transmitted from father to son (see Figure 1.1) (Gelehrter & Collins, 1990, p. 41).

1.3   Dystrophin gene

The discovery of the dystrophin gene by Kunkel and his co-workers, (1986) has resulted in a substantial increase in research into both the structure and function of the dystrophin gene (Hoffman et al, 1987; Beggs et al, 1990). The dystrophin gene was first sub-localised on the X chromosome by cytogenetic analysis of several DMD patients with deletions and translocations of the X chromosome (Francke et al, 1985; Boyd et al, 1986; Beggs et al, 1990). These cytogenetic analyses demonstrated abnormalities in the middle of the short arm of the X chromosome (Xp 21). The localisation was then confirmed by linkage analysis, using polymorphic markers on the short arm of the X chromosome. These markers were inherited with the disease causing mutation more than 50% of the time, as would be expected with linked markers (Laing, 1993). Once the gene that resulted in DMD was isolated it was then possible to use genomic deoxyribonucleic acid

2

Figure 1.1

X-linked recessive inheritance as in Duchenne/Becker muscular dystrophies. This pedigree shows how the probability of inheriting an X-linked gene is 0.5. Males because of their XY hemizygous condition, have a 50% chance of being affected by an X-linked disorder. The XX genotype of females provides them with protection from phenotypic manifestations of X-linked recessive disorders, however in affected families they have a 50% chance of inheriting an X-linked disease gene and therefore have a 50% chance of being carriers of such X-linked disease genes.

Figure 1.2

Four domains of dystrophin are represented on this tentative schematic diagram. The conformation of the actin-binding, cysteine-rich and C-terminal domains is purely speculative and are represented as broad loops simply to illustrate that they may adopt a more globular structure, than the rod section. The rod section is formed by 25 triple helical segments (Koenig et al, 1988).

A			
XX			XY

A		A	
XX	XX	XY	XY
CARRIER	NORMAL	AFFECTED	NORMAL

'actin-binding'
domain

triple helical segments
$\approx$ 125 nm

cystein-rich   C-terminal
domain   domain

EF

EF
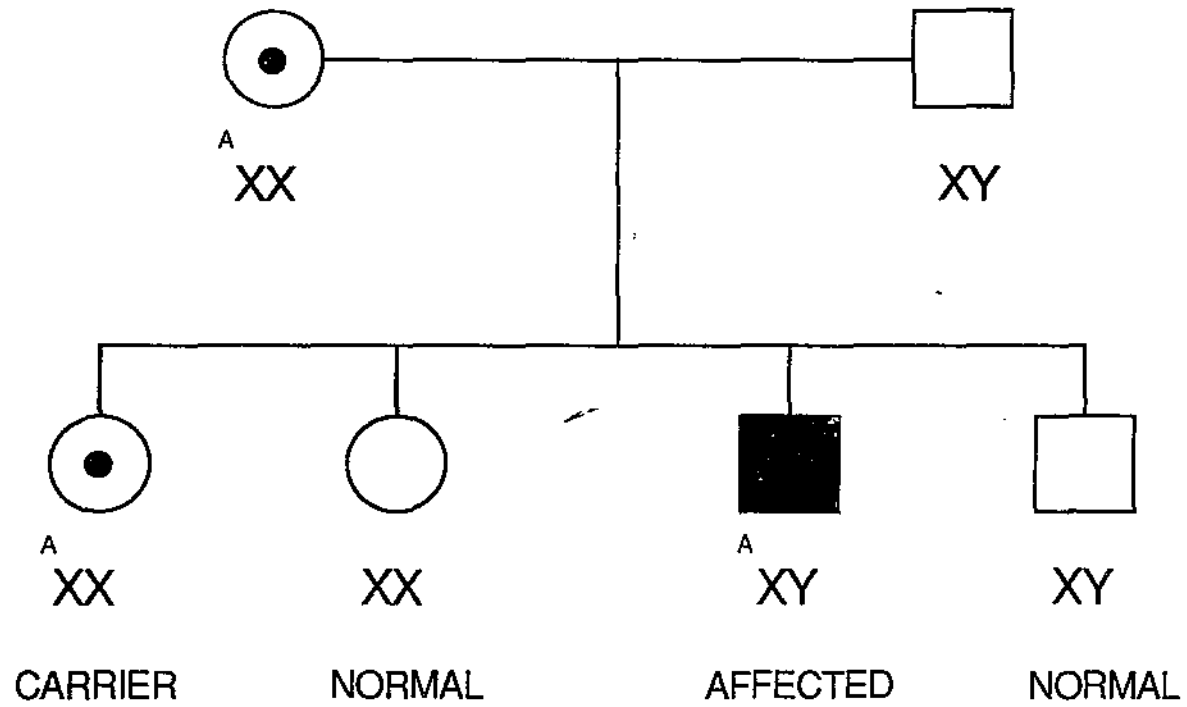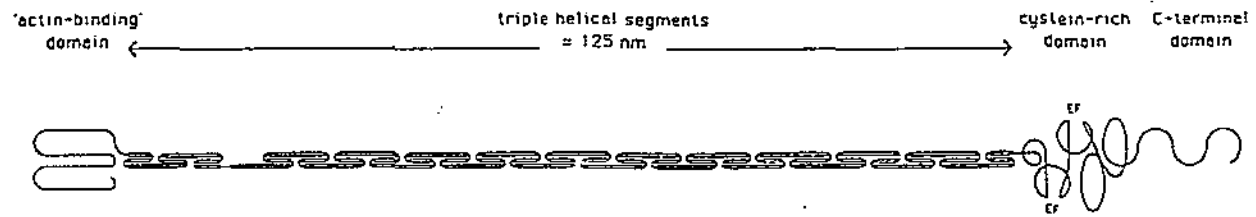
Carpenter et al (1990) and Watkins et al (1988), both localised dystrophin at or close to the plasma membrane of human skeletal muscle. The use of both light and electron microscopy has shown dystrophin to be located in the sarcolemma of the muscle and immunostaining showed particular prominence of dystrophin at the myotendinous junction (Campbell et al, 1989; Knudson et al, 1990).

Some of the proposed functions of dystrophin are:-

1) A role in excitation and contraction coupling. The early localisation studies showed dystrophin to be enriched on the T-tubules at the triadic junction of skeletal muscle (Knudson et al, 1990; Harris et al, 1992).

2) Involvement in the regulation of Ca^{++} movement. In this case it is said the dystrophin is bound to stretch-inactivated channels. In a dystrophin deficient state, these channels remain open allowing a continual influx of Ca^{++}. It must be noted that it is not known by what mechanism dystrophin affects the Ca^{++} channels (Turner et al, 1988; Harris et al, 1992).

3) Maintenance of flexibility and stability of the plasma membrane. It is thought the C-terminus is bound tightly to the plasma membrane glycoproteins, while the amino terminus is attached to the cytoskeleton. This provides the plasma membrane with protection from stretching. (Watkins et al, 1988; Harris et al, 1992).

1.5   Reading frame theory

Irrespective of the role of dystrophin it has been established that the size of the deletion does not provide information as to the severity of the disease (Hodgson et al, 1989). It was proposed by Koenig et al

(1989), that mutations which maintained the reading frame of mRNA resulted in the milder BMD, while DMD occurs when the reading frame is disrupted, due to the insertion of a stop codon. This stop codon in DMD patients resulted in a severely truncated protein (Hodgson et al, 1989; Koenig et al, 1989). It was subsequently shown by Koenig et al (1989), that this was not always the case. In 60 out of 70 BMD patients, deletions in the Dystrophin gene had maintained the reading frame and in 178 out of 188 DMD patients, deletions had disrupted the reading frame. It was evident from these results that approximately 92% of DMD and BMD patients fitted the reading frame model. Western blot analysis also showed that DMD muscle, when tested with specific carboxy terminus antiserum, gave negative results. BMD when tested with the same carboxy antiserum gave a positive result, but with reduced protein size and expression (Tanaka et al, 1990; Harris et al, 1992). This was an indication of a mutation that has maintained the reading frame. Hodgson et al (1989), showed that the same deletion in different patients could result in different phenotypic manifestations. This suggested that other factors might influence the phenotypic response to a mutation. Chelly et al (1990), suggested that alternate splicing might explain the exceptions to the reading frame theory.

1.6   Splicing

In higher eukaryotes some pre-mRNAs undergo alternate splicing, which increases the number of protein products of a single gene and provides additional mechanisms for regulating gene expression (Green, 1986). Alternate splicing can result in the skipping of specific exons, therefore generating multiple mRNAs from a single pre-mRNA (Green, 1986).

The process of transcription involves the synthesis of a single-stranded mRNA molecule from the double-stranded DNA template in the cell nucleus. Transcription of DNA is selective, with specific regulatory sequences indicating the beginning and the end of the segments of DNA to be transcribed. Transcription of a gene results in many identical single-stranded transcripts containing both introns and exons. This pre-mRNA then needs to have the intronic (non-protein coding region) mRNA spliced out before it can become mature mRNA and subsequently leave the nucleus (Green, 1986; Padgett et al, 1986).

The process of splicing intronic RNA out of pre-mRNA to make mature mRNA is not well understood (Ruby & Abelson, 1991). Small nuclear RNA's (snRNA) are involved in this splicing. In particular a subset of these snRNA's known as U1, U2, U4, U5, U6, U7, U8, U9, U10, have been shown to form complexes with small nuclear ribonucleoproteins (snRNP's). The snRNA U1 is the first to bind to the pre-mRNA and is complementary to the consensus sequence at the 5' splice site. Consensus sequences play an important functional role in gene expression and although the sequence may not be identical from gene to gene, they do tend to share certain features. U5 is complementary to the 3' splice site. These two snRNA's along with the other snRNA's and the associated snRNP's form a complex known as a spliceosome, which causes a looping of the intronic mRNA and subsequent splicing out of the intron, to form the mature mRNA (Ruby, 1991). It does this by acting as a temporary clamp to hold the two consecutive exons together so that enzymatic splicing occurs at the correct point. The process of spliceosome formation requires ATP (Green, 1986; Padgett et al, 1986; Gunzel et al, 1992).

The 5' and 3' ends of introns contain only two universally conserved dinucleotides, the first and last two nucleotides of the intron. The first two nucleotides, known as the donor splice site are located at the 5' end of the intron and consists of a Guanine and a Uracil. The last two nucleotides, known as the acceptor splice site are located at the 3' end of the intron and consist of an Adenine and a Guanine (see Figure 1.3) (Green, 1986; Padgett et al, 1986).

At the exon-intron boundaries, there is also a conserved sequence which provides binding sites for snRNA's. At the Donor site 5' end there is an eight nucleotide semi conserved consensus sequence, two residues 5' and six residues 3' from the splice point. The actual nucleotide frequencies upon which the consensus is based are A 64%, G 73%, / G 100%, U 100%, A 62%, A 68%, G 84%, U 63%. Beyond this region the sequence is again random (Green, 1986). At the Acceptor site 3' end of this intron, the consensus sequence is of variable length (greater than ten nucleotides), 1 residue 3' and a variable number of residues 5' from the splice point, which includes a pyrimidine rich region and the universally conserved "AG" dinucleotide (see Figure 1.3) (Green, 1986; Padgett et al, 1986).

The function of introns is not well understood, although they may provide extra genetic flexibility. This versatility is conferred by alternate patterns of mRNA splicing which can generate different proteins from the same mRNA transcript although there is no apparent mutation at the donor or acceptor splice sites. Alternative splicing of mRNA can be related to tissue-specific splicing ie. different mature mRNA products are spliced in different tissue samples (Inazu et al, 1992). Alternate

Figure 1.3

Splicing of pre-mRNA results in mRNA, which then leaves the
nucleus. Donor and Acceptor splice sites indicate the region of
RNA to be spliced out and are universally conserved. The
donor consensus sequence is semi-conserved and necessary
for accurate splicing to occur.

pre-mRNA

EXON   INTRON   EXON   INTRON   EXON

5' [⎕]————————————[⎕⎕]————————————[⎕⎕] 3'

AGGTAAGT   AG   GT   AG

Donor consensus sequence

Acceptor splice site

Donor splice site

Acceptor splice site

mRNA

EXON   EXON   EXON

5' [⎕|⎕|⎕] 3'
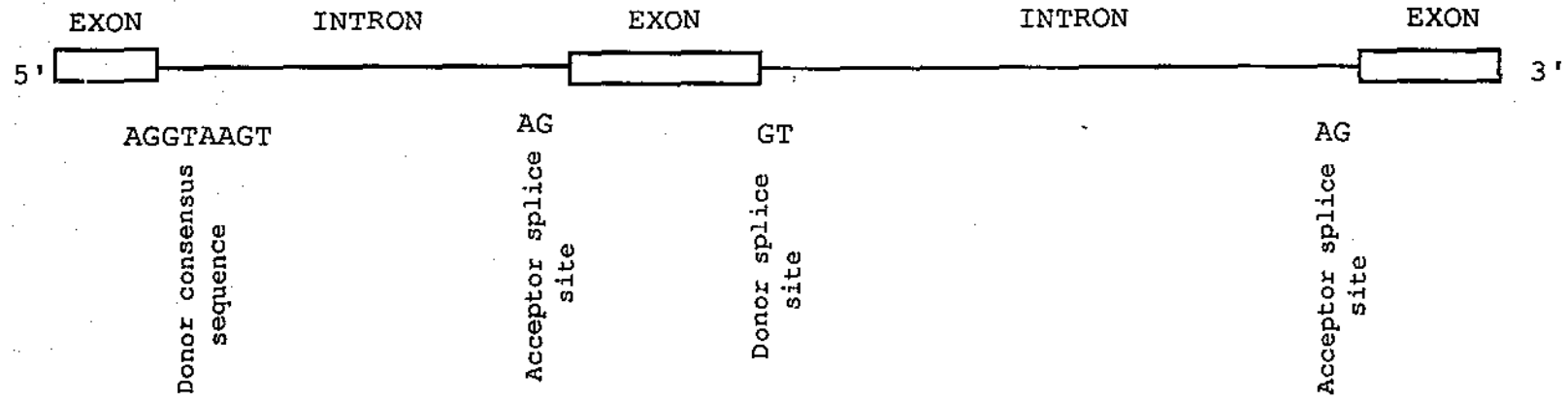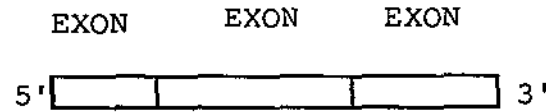
splicing can also occur at different times during development, resulting again in changes in the mature mRNA transcript (Green, 1986). It is also possible to have non-regulated splicing. For example this occurs in the adenovirus allowing the virus to produce multiple protein products from a single transcription unit (Green, 1986).

1.7   Mutations in the dystrophin gene and disease detection

Mutations at the donor and acceptor splice sites of a gene have been shown to produce various effects in the formation of mature mRNA. These include exon skipping, insertion of intronic sequence in mRNA and partial insertions and deletions of both exons and introns into mRNA which may or may-not affect the reading frame (Rees et al,1985; Cladaras et al, 1987; Mounir et al, 1990; Takahashi et al, 1990; Carstens et al, 1991).

In 30% of DMD families and 14% of BMD families there is no easily detectable mutation (Laing, 1993). These DMD and BMD patients therefore have point or minor mutations of the dystrophin gene, which conventional southern analysis or multiplex polymerase chain reaction (PCR) are unable to detect. These families must rely on linkage analysis for prenatal diagnosis. The accuracy of diagnosis based on linkage analysis varies, depending on the distance of the polymorphic marker from the disease-causing mutation. Linkage analysis is also limited by the number of affected people in a family. For example, if only one affected person is seen in the family it is not possible to establish linkage between markers and the disease gene and so linkage analysis becomes useless in these circumstances. The large size of the dystrophin gene 2.5 Mbp and other undefined factors result

in 11% to 12% recombination across the dystrophin gene (Abbs et al, 1990). Prenatal diagnosis of DMD/BMD families by linkage analysis could result in errors unless tightly-linked highly polymorphic flanking markers are used (Laing, 1993). PCR techniques have been used to analyse microsatellites (polymorphic markers) at either end of the dystrophin gene, to determine linkage. This method of linkage analysis is both extremely informative and fast (Webber et al, 1990). The problem with linkage analysis is most family pedigrees are not large enough to show linkage of the disease with a polymorphic marker. If the mutation is *de-novo*, which is estimated to be the case in 33% of DMD/BMD patients, then linkage analysis is of no value (Laing, 1993). The best method of offering these families accurate prenatal diagnostic testing, would be to identify the actual point or minor mutation in these families that results in DMD/BMD. It is then possible to devise an accurate prenatal diagnostic test based on the disease causing mutation.

1.8   Project outline

In 70% of DMD and BMD patients the conventional restriction fragment length analysis of the dystrophin gene by southern blots, show notable deletions, duplications or translocations. This provides accurate prenatal diagnostic tests for these families. In the other 30% of DMD and BMD patients there is no known deletion or translocation, therefore for these families linkage analysis is the only means of providing a prenatal diagnostic test and this may not always be informative. This 30% of patients with DMD and BMD must therefore have a small mutation, even a point mutation which is not able to be detected by southern analysis (Richards et al, 1991; Laing et al, 1992).

The project involves looking for small or point mutations, in one Western Australian family which does not have a notable deletion and/or translocation. If a point or small mutation is identified this will provide the basis for an accurate prenatal diagnostic test for this family. It will also enable carrier females to be identified. To do this I will perform a dystrophin gene nested amplification of complementary DNA (cDNA) from seven affected individuals belonging to seven different families. This may give an indication of which family is best to investigate further, with a view to developing a diagnostic test for their form of muscular dystrophy. In doing the project I will become familiar with a large number of molecular techniques, polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP), amplification refractory mutation system (ARMS) and genomic sequencing.

1.9   Techniques used to identify mutations

Roberts et al (1991) and (1992) have described a method for finding point mutations in the dystrophin gene. Their method of detecting point mutations will be used in this project with some slight modifications. Messenger RNA will be extracted from muscle instead of peripheral blood lymphocytes. Reverse transcription of mRNA to produce cDNA and nested PCR of the dystrophin gene will be carried out using the nested primers specified by Roberts et al (1991).

If no mutation is evident after nested PCR, then SSCP analysis will be attempted. Richards and Friend (1991), showed SSCP analysis of DNA could be used to determine carrier status of dystrophic women. Orita et al (1989a), noted that SSCP analysis could be used to detect

single base changes in sequences under 200 base pairs (bp). SSCP is used to analyse the cDNA after it has been amplified [PCR], because single strand DNA migrates according to the nucleotide sequence which in turn determines the conformation of the DNA and subsequently the speed of migration. Therefore a single base change in single-stranded DNA may cause a conformation change, which will lead to a change in migration speed during electrophoresis. A single base change in double-stranded DNA would not cause a change in migration with normal electrophoresis (Orita et al, 1989b; Baldwin et al, 1992).

Once nested PCR or SSCP has determined an area of the dystrophin gene to be polymorphic, it is necessary to sequence the genomic DNA for that area and determine whether or not there is a sequence change from the published cDNA sequence and whether that particular change could result in the clinical manifestation of DMD or BMD in the individual (Rao & Sanders, 1992).

The final phase of the project will involve providing the families with an accurate prenatal diagnostic test. This could be either using SSCP to characterise the effect of the mutation on all the family members, or another diagnostic test, known as amplification refractory mutation system (ARMS). The ARMS method uses primers specific for the mutation, to amplify the mutant allele or the normal allele (Wenham et al, 1991).

CHAPTER 2: RNA EXTRACTION AND cDNA SYNTHESIS

2.1   Introduction

During ribonucleic acid (RNA) extraction from tissue there are many opportunities for contamination with RNAases. RNAases are enzymes which can degrade the RNA in the tissue and during the extraction procedure (Sambrook et al, 1989). The history of the frozen tissue used in this project and its handling at the time before freezing was not known and may have resulted in contamination with RNAases. It was therefore important to minimise the introduction of RNAases in the extraction procedure, to limit degradation of the RNA still present in the tissue. This was done using sterile technique and RNA dedicated equipment and solutions. All further analysis carried out on this RNA, was done using the polymerase chain reaction (PCR) technique. PCR requires only small amounts of mRNA to be converted into cDNA for subsequent amplification to occur successfully. As the products being amplified were approximately 1250 bp long, it was necessary that RNAase activity/presence was minimised so as to allow the production of this relatively large cDNA transcript.

The RNA extracted from the muscle biopsies does not have any intronic sequence and is used as a template in the production of cDNA. Converting the mRNA to cDNA, by using the enzyme reverse transcriptase, reduces the sequence information, making it easier to find minor mutations. Genomic DNA coding for the dystrophin protein consists of 2500 kb whereas the cDNA which codes for dystrophin is only 11 kb long or 14 kb if promoters and 3' untranslated regions are included (Koenig et al, 1987).

The conversion of RNA to DNA occurs within a group of viruses, known as retroviruses. These viruses have a genome composed of single-stranded RNA. Upon entering a cell the retrovirus is uncoated and the RNA is copied into DNA by a unique viral enzyme called reverse transcriptase (Gelehrter and Collins, 1990, p.238). This retroviral enzyme can also be used to convert mRNA extracted from cells to cDNA, resulting in a DNA transcript with no intronic sequence. This process of converting single-stranded RNA to double-stranded DNA is known as reverse transcription (Watson et al, 1987, p.90).

Reverse transcription of mRNA extracted using RNAzol (Biotecx lab) or the GuHCl (guanidine hydrochloride) methods, was carried out using avian myeloblastosis virus reverse transcriptase (AMV-RTase), supplied by Promega Corporation. This enzyme synthesises DNA in the 5' to 3' direction from a single stranded RNA template, using a short primer to initiate synthesis. AMV-RTase works efficiently at $42^{o}C$, which allows RNA's rich in secondary structures to be copied more efficiently than the murine enzyme which works at $37^{o}C$ (Sambrook, 1989). The problem associated with both these reverse transcriptase enzymes is the lack of 3' - 5' exonuclease activity, which provides an editing function and therefore the presence of high concentrations of dNTP's and Mg^{++} may lead to mis-incorporation (Sambrook et al, 1989). AMV-RTase enzyme and associated 5x buffer were supplied by Promega Corporation. The 5x buffer supplied has the concentration of dNTP's and Mg^{++} optimised and therefore reduces any chance of mis-incorporation during cDNA synthesis.

2.2   Materials and Methods

Two methods of RNA extraction were used. RNAzol (Biotecx lab) and a modified GuHCl method (Chirgwin et al, 1979).

Note: all reagents used in this honours project were of analytical grade.

2.2.1   RNAzol (Biotecx lab)

This is a new method of RNA extraction and the ingredients which make up RNAzol remain a closely guarded secret of Biotecx Inc. The benefit of this method of RNA extraction is that it requires minimal steps and time in order to get good RNA yields. The RNA extraction consists of four steps :-

> 1.   Homogenisation (tissue)
>
> 2.   RNA extraction
>
> 3.   RNA precipitation
>
> 4.   RNA wash

The RNAzol method of extracting RNA can be completed within 2 hours. The methodology used for the extraction of RNA in this case was in compliance with the RNAzol product information pamphlet and is as follows:-

A muscle sample (5-10mg) was placed in a 1.5ml reaction tube and 200ul of RNAzol was added. The muscle was then homogenised using a sterile, disposable, plastic homogeniser. 20ul of chloroform was then added to the homogenate and the sample shaken vigorously for fifteen seconds. The sample was then left on ice for five minutes and then spun down at 11500g (4°C) for 15 minutes. At this stage two phases

formed, an upper colourless phase and a lower blue phenol-chloroform phase. RNA remains in the upper colourless phase. The upper phase was then transferred to a fresh 1.5ml reaction tube, with an equal volume of isopropanol and then stored for 15 minutes at 4°C. The sample was then spun in a centrifuge at 11500g (4°C) for 15 minutes. The RNA formed a white-yellow pellet at the bottom of the tube. The supernatant was then removed with a micro-pipette and the pellet washed in 500ul of 75% ethanol, by vortexing and subsequent centrifugation at 7100rpm (4°C) for 8 minutes. The 75% ethanol was removed and the RNA pellet was dried under vacuum for 10 minutes. Finally, the dried RNA was resuspended in 50ul of double distilled water (DDW).

2.2.2 Modified GuHCl RNA Extraction

This method of RNA extraction is described in Chirgwin et al (1979) and requires more preparation and time than the RNAzol method. The first step involves making up the three solutions required in the extraction procedure.

2.2.2.1 Solutions For RNA Extraction

Solution 'A'	6M	GuHCl
	200mM	NaAc pH 5.2 - 6.0
	10mM	B-ME

Solution 'B'	6M	GuHCl
	200mM	NaAc pH 5.2 - 6.0
	10mM	EDTA

Solution 'C'	7M	Urea
	100mM	Tris pH 8.0
	200mM	NaAc pH 5.2 - 6.0
	0.5 %	SDS
	10mM	EDTA

2.2.2.2 GuHCl RNA Extraction Procedure

The volumes of each solution required for the extraction of RNA are based on a starting muscle sample of 5 to 10mg. Throughout the RNA extraction the samples and solutions were kept on ice, whenever possible.

The muscle sample was homogenised in a 1.5ml reaction tube, with 500ul of solution 'A', then 500ul of 100% ethanol was added. This solution was then mixed well and chilled at -80°C for 15 minutes. The sample was then spun at 13650g (4°C) for 15 minutes, the supernatant discarded and the pellet resuspended in 300ul of solution 'B'. The pellet was then homogenised again in solution 'B', after which 300ul of 100% ethanol was added. This solution was then mixed well and chilled at -80°C for 15 minutes. Next, the sample was then spun at 13650g (4°C) for 15 minutes, the supernatant was discarded and the pellet resuspended in 400ul of solution 'C' after which 400ul of buffered phenol and 400ul chloroform:isoamyl alcohol 24:1 was added to the pellet in solution 'C'. This solution was mixed well and left on ice for 10 minutes. Spinning the solution at 13650g (4°C) for 15 minutes resulted in two phases, with the top aqueous phase containing the RNA. This top aqueous phase was transferred to a fresh 1.5ml reaction

tube containing 800ul of 100% ethanol. The ethanol and RNA aqueous phase were mixed well and chilled at -20°C overnight. The following day, the aqueous phase with ethanol was spun at 13650g (4°C) for 15 minutes and the supernatant discarded. The RNA pellet was washed in 300ul of 70% ethanol, the 70% ethanol removed and the pellet dried under vacuum. The RNA pellet was then resuspended in 50ul of DDW.

2.2.3   cDNA Synthesis from mRNA

Reverse transcription is the process of converting single stranded mRNA into double stranded cDNA using the retroviral enzyme reverse transcriptase. This cDNA doesn't include any intronic sequence as this was spliced out of the mature mRNA (Sambrook et al, 1989).

Using the RNA extracted by the RNAzol or modified GuHCl methods, 4ul of RNA was added to 4 units of AMV-RTase, 250ng of random hexamers, 5ul of 5x buffer as supplied by Promega and then the reaction was brought to volume with DDW. The reaction was then incubated for 5 minutes at room temperature followed by 60 minutes at 42°C. The cDNA was then diluted 1:20 in DDW and stored at 4°C.

2.2.4   Gel Electrophoresis

DNA and RNA migrates according to descending size, from the cathode (-ve) towards the anode (+ve) in response to an electric current in either an acrylamide or agarose gel. In order to increase separation of the different sized DNA and RNA it is necessary to vary the gel concentration, running time and voltage (Sambrook et al, 1989).

2.2.4.1 Making Agarose gel

Increasing the agarose concentration makes for a denser gel. The greater the agarose concentration, the slower the DNA travels through the gel matrix. Also the size of the DNA sample being fractionated determines the agarose concentration needed, the smaller the DNA sample the greater the concentration of agarose required (Sambrook et al, 1989).

1% Agarose gel

     1g of agarose in 100ml of 1x TAE buffer

2% Agarose gel

     2g of agarose in 100ml of 1x TAE buffer

3% Agarose gel

     3g of agarose in 100ml of 1x TAE buffer

Agarose and 1x TAE buffer were added to a stock bottle, with the lid on loosely. The solution was boiled in a microwave oven and agitated until all the agarose had dissolved, then stored in a dry oven at $65^{o}C$ until ready to pour.

2.2.5 Running RNA on Agarose gel

A 1% agarose solution was poured onto a gel tray to a depth of 5 to 10mm. A gel comb was then placed in the solution, 1cm in from one end and to a depth of approximately 1mm from the base of the tray. The agarose solution was then left 15 minutes at room temperature to

set. Once set, the comb was removed and the gel placed in a gel tank with 1x TAE buffer covering the gel. 10ul of the RNA sample, along with 2ul of ficoll loading buffer (6x), was loaded into each well. The gel was then run for 45 minutes at 100 volts(v) and stained with ethidium bromide, which binds to DNA/RNA. After staining, the gel was observed under an ultra-violet (UV) light (305nm Transilluminator), which fluoresces the DNA and RNA.

2.3   Results

The RNA extraction was carried out using two methods, (i) RNAzol system and (ii) modified GuHCl method. The RNAzol method, although very quick (total extraction of RNA in less than three hours) did not yield RNA of sufficient size and quantity as subsequent nested PCR analysis showed. In Figure 2.1 the "RNAzol 1" and "RNAzol 2" lanes show faint bands, indicative of 18s ribosomal RNA. The second method of RNA extraction was a modified GuHCl method (Chirgwin et al, 1979). This method also extracted DNA. The "GuHCl 1" and "GuHCl 2" lanes in Figure 2.1 shows the DNA extracted with no sign of RNA. However, PCR analysis showed this RNA extraction procedure worked well. A third method of RNA extraction, a hybrid of RNAzol and the modified GuHCl methods was tried in the hope of providing a faster and more efficient method of extracting RNA. This method did not prove successful, as the yield of RNA was not sufficient to allow further nested PCR analysis (data not shown).

cDNA synthesis was achieved using AMV-RTase. The conditions remained constant for all RNA transcripts whether extracted by RNAzol or the modified GuHCl method. It was only after the amplification stage of the cDNA by PCR that it was possible to determine whether the RNA

Figure 2.1

A 1% agarose gel loaded with 10ul aliquots of the RNA
extracted from normal muscle by the RNAzol and GuHCl
methods. The numbers 1 and 2 for RNAzol and GuHCl
represent two different muscle samples, which are normal for
dystrophin. The bands in sample GuHCl 1 and GuHCl 2
represent DNA extracted in this procedure. Lamda marker
digested with Pst I.
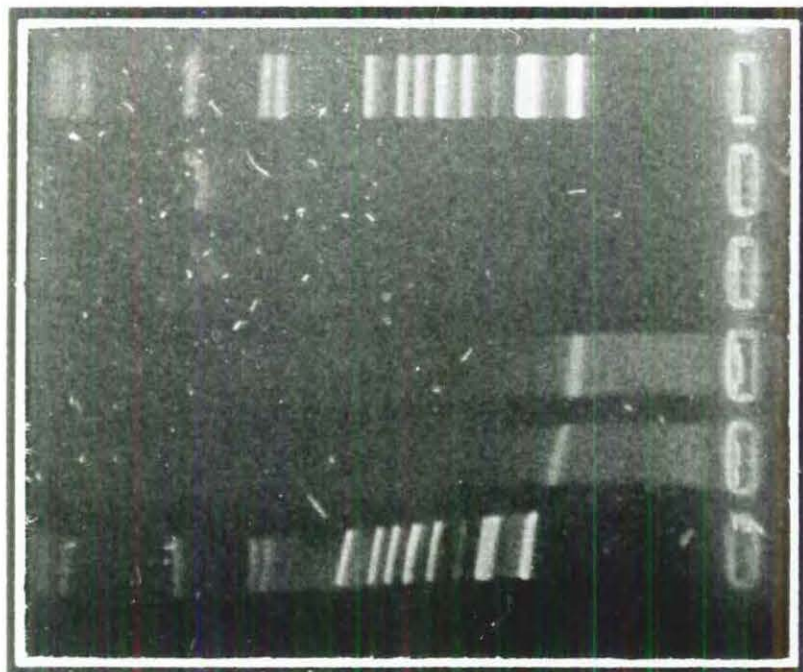
Lambda Marker
RNAzol 1
RNAzol 2
GuHCl   1
GuHCl   2
        Lambda Marker

extraction and the cDNA synthesis actually worked. The best method of extracting RNA from small amounts of frozen tissue proved to be the GuHCl method although it was more time consuming than the RNAzol method.

Extraction of RNA from 5 to 10mg of muscle tissue resulted in very few RNA transcripts. It was not possible to determine the presence of tRNA and rRNA by running an aliquot of the RNA on a 1% agarose gel (see Figure 2.1). All RNA samples were converted to cDNA (reverse transcription), in order to perform further analysis. This was done because PCR amplification requires only a few full length transcripts to produce multiple copies of cDNA required for further analysis.

2.4   Discussion

Factors that influence the quality of RNA extraction include the type of sample, storage of the sample and the extraction procedure. As muscle samples from affected DMD/BMD patients were in such small quantities, it was necessary to optimise the extraction procedure by trying various RNA extraction techniques on normal muscle samples which had been stored in the same manner as the affected muscle samples (-80° C). Once the RNA extraction and cDNA synthesis were optimised it was then decided to proceed with the affected samples. Three methods of RNA extraction were tried on normal muscle samples, (i) RNAzol (ii) modified GuHCl and (iii) a hybrid of RNAzol and GuHCl methods. The RNAzol method and the hybrid RNAzol/GuHCl methods when used on small amounts of frozen tissue (5-10 mg), did not provide sufficient good quality mRNA to do further PCR analysis using the nested primers specified by Abbs et al (1991). The modified GuHCl method was able to produce RNA of sufficient

quality to allow further PCR analysis. When this RNA was extracted and an aliquot was run on a 1% agarose gel to determine (i) whether there was RNA present and (ii) if it was present whether it was degraded. The small amounts of tissue being used 5-10mg in these RNA extraction procedures, did not produce sufficient RNA for the bands to be visualised. RNA when run on an agarose gel should give three prominent bands, representing 5s, 18s and 28s ribosomal and transfer RNA (Alberts et al, 1983 p.209). It was necessary to go on and produce the cDNA and do a nested amplification in order to determine whether the RNA extraction had worked efficiently.

Not all DMD/BMD patients will submit to a biopsy, therefore, although muscle may provide the most abundant quantity of dystrophin mRNA transcript (Chelly et al, 1988), it is possible to obtain dystrophin mRNA from lymphocytes (Roberts et al, 1990). This may prove to be a much more practical when looking for mutations in other Western Australian dystrophic families.

CHAPTER 3: NESTED AMPLIFICATION

3.1 Introduction

Polymerase chain reaction (PCR) is a new development in DNA technology, with the ability to amplify a short sequence of DNA from a complex mixture containing:-

    1)   Target DNA

    2)   Taq Polymerase

    3)   5x mix (buffer, nucleotides and $MgCl_2$)

    4)   Primer

    5) H_2O

    6)   Paraffin overlay (stops evaporation)

It is first necessary to synthesise two short oligonucleotide primers based upon the known sequence of the region. These primers are chemically synthesised and are usually between 20-35 nucleotides long. Polymerase chain reaction takes place by heating the target DNA, which denatures it into single strands. The reaction is then cooled to a preset annealing temperature which allows the oligonucleotide primers, present in high concentration, to anneal to their complementary DNA strands (Bej, 1991). Once annealing is completed, extension of the DNA occurs with nucleotides being added to the primer by the enzyme Taq polymerase. The Taq enzyme is able to withstand temperatures as high as 95^oC and is derived from a bacteria found in hot springs (*Thermus aquaticus*) (Erlich, 1989). Extension only occurs when the primers anneal to the target DNA, which then allows the Taq polymerase to synthesise a DNA strand using the target DNA as a template. This whole procedure is easily

cycled, with each cycle taking about 5 minutes and the synthesised product in one cycle can serve as a template in the next. At the completion of each cycle the DNA sequence specified by the primers, will have doubled. As many as 30 to 40 cycles can be carried out without the need to add fresh enzyme or primers. 30 cycles results in approximately 1 billion copies of the target DNA and all this occurs in a single PCR tube (Gelehrter & Collins, 1990, p. 82).

Nested amplification is a modified PCR. It involves two sets of primers, used in two separate PCR reactions. The first reaction is a standard PCR, usually carried out for 30-35 cycles. The nested amplification, uses primers which are internal to the first round primers. It also uses a diluted first round amplification product as target DNA in the nested amplification. The product that results is the same size as one obtains, when only the nested primers and the original target DNA are used in a PCR reaction (see Figure 3.1) (Roberts et al, 1991).

The nested amplification method provides both a double check on the sequence being amplified and results in a much more specific amplification. The conditions of amplification are not extremely stringent, but because the second round amplification is of a product slightly smaller and contained within the first round amplification, this increases the specificity of the bands produced.

The nested PCR method of amplification is able to amplify up extremely small amounts of the template DNA billions of times, if the cDNA produced by reverse transcription was of sufficient quality. It is then possible to run the amplified DNA on an agarose gel and after ethidium bromide staining, visualise it under a UV light (Bej, 1991).

Figure 3.1

Nested Amplification: The cDNA is amplified primarily with a
primer mix consisting of primer A and D, this amplified product
is then diluted and used as target for the nested amplification.
A primer mix consisting of primer B and C is then used in the
nested amplification, this nested product can then be
electrophoresed on an agarose gel, stained with ethidium
bromide and viewed under a UV light source.

Primer C

Primer D

5' ——————————————————————————————— 3'

3' ——————————————————————————————— 5' cDNA

Primer A

Primer B

Nested amplification product

Primary amplification product
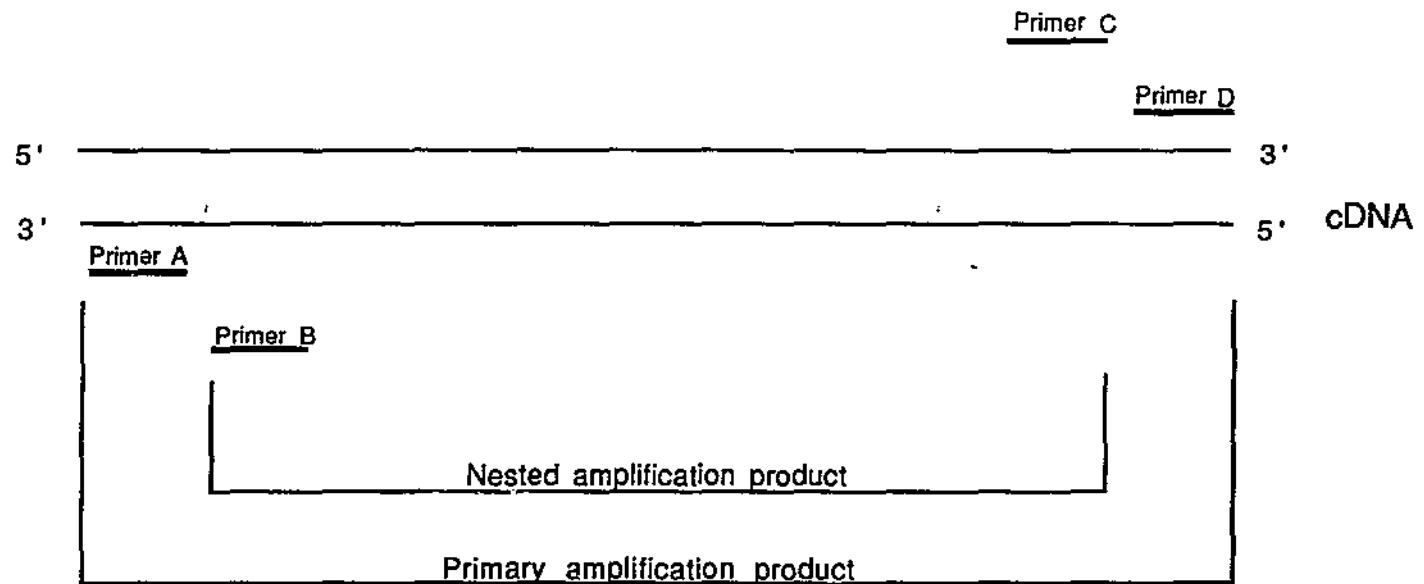
The accuracy of PCR analysis depends upon a number of factors including the quantity of template, the concentration of the dNTP's, primer concentration, Taq Polymerase concentration and $MgCl_2$ concentration (Innis et al, 1990). The conditions used in the nested PCR and all the other PCR reactions were chosen in order to minimise the chance of mis-incorporation during amplification.

3.2 Materials and Methods

All PCR reactions used the following 5x Amplification mix unless otherwise stated.

The 5x Amplification mix consists of:-

       1mM dNTP

       5x Reaction Buffer (Biotech)

       10mM $MgCl_2$

Reaction Concentration:-

       200uM dNTP

       1x Reaction Buffer (Biotech)

       2mM $MgCl_2$

3.2.1 Amplification of dilute cDNA

Amplification of the cDNA (dilute) involved a primary amplification with external primers. This reaction was subsequently diluted 1:200, so as to reduce carry over effect of external primers, with DDW and a 5ul aliquot used in the secondary amplification in which primers internal to the primary amplification primers were used. The approximate length of the final amplified product was 1250 bp. This procedure is known as nested amplification. Table 3.1 indicates the primers used in order to

Table 3.1

Primers used in nested amplification

Sequence (5' to 3')	Primary/Nested	Position	Exon
ATGCTTTGGTGGGAAGAAGTAG	P	208-230	2-10
GAGAGGTAGTTACTTGACGGT	P	1197-1218	2-10
TTATGAAAGAGAAGATGTTCAA	N	237-259	2-10
CTTGTCTTCAGGAGCTTCCAA	N	1171-1192	2-10
CGATTCAAGAGCTATGCCTAC	P	1091-1111	9-18
GCGAGTAATCCAGCTGTGAAG	P	2399-2419	9-18
CCTCTGACCCTACACGGAGC	N	1134-1153	9-18
CAGTTATATCAACATCCAACC	N	2376-2396	9-18
CATGCTCAAGAGGAACTTCC	P	2300-2319	17-25
CTGAGTGTTAAGTTCTTTGAG	P	3599-3619	17-25
GCAGATTACTGTGGATTCTG	N	2344-2363	17-25
GTCTCAAGTCTCGAAGCAAAC	N	3574-3594	17-25
CTAGAAATGCCATCTTCCTTG	P	7583-7603	51-58
CTCAGGAGGCAGCTCTCTGG	P	8871-8890	51-58
CTGCTCTGGCAGATTTCAAC	N	7617-7636	51-58
CTCCTGGTAGAGTTTCTCTAG	N	8849-8869	51-58

(Roberts et al, 1991)

do the nested amplification of exons 1-10, 9-18, 17-25, 51-58. All primers were synthesised at this laboratory on a Cyclone Plus DNA Synthesizer (Milligen / Biosearch).

3.2.2 Primary amplification of the dystrophin transcript

To a 0.5ml PCR tube was added 5ul of 5x buffer, 13.5ul DDW, 1ul of the outer primers (70ng), 0.5 units of Taq polymerase (Biotech) and 5ul of dilute cDNA. The reaction mix was then vortexed for one second and a drop of paraffin added to overlay the reaction. The primary amplification was for 35 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes (the initial denaturation step at 94°C was carried out for 5 minutes). At the end of the primary amplification the PCR product was diluted 200 fold by adding DDW to the PCR tube and then vortexing. This provided the target for the secondary amplification.

3.2.3 Nested amplification of the dystrophin transcript

The second round polymerase chain reaction was essentially the same as the primary amplification except 5ul of the diluted primary amplification mix was used as the target instead of the 5ul of dilute cDNA that was used in the primary amplification. Also the number of cycles used in the secondary amplification was reduced to 30 cycles.

3.2.4 Molecular weight markers

In order to determine the size of the DNA fragments during gel electrophoresis it was necessary to run standards which indicate the approximate size of the DNA samples run on the gel. Lambda phage

36

when cut with the restriction enzyme Pst I provided a high molecular weight marker and pUC 19 cut with Msp I provided a low molecular weight marker (see Figure 3.2).

3.2.4.1 Lambda cut with Pst I

To a 1.5 ml reaction tube was added; 150ul of 10x buffer "3", 30ul of 0.1 M spermidine, 15ul of 10mg/ml BSA, 9ul of 20 units/ul Pst I enzyme, 996ul of DDW and 300ul uncut lambda 500ng/ul. The tube was then vortexed lightly and incubated for 8 hours at 37°C to allow digestion to take place. The lambda cut with Pst was then ethanol precipitated and resuspended in 6ml of Ficoll 1x loading buffer. 5ul of marker was loaded on all agarose gels.

3.2.4.2 pUC 19 cut with Msp I

To a 1.5 ml reaction tube was added; 150ul of 10x buffer "A", 30ul of 0.1 spermidine, 15ul of 10mg/ml BSA, 9ul of 20 units/ul Msp I enzyme, 996ul of DDW and 300ul of uncut pUC 500ng/ul. The tube was then vortexed lightly and incubated for 8 hours at 37°C to allow digestion to take place. The pUC cut with Msp I was then ethanol precipitated and resuspended in 1.5ml of Ficoll 1x loading buffer. The DNA concentration was much less than 500ng/ul, due to the presence of RNA in the uncut pUC sample. The RNA in the pUC sample resulted in a much greater DNA reading when measuring absorbance at 260nm in a spectrophotometer. 5ul of the marker was loaded on all agarose gels.

Figure 3.2

Molecular weight markers: Lambda cut with the enzyme Pst I (High molecular weight marker) and pUC 19 cut with Msp I (Low molecular weight marker).

Lambda cut with Pst I

	————	11500
4789 - 4749	————	
2556 - 2443	≡≡≡	2838
1886	≡≡	2190
	————	1700
1159		
	≡≡	1093
805	————	
468	≡≡	514
	≡≡≡	448
339	————	

Number of
Base Pairs

pUC cut with Msp I

————	501 - 489
————	404
————	331
————	242
————	190
————	147
————	111

Number of
Base Pairs

3.2.5 Electrophoresis of nested products

The nested amplification products were run on a 2% agarose gel, for one and a half hours at 100 volts in 1x TAE buffer. 2ul of the sample was mixed with 2ul of ficoll loading buffer (1x) and loaded to each lane. The gel was visualised by staining with ethidium bromide, which will fluoresce the DNA/RNA under UV light (305nm Transilluminator).

3.3 Results

Figure 3.3 shows the nested amplification of cDNA, exons 1 to 10, derived by reverse transcription of RNA extracted using GuHCl method on normal muscle samples. Figure 3.3 is also evidence that the RNA extraction and cDNA synthesis worked with normal muscle samples (no known deletions). Although the primary amplification lanes in Figure 3.3 samples show no evidence of any amplified product, there is an ion front which is a characteristic of the loading buffer that was used. Subsequent dilution of this primary amplification product followed by nested amplification resulted in a strong signal for the nested products corresponding to exons 2 to 10 of the dystrophin gene.

Figure 3.4 shows the nested amplified products of seven individuals from different families (A - G) who have point or minor mutations in the dystrophin gene. Amplification of exons 9-18 show patient D as having no product, this is consistent with the amplification of exons 17-25 and 51-58 which also indicate no products for patients D (see Figure 3.4). This indicates either the RNA extraction or the cDNA synthesis hasn't worked, or that this patient lacks all these exons. As patient D has already been tested using conventional southern blot analysis, which

Figure 3.3

Nested amplification of exons 2 to 10 from cDNA normal for dystrophin. 1 and 2 signify two different cDNA samples, which are normal for the dystrophin gene. Amplification was done using nested primers to amplify exons 2 to 10 of the dystrophin gene. The samples were run on a 2% agarose gel at 100 volts for 1 hour. Lambda marker digested with Pst I.
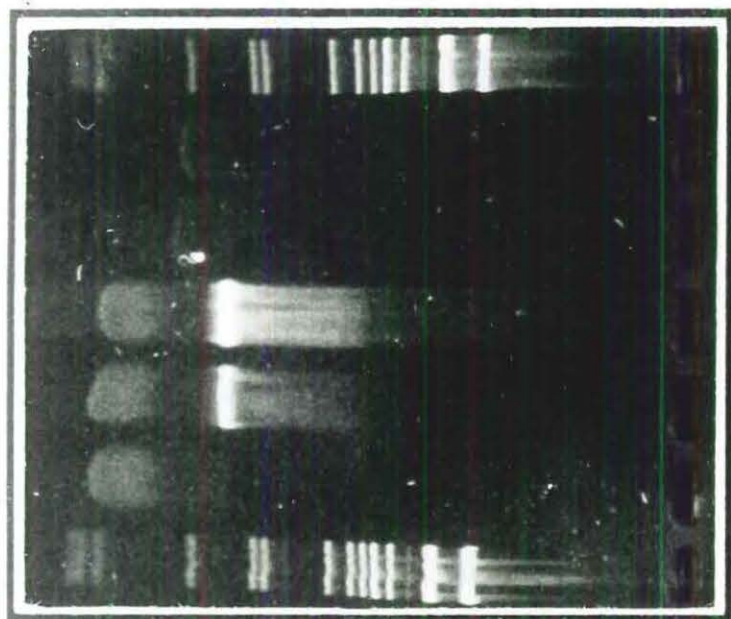
Lambda Marker
Primary Amplification 1
Primary Amplification 2
Nested Amplification 1
Nested Amplification 2
-ve Control
Lambda Marker

Figure 3.4

Nested amplification of cDNA derived from patients from different families who have either DMD or BMD. A to G signify different patients, the exons amplified for each of these patients are 9-18, 17-25 and 51-58 respectively. The samples were run on a 2% agarose gel at 100 volts for 1 hour and 15 minutes. Lambda marker digested with Pst I.
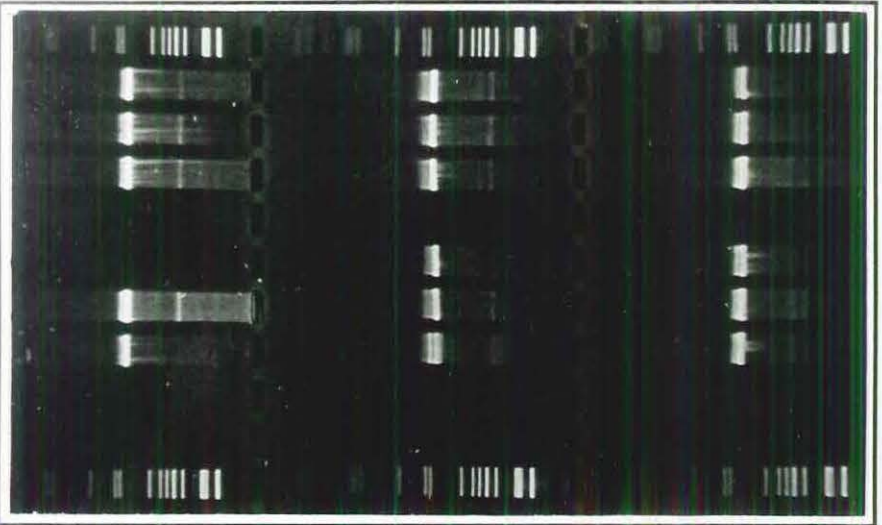
Lambda Marker

A
B
C
D
E
F
G
-ve Control 1
-ve Control 2
Lambda Marker

Exon 51-58 (1252bp)

Exon 17-25 (1251bp)

Exon 9-18 (1263bp)

did not indicate any major deletions, it was therefore assumed that the RNA extraction or cDNA synthesis had not worked. No product is evident for patient E exons 51-58 (see Figure 3.4). As the cDNA of patient E amplified exons 9-18 and 17-25 this would indicate either; that the cDNA transcript for exons 51-58 was not in sufficient quantity to provide a target in the primary amplification, there was human error in the setting up of the amplification, there was a mutation in the primer binding sites, or a major deletion of exons 51 to 58. A major deletion of this size would have been picked up using conventional southern blot analysis. Excessive amplification of exons 51-58 for patients (A to G) has resulted in a strong signal for all patients, with some non-specific bands. Figure 3.4 shows patient G as having two distinct bands for exons 17-25, one band corresponding to the normal sized product and also a smaller product which is of the same intensity yet approximately 90 bases smaller.

3.4 Discussion

It was shown in Figure 3.3 that by using nested amplification it is possible to amplify small amounts of target. Although at the end of primary amplification no signal is evident, this primary amplified product was then diluted prior to being used as target DNA for the nested amplification. As noted earlier in 3.2.1 dilution takes place so that the primary amplification primers do not interfere in the nested amplification otherwise this could result in a second larger product specified by the external primers also being amplified.

When nested amplification of seven individuals (figure 3.4; A to G) who had either DMD or BMD was done using primers that amplified exons 9-18, 17-25 and 51-58 of the dystrophin gene, it was evident

that patient G had two amplified products for exons 17-25, one a normal size product and a second product approximately 90 bp smaller. The doublet band in Figure 3.4 patient G, exons 17-25 indicates that the two transcripts are found in equal volume in the muscle. Dr Steve Wilton showed by repeating this nested reaction with a decrease in the number of PCR cycles that the quantity of the alternately spliced transcript was approximately nine fold higher than the normal transcript (data not shown). In order to reduce the non-specific banding in Figure 3.4 and give a clearer quantitative indication of both transcripts, it would be necessary to reduce the number of cycles either in the primary or secondary amplification or increase the dilution of the primary amplification, prior to using it as target in the nested amplification.

Exon 19 is 88 bp long and it was considered a good location for looking for a mutation, as all the other exons from 17 to 25 were over 100 bp (Koenig et al, 1988).

It is also important to note that not all muscle samples from the affected DMD/BMD patients were able to be amplified using nested PCR. This indicates the difficulty of trying to extract RNA and then produce viable cDNA in order to amplify transcripts of the dystrophin gene, which are over 1200bp.

Chapter 4: SEQUENCING

4.1 Introduction

The nested amplification of exons 17-25 indicated that the mutation in patient G was under 100 bp. Analysis of the cDNA transcript for exons 17 to 25 indicated exon 19 as an ideal location to search for this mutation as it was 88bp, while all the other exons in the area were over 100 bp (Koenig et al, 1988). It was decided to sequence exon 19 and part of intron 18 and 19, from genomic DNA in an attempt to determine the site of the mutation. The entire sequence of the cDNA transcript for the dystrophin gene is known (Koenig et al, 1988), making comparison of our exon sequences with the normal transcript easy. It was also necessary to sequence genomic DNA from a patient who is normal for dystrophin, in order to determine differences in intronic sequence that may also have occurred.

The Sanger sequencing method involves using four separate stop reactions, one for each nucleotide 'A', 'C', 'G', 'T', in four separate PCR reactions. Included in the 'A' stop reaction are the normal dinucleotide triphosphates (dNTP's), which are required for normal amplification reactions, with the addition of dideoxynucleotide ATP (ddATP) (Gelehrter and Collins, 1990, p. 87). In the 'A' stop reaction the end labelled primer anneals to the target DNA and the complementary strand is synthesised using Taq Polymerase. When the ddATP is incorporated into the growing chain, during PCR, synthesis of that molecule stops. The ddATP is randomly incorporated into the growing chain producing amplified products of different sizes, dependant on when the ddATP is incorporated during each PCR cycle. The same process takes place for the other three nucleotide stop reactions ('C',

47

'G', 'T'), but these reactions have dideoxynucleotide CTP (ddCTP), dideoxynucleotide GTP (ddGTP), dideoxynucleotide TTP (ddTTP) respectively, to stop each reaction (Gelehrter and Collins, 1990, p.87). The four stop reactions, if run in separate lanes on a polyacrylamide gel and then exposed to X-ray film, will produce a ladder of oligomers of different lengths from which the DNA sequence can be read. Reading from the bottom of the gel upwards, each band represents the complementary base found on the target DNA moving in a 5' to 3' direction away from the primer (see Figure 4.1) (Erlich, 1989).

The Genesis sequencing system (Biotech) was chosen because of its simplicity in setting up the four stop reactions. The inclusion of a single dideoxynucleotide in each of the stop reactions effectively terminates the DNA chain elongation at the base complementary to the ddNTP. This is due to the ddNTP lacking the 3'-OH group that is necessary for continued chain elongation (Watson et al, 1987, p. 276). The use of PCR in the sequencing increases the speed and sensitivity of sequencing.

4.2   Materials and Methods

A 500ml stock of 6% denaturing acrylamide solution, for sequencing, was made by adding; 28.5g acrylamide, 1.5g bis-acrylamide, 210g urea and 425ml DDW to a beaker and the contents stirred until all the reagents were dissolved. Once dissolved, 40g of mixed bed resin was added and stirred slowly for 30 minutes at room temperature. A funnel and filter paper were then used to filter the acrylamide from the mixed bed resin. DDW was then added to the mixed bed resin and filter to bring the volume of acrylamide up to 450ml. Finally, 50mls of 10x TBE

48

Figure 4.1

"The DNA sequencing method developed by Sanger. Using a primer homologous to one end of the DNA strand to be sequenced, the complementary strand is synthesized using DNA polymerase. Small amounts of dideoxynucleotide (ddATP, ddCTP, ddTTP, ddGTP) are included in four separate such reactions; when a dideoxynucleotide is incorporated into a growing chain, synthesis of that molecule stops. Separation of the resultant labelled fragments in four lanes of a polyacrylamide gel, followed by exposure to X-ray film, allows the sequence to be read off directly" (Gelehrter and Collins, 1990, p. 87).

SINGLE-STRANDED DNA
OF UNKNOWN SEQUENCE

5' C T G A C T T C G A C A A 3'

RADIOACTIVELY LABELED 3' T G T T ● 5'
PRIMER

DNA POLYMERASE I
dATP
dGTP
dCTP
dTTP

(P)(P)(P)—O—CH₂ O BASE

H H
H H
H H

DIDEOXYNUCLEOTIDE (ddNTP)

ddATP   ddCTP   ddTTP   ddGTP

REACTION
MIXTURES

C T G A C T T C G A C A A

ddG O——————●
ddG O——————●
ddG O——————●

PRODUCTS IN ddGTP REACTION
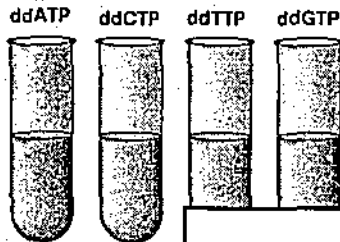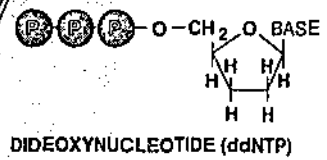
GEL
ELECTROPHORESIS

AUTORADIOGRAPHY
TO DETECT
RADIOACTIVE BANDS

ddATP ddCTP ddTTP ddGTP

LARGER
FRAGMENTS

C
T
G
A
C
T
T
C
G
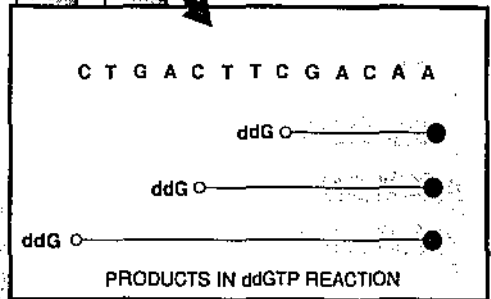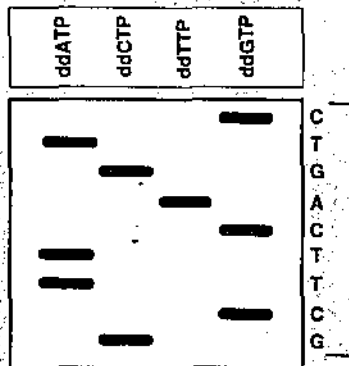
READ SEQUENCE OF ORIGINAL
SINGLE-STRANDED DNA
(COMPLEMENT OF PRIMER-
GENERATED SEQUENCE LADDER)

SMALLER
FRAGMENTS

was added to the acrylamide mix which was then stored at 4°C in a bottle wrapped in aluminium foil.

4.2.1 6% Denaturing acrylamide gel for sequencing

80mls of 6% denaturing acrylamide solution was prepared to pour a Poker Face acrylamide gel for sequencing analysis.

80mls of the 6% denaturing acrylamide stock solution was added to a 100ml beaker. 170ul of TEMED and 170ul of 25% ammonium persulphate (APS), which had both been stored at 4°C, were added to the beaker and mixed gently. The TEMED and the 25% APS catalyse the polymerisation of liquid acrylamide into gel acrylamide within 5 minutes. The acrylamide solution was then drawn up in a 100ml syringe and injected between the two glass plates used in the Poker Face electrophoresis system (Hoefer), making sure not to leave air bubbles between the plates. The comb was then inserted approximately 5 to 10mm into the gel mix. Once the gel had formed, a moist piece of paper was placed over the comb and the top of the gel wrapped in gladwrap to keep in the moisture. This gel was then stored at room temperature for up to one week.

4.2.2 Cycle sequencing (Biotech)

The Biotech Genesis sequencing kit provided a simple and effective means of analysing DNA sequences. The sequencing protocol was carried out according to the Biotech Genesis information pamphlet with some slight modifications. The volume of sterile water was reduced to 14.25ul, 2.25ul of template DNA was used which is approximately 20ng of DNA and only 1.5ul of end labelled primer was used. These changes in the Biotech protocol were made to rectify the master mix

concentrations, which were incorrect in the Biotech protocol pamphlet. All solutions except for the end labelled primer and target DNA wereprovided in the Genesis sequencing kit. Genomic DNA was extracted from lymphocytes (Jacobsen, 1992). The end labelled primer was donated by Dr Steve Wilton. This primer sequence was specified by Abbs et al, (1991) and designated primer sequence:-

(19 F)orward   [5' TTCTACCACATCCCATTTTCTTCCA 3']

Four 0.5ml PCR tubes were labelled A, C, G, T, and then 4ul of each stop mix was added to the appropriate tubes. The tubes were then placed on ice until required. Next, a master mix was made up in a 1.5 ml reaction tube, by adding in order the following components: 14.25ul DDW, 10ul 5x sequencing buffer, 2.25ul of template DNA (20ng), 1.5ul of end labelled primer (30ng/ul) and 2ul of Taq polymerase (2units/ul). The master mix was gently mixed and 6ul transferred to each of the four stop mixes. Then each of the stop reactions was overlaid with one drop of paraffin oil. The four stop reactions were then placed in a thermal cycler and the following program was run :-

$94^{o}C$ for 2 minutes   x1

$94^{o}C$ for 30 seconds   x20
$55^{o}C$ for 30 seconds
$70^{o}C$ for 1 minute

$94^{o}C$ for 30 seconds   x10
$70^{o}C$ for 1 minute

5ul of Formamide loading buffer was then added to each of the stop reactions ("A", "C", "G", "T") through the paraffin overlay and gently mixed by pipetting up and down.

4.2.3   Running sequencing gel

The sequencing products from the Genesis system were fractionated on a 6% denaturing polyacrylamide gel, using the Poker Face electrophoresis system.

The 6% denaturing polyacrylamide gel was pre-run (to warm gel to $45^{o}C$) for 30 minutes in 1x TBE buffer at 1400 volts and the lanes marked before the comb was taken out. The lanes were then flushed out with the 1x TBE, using a syringe, in order to remove urea and bubbles from the wells. Stop mixes made using the Genesis sequencing kit (Biotech) were incubated at $94^{o}C$ for 5 minutes and 2ul of each stop mix ("A", "C", "G", "T") was loaded into consecutive lanes. The gel was then run at 1400 volts for 4 hours.

4.2.3.1 Fixing, drying and exposing polyacrylamide gels.

The polyacrylamide gel was then fixed in a 10% acetic acid, 10% methanol solution, by pouring 1 litre of the fixative over the gel in 15 minutes. Once the gel was fixed it was removed from the glass plate with 3mm blotting paper. The blotting paper was placed on the gel and lifted off taking the gel off the glass plate. Gladwrap was carefully placed over the gel. The gel was then put in a gel dryer (Hoefer) for 3 hours at $80^{o}C$ at which time the gel had dried to the 3mm blotting paper. An X-ray film (Cronex 4 - DU PONT) was then exposed to the gel for 10 to 24 hours at $-80^{o}C$, dependant on how much ^{32}P was in the reaction, with an intensifying screen.

4.3   Results

In Figure 4.2 there is some cross-banding close to the mutation site making some of the sequence hard to distinguish. The "A" to "C" base change is clearly visible and had the gel been allowed to run further, this would have increased separation of the relevant area. Initially though, it was not known exactly where the mutation would lie (if it was present at all), therefore this gel provided the first indication of the point mutation. At the bottom of Figure 4.2 the sequence separation is much greater and easier to read and so in subsequent sequencing of the mutation the gel was run much further. In order to confirm the mutation at the 5' end of intron 19, one base 3' to the universally conserved donor splice site, the complementary strand of genomic DNA was sequenced, by Dr Steve Wilton. This indicated a "T" normal for dystrophin changed to a "G" in the BMD patient. This provided supporting evidence for a mutation in the consensus sequence at the 5' end of Intron 19.

4.4   Discussion

Sequencing of genomic DNA of a subject normal for dystrophin and patient G with BMD indicated an "A" to "C" change shown in Figure 4.1. This indicates a mutation at the 5' end of Intron 19, one base pair 3' to the universally conserved "G", "T" donor splice site, but in the donor consensus sequence, necessary for splicing to occur (see Figure 4.3). Mutations at the donor (5' end of intron) and acceptor (3' end of intron) splice sites of genes have been shown to produce varied effects in the formation of mature mRNA, including the complete exclusion of exons, as seen in patient G with BMD.

Figure 4.2

Sequencing of normal and affected (Patient G) genomic DNA on a 6% denaturing acrylamide gel. The mutation is evident at the base after the universally conserved donor splice site "GT" and results in an "A" to "C" mutation in the affected individual.
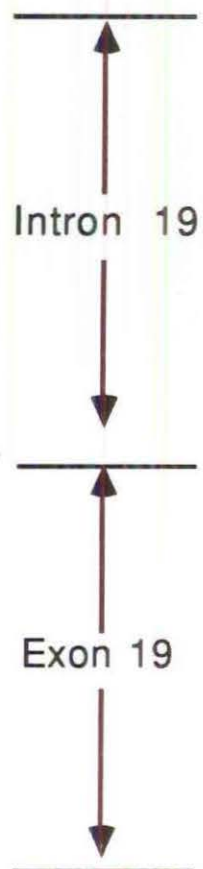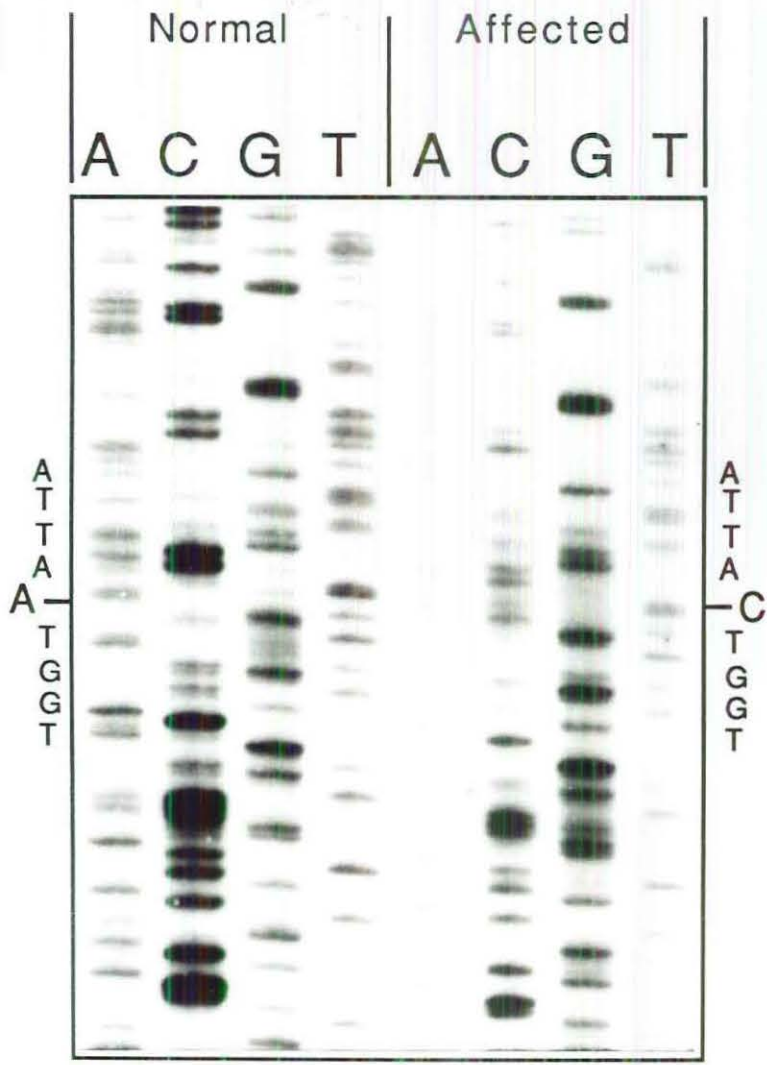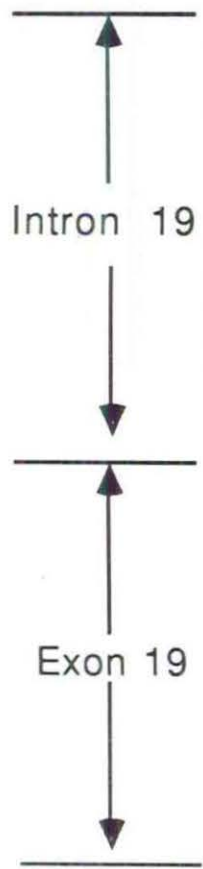
Figure 4.3

Sequencing information derived from the normal and BMD patients. " * " indicates the site of the mutation, while the arrow delineates the exon intron boundary.

Exon 19 | Intron 19

5'   3'

TGGAACAAGATGGTGAATG GTAATTACACGAGTTGATTTAGATA   Normal sequence

                              *

TGGAACAAGATGGTGAATG GTCATTACACGAGTTGATTTAGATA   BMD sequence

AG GTAAGT Consensus splice site
           (semi-conserved)

A G G T A A G T

64 73 100 100 62 68 83 63

Consensus Sequence Frequencies (%)

Rees et al (1985), showed in a haemophilia B patient, that the only significant sequence difference occurred at a donor splice site and consisted of a single base substitution of a "G" to a "T". Cladaras et al (1987), showed that an "A" to "G" substitution in the acceptor splice site of the third intron of a deficient Apo-E gene can cause aberrant splicing of the third intron. This aberrant splicing led to two mature mRNA's containing either a portion or the entire 3rd Intron of the Apo-E gene. Cladaros et al (1987), also mentions a similar "A" to "G" substitution of the second intron of the B globin gene. This mutation, however only generates a single larger size B globin mRNA that contains a portion of the second intron.

Mounir et al (1990), showed that a mutation at the JH6 Donor splice site in the JBL2 gene causes Burkitts Lymphoma. Splicing therefore occurred at an alternate splice site which was 5' to the normal Donor splice site. Carstens et al (1991), identified two point mutations in the Ornithine Transcarbamylase (OTC) gene which is an X-linked recessive gene like DMD and BMD. One patient who had deficiency of OTC had a "T" to "C" substitution at the Donor splice site of intron 7, this substitution caused exon 7 to be skipped (see Figure 4.4). This mutation is similar in effect to the mutation described in our BMD patient, both are mutations of the donor consensus sequence although the OTC mutation is at the donor splice site, which results in the previous exon being skipped. Another patient had an "A" to "T" substitution at the Acceptor splice site, at the end of Intron 4. This led to a cryptic 3' Acceptor splice site being formed in exon 5 resulting in a deletion of 12 bases in the mature mRNA.

Figure 4.4

Ornithine transcarbamylase gene: A single base mutation, at the universally conserved donor splice site of intron 7, results in exon 7 being skipped in the mRNA transcript (Carstens et al, 1991). Note that the universally conserved donor splice site is GU as compared to GT in DNA.

pre-mRNA

Exon 6   Intron 6   Exon 7   Intron 7   Exon 8   Intron 8   Exon 9   Intron 9   Exon 10

5' ━━GU...AG ━━━ GU...AG ━━━ GC...AG ━━ GU...AG ━━ 3'

Mutation

mRNA

Exon 6   Exon 8   Exon 9   Exon 10
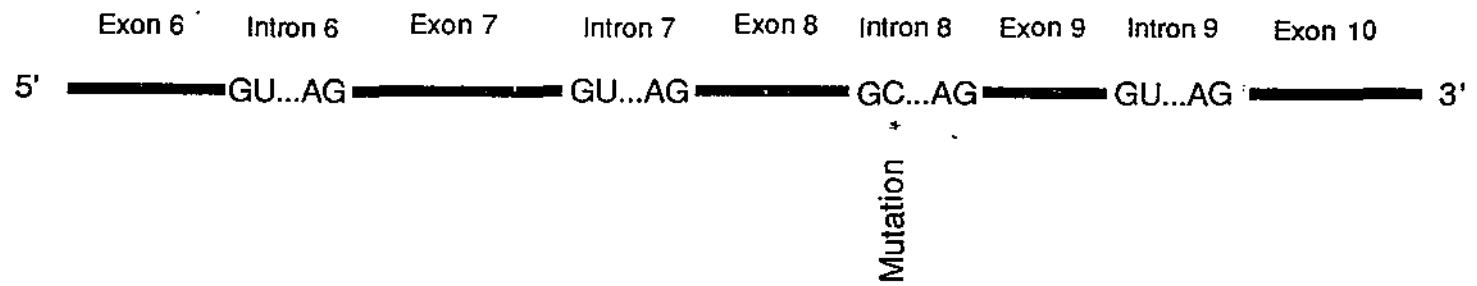
Takahaski et al (1990), working with the p53 tumor suppressor gene showed that point mutation at the acceptor and donor splice sites can both result in the inactivation of p53 tumor suppressor gene. A point mutation at the acceptor splice site "G" to "C" at the 3' end of intron 3 can generate two forms of mature mRNA. The first form uses a cryptic acceptor splice site in Exon 4, therefore producing a mRNA with part of exon 4 incorporated. The second form uses the normal acceptor splice site at the 3' end of Intron 4. This results in mRNA which has exon 3 connected to exon 5 ie. the complete exclusion of exon 4. The other mutation noted by Takahashi et al (1990),was a point mutation at a donor splice site of intron 7, a "G" to a "T". This resulted in the entire intron 7 sequence remaining in the mature mRNA.

The above examples show that the outcomes of splice site mutations are varied and result in different mature mRNA transcripts being produced. It must also be noted that in these cases a mutation of the donor or acceptor splice sites is significant enough to cause structural changes in the mature mRNA, which in turn results in phenotypical changes in protein synthesis. The nested PCR of patient "G" indicated a deletion of under 100 bp, exon 19 is 88 base pairs long, providing a candidate area to sequence. This splice site mutation resulted in alternate splicing of exon 19, with two mRNA transcripts being produced, one with exon 19 the other without.

The identification of a point mutation in the affected individual by sequencing, although accurate, is inefficient when analysing all members of the family for the mutation. Demonstration of fragment length polymorphism of cDNA, which identified the point mutation initially, is an extremely difficult procedure and inefficient when analysing all members of the family for the mutation. The requirement

of a muscle biopsy makes this method highly invasive and impractical when doing chorionic villi sampling or amniocentisis in order to provide the family with a pre-natal diagnostic test. Extracting mRNA from lymphocytes would make this a much more viable method of characterising the mutation in this family (Roberts et al,1990).

In order for a diagnostic test to be minimally invasive and provide this family with an accurate pre-natal diagnosis, the test would have to use genomic DNA extracted from whole blood. This diagnostic test would also have to distinguish a single base substitution in genomic DNA of affected males and carrier females. One method considered was Southern blot analysis. If the point mutation introduced or deleted an enzyme cutting site, this would lead to a simple and effective Southern analysis test. Similarly if the mutation introduced or deleted an enzyme cutting site, then exon 19 with part of intron 19 could be amplified (PCR) and a restriction digest would identify the affected allele. Although the site is palindromic no known restriction enzyme cuts at that particular site, so this approach was not an option.

Another method using restriction enzymes resulted involves the introduction of a restriction site by polymerase chain reaction. In this method a mis-match is introduced close to the mutation by a mis-matched primer which anneals to the target DNA. The primer which introduces a mutation into the amplified product, is designed to introduce a enzyme cutting site, when matched with the mutant allele. The normal allele would not have the correct base sequence for the enzyme to cut. This test amplifies and gives a signal for both the mutant and the affected allele, but only the mutant allele will be digested using the appropriate restriction enzyme. This method has

the advantage of providing a signal but of different sizes depending on the status of the individual (Schwartz et al, 1991). This would be a viable method of characterising this point mutation, but due to time constraints it was not tested.

It was decided to use SSCP and the ARMS techniques to characterise this "A" to "C" point mutation. Although SSCP analysis is generally used in the identification of point mutations, in this case by having a point mutation it was possible to optimise the conditions for detecting, this point mutation and in the process become familiar with the SSCP technique. The SSCP analysis could be then used to find more point or minor mutations, in Western Australian dystrophic families (Hayashi, 1991). The ARMS method of characterising this point mutation, involved designing primers specific for the mutant and normal alleles and then optimising the PCR conditions. These primers, normal/affected, would only vary at the end base "A" normal and "C" in the affected primer (Billadeau et al, 1991).

Chapter 5:   SINGLE STRANDED CONFORMATION POLYMORPHISM

5.1   Introduction

This is a method by which the migration of the single stranded DNA does not only depend on the number of nucleotides, instead the shape of the single stranded DNA determines its rate of migration through a non-denaturing polyacrylamide gel. In normal double stranded DNA electrophoresis, even on a polyacrylamide gel, it is only possible to notice small deletions or insertions of DNA. It is not possible to detect substitutions which do not affect the size of DNA and therefore do not affect its migration during electrophoresis (Hayashi, 1991). Single stranded DNA under non-denaturing conditions has a stable shape-conformation, which tends to vary depending on the sequence of the DNA and the intrastrand reactions. This in turn will cause changes in electrophoretic migration depending on the shape of DNA (see Figure 5.1) (Hayashi, 1991; Baldwin et al, 1992).

SSCP analysis was carried out on genomic DNA from normal, carrier and affected individuals in the affected family. Although this method is conventionally used in the detection of point or minor mutations, in this case we had already identified a point mutation and SSCP analysis was done in order to characterise the mutation and provide an easy screening method for the family. As the mutation consisted of a single base substitution, fractionation on a polyacrylamide gel with its high resolution was determined to be the best method (Orita et al, 1989b). Silver staining of agarose gels provided a simpler method, but would not have provided the resolution of the polyacrylamide gel (Bassam et al, 1991). Orita et al (1989a),

Figure 5.1

SSCP: Migration of double stranded (ds) DNA compared to single stranded (ss) DNA during electrophoresis. The ds DNA forms a stable conformation, double helix, and migrates uniformly dependant on the number of nucleotides. ssDNA has different conformational forms dependant on the sequence of the strand and the running conditions. The complementary strands of DNA when run under appropriate SSCP conditions should result in two distinct bands.

ss DNA

ds DNA

Normal　Carrier　Affected

- ve

+ ve
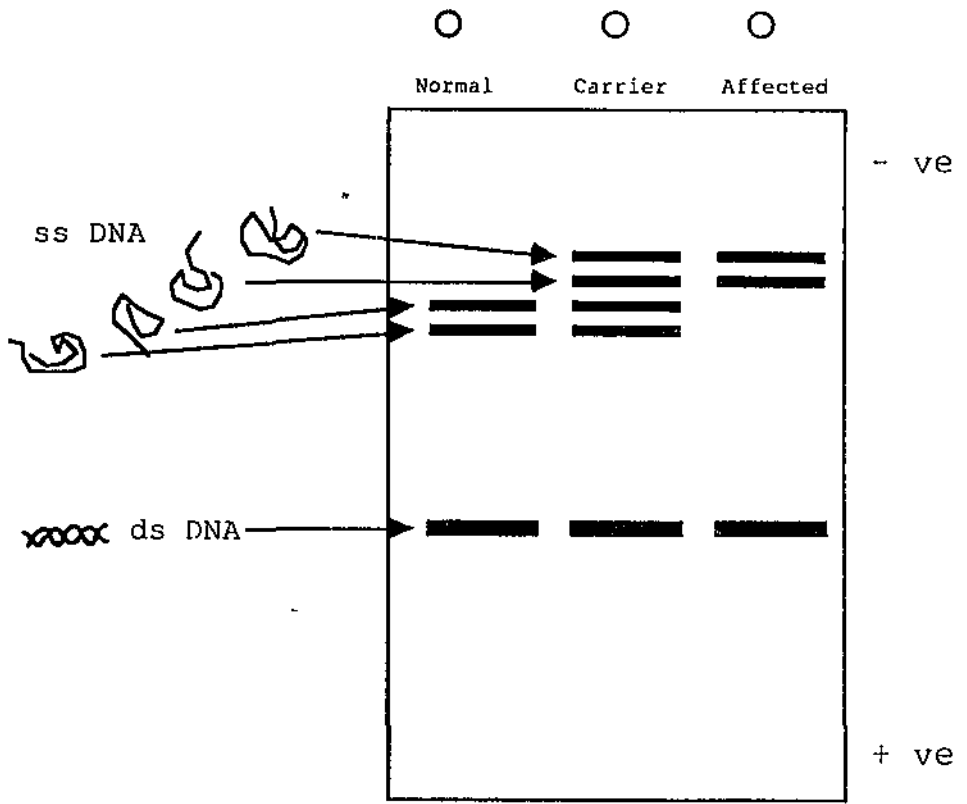
6% Acrylamide Gel

noted that most point mutations could be detected when analysing products less than 500 bases long. As the amplified product was just under 500 bases long, approximately 460 bases, it was decided to digest the amplified product with restriction enzymes (frequent cutters) in order to reduce the fragment size being analysed and therefore increasing the chance of detecting a polymorphism .

5.2   Materials and Methods

5.2.1   Acrylamide stock for SSCP analysis

Volumes of 100ml of 40% Acrylamide gel stock were made up for use in SSCP analysis. 100mls of 40% acrylamide contains 39g of Acrylamide, 1g of Bis-Acrylamide and double distilled water to bring the volume of the solution to 100ml. This mixture was stirred slowly, with a magnetic stirrer, until dissolved. 10g of mixed bed resin was then added to the beaker containing the acrylamide solution and stirred slowly for 30 minutes at room temperature. The mixed bed resin was then filtered from the acrylamide solution, with funnel and filter paper. DDW was then added to the mixed bed resin, located in the filter, to bring the volume of acrylamide stock up to 100mls. The acrylamide stock solution was stored at $4^{o}C$ in a bottle wrapped in aluminium foil. Since acrylamide is a neurotoxin appropriate safety precautions were taken whenever it was handled. This included wearing two sets of gloves, a mask and using the fume hood where possible.

5.2.2   6% Acrylamide gel for SSCP analysis

SSCP analysis employs a 6% acrylamide solution and 80mls of the solution are required to pour Poker Face gel. 80mls of 6% acrylamide

68

solution contains; 12ml of 40% acrylamide stock, 8ml of 10x TBE and 60mls of DDW. Then 170ul of TEMED and 170ul of 25% Ammonium persulphate, which had been stored at 4°C, were also added to the solution and mixed quickly. The TEMED and 25% Ammonium persulphate catalysed the polymerisation of acrylamide, turning the acrylamide into a gel within 5 minutes. The acrylamide solution was drawn up in a 100ml syringe and injected between the two glass plates used in the Poker Face system, making sure not to leave air bubbles between the plates. A comb, which forms the loading wells, was then inserted approximately 5 to 10mm into the gel mix. After the catalysts had been added, the acrylamide solution was poured quickly and the comb inserted between the plates, prior to the gel setting. Once the gel had solidified a moist piece of paper was placed over the comb and the top end of the gel wrapped in gladwrap to keep in the moisture. The gel could then be stored for up to a week at room temperature, prior to being used.

5.2.3   Genomic amplification of exon 19

Primers used in the amplification of exon 19 from genomic DNA were:-

(19F)orward   5' TTCTACCACATCCCATTTTCTTCCA   3'

(19R)everse   5' GATGGCAAAAGTGTTGAGAAAAAGTC   3'

These primers were specified by Abbs et al (1991).

Each PCR reaction consisted of 5ul of 5x buffer, 75 ng/ul primer mix, 0.5 units of Taq Polymerase, 50ng of template genomic DNA, 0.15ul of dCTP-32 (50uCi Amersham) and the reaction was brought to volume with DDW and overlaid with one drop of paraffin. Thermal cycling of samples involved 35 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes (the initial denaturation step at 94°C was

carried out for 5 minutes). An aliquot of 4ul of the samples was taken and mixed with 1ul 5x ficoll loading buffer. This was run on a 3% agarose gel at 100 volts for 45 minutes. The gel was then stained with ethidium bromide and fluoresced under a UV light. This indicated whether the samples had amplified. If the amplification worked, then the samples were digested with restriction enzymes.

5.2.4 Digestion of amplified products

After amplification of the genomic DNA it was digested with three known frequent cutters (Hae III, Hha I and Hinf I). The only variation in digestion conditions for the three enzymes was that Hinf I required buffer "B", while buffer "C" was used for Hae III and Hha I (Promega).

The digested reactions consisted of 5ul of 10x buffer "C" or "B" (depending on the enzyme used), 1ul of 0.1M spermidine, 1ul of enzyme (Hae III, 11 units/ul or Hha I, 12 units/ul or Hinf I, 10 units/ul), 0.5ul of 10mg/ml BSA, 5ul amplified DNA and then brought to volume with DDW (Promega). The digest reaction was then incubated for 8 hours at 37^0C, ethanol precipitated, and resuspended in 10ul of DDW and 10ul of formamide loading buffer.

5.2.5 Ethanol precipitation

To the DNA sample was added, 3M NaAc (1/10th the volume of the DNA sample) and then 100% cold ethanol (3 times the volume of the DNA sample). The sample was then vortexed lightly and left at -80^0C for 2 hours to precipitate. After the DNA had precipitated, the sample was then spun down at 13650g for 15 minutes and the supernatant poured off. The DNA pellet was then washed by adding 70% cold

ethanol (3 times the total volume of original DNA sample) and then the pellet was spun down for 5 minutes at 15000rpm. The 70% ethanol was then poured off the DNA pellet, which was then vacuum dried and resuspend in DDW.

5.2.6   Running 6% polyacrylamide gel for SSCP analysis

5ul of the digested DNA was aliquoted into a fresh tube to be run under non-denaturing conditions. The rest of the digested DNA was run under denaturing conditions for SSCP analysis. This involved heating the sample to 80°C which denatured the DNA. After 10 minutes 2ul of the hot, denatured DNA was loaded onto a 6% non denaturing polyacrylamide gel (Poker Face) and run in 1x TBE buffer. 2ul of the non-denatured DNA was also run on the same gel. The gel was then run for 8 hours at 400 volts. Prior to loading the samples, the polyacrylamide gel was pre-run for 30 minutes at 400 volts. The final step involved fixing, drying and exposing the polyacrylamide gel as described in 3.6.2.

5.3   Results

Single strand conformation polymorphism analysis of exon 19 using genomic DNA amplified with primers 19F and 19R, indicated a polymorphism, therefore providing a means of characterising the point mutation found in this BMD family. In Figure 5.2 the uncut DNA showed no polymorphism in samples 1 to 4 both in the normally electrophoresed and those samples in which SSCP analysis was performed. It is important to note that single stranded DNA (ssDNA) migrates much slower than double stranded DNA (dsDNA), during electrophoresis. Therefore for all digests in Figure 5.2 sample 1 has

71

Figure 5.2

SSCP analysis: Autorad showing amplified exon 19 samples for "1" normal female, "2" carrier female, "3" BMD male, "4" normal male. These samples were run uncut and also cut with the restriction enzymes Hae III, Hha I, Hinf I. pUC 19 marker digested with Msp I. Sample 1 was loaded twice for each digest first as double stranded DNA and then as single stranded DNA.
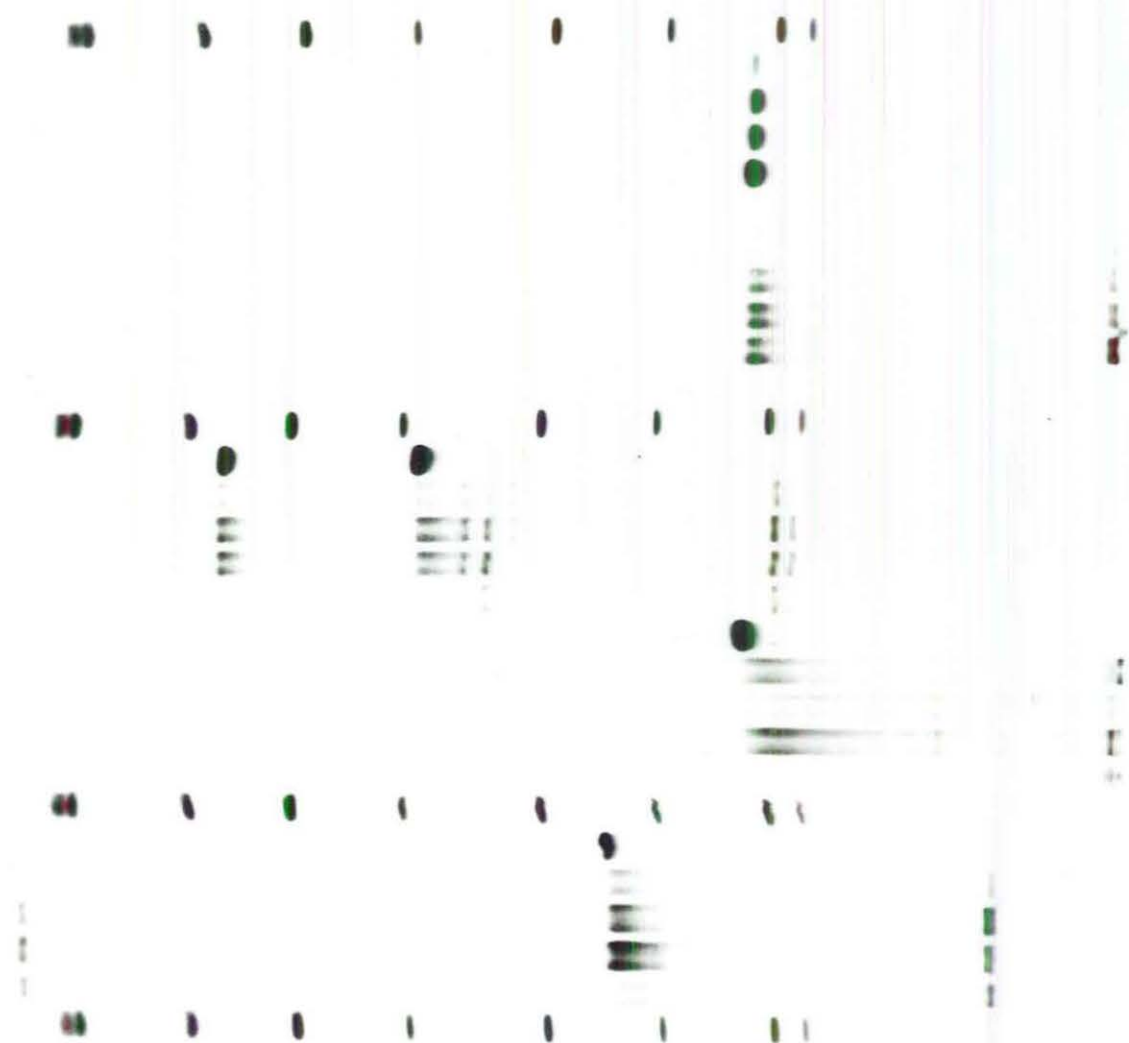
pUC Marker		
1		
2		
3		UNCUT
4		
-ve Control		
1		
2		
3	SSCP	
4		
-ve Control		
pUC Marker		
1		
1		
2	SSCP	Hae III
3		
4		
1		
1		
2	SSCP	Hha I
3		
4		
pUC Marker		
1		
1		
2	SSCP	Hinf I
3		
4		
pUC Marker		

been loaded twice. The first time as dsDNA and the second time under SSCP conditions. This gives an indication of how the double stranded DNA has migrated in relation to the single stranded (SSCP) DNA.

The Hae III digested DNA showed no polymorphism with SSCP analysis (see Figure 5.2). The DNA digested with Hha I showed a polymorphism with SSCP analysis; samples 2 and 3 have only two bands corresponding to carrier female and affected male, samples 1 and 4 have three bands corresponding to normal female and normal male (see Figure 5.2). In Figure 5.2 Hha I has either cut the amplified exon 19 at the very end of the amplified product ie. in the primer sequence or the enzyme has not cut at all. As there is no Hha I restriction site in the primers (19F and 19R), this indicates that Hha I has not cut exon 19, as any cutting of exon 19 inside the primers would result in a different sized product compared to the uncut samples (see Figure 5.2). The only variation between the uncut SSCP and the Hha I samples was that the Hha I samples were ethanol precipitated where as the uncut samples were loaded straight from the amplification reaction. The uncut SSCP samples have salts and $MgCl_2$ from the amplification buffer, these would not be present in the Hha I SSCP samples, this could account for the variation with the Hha I SSCP samples as seen in Figure 5.2. The Hinf I digest of the four amplification products resulted in at least two products. The smaller SSCP product can just be seen on Figure 5.2 at the bottom of the autorad. The second larger product shows a polymorphism using SSCP analysis. Samples 2 and 3 show two distinct bands indicating carrier female "2" and affected male "3" while normal female "1" and normal male "4" have only one band (see Figure 5.2).

5.4   Discussion

Orita et al (1989b), indicated that a number of factors can influence the migration of single stranded DNA. For example, the sequence and the size of the DNA fragments (determined by restriction enzyme), the temperature, the pH and gel concentration are all important in determining rate of migration through a non-denaturing polyacrylamide gel. By varying the conditions of SSCP analysis it is possible detect most base changes in DNA under 200bp. Optimisation of the DNA samples 1 to 4 in order to show a polymorphism included varying the current, the temperature and the running time of gels (data not shown). Once conditions had been optimised it was possible to detect a single base change in an amplified product which was just over 460 bases (see Figure 5.2). This could be due to a single strand of DNA having more than one stable conformation under a given set of conditions. This would result in 2 bands from single stranded DNA with the same sequence as described by Orita et al, (1989b).

Once the conditions for SSCP analysis were optimised it was possible to determine which individuals had the single base substitution. One problem was that there was no distinction between carrier female and affected male, therefore a sex determination test must also be done in order to provide the family with adequate information to make an informed decision based on the prenatal tests. This may be a characteristic of this specific test as Orita et al (1989a), demonstrated different conditions change the characteristics of migration. It may therefore be possible to improve on this test, by changing the running conditions, resulting in a test which is able to distinguish between affected males and carrier females.

This test provided a relatively simple method of analysing members of the family for the point mutation, although time consuming and requiring the use of ^{32}P, it must be considered an option for prenatal diagnostic testing.

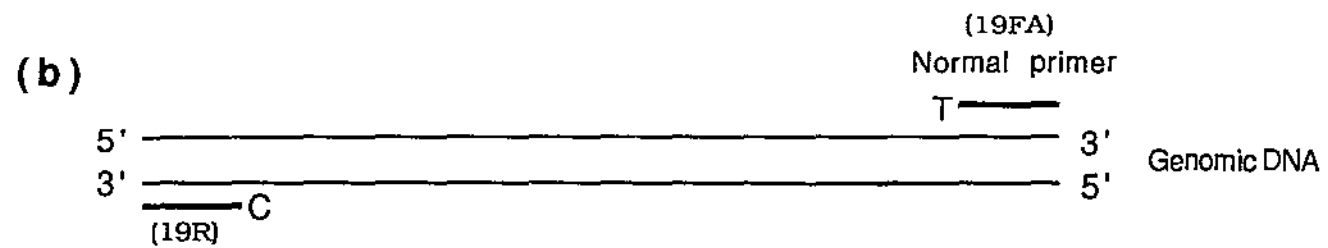# Chapter 6: AMPLIFICATION REFRACTORY MUTATION SYSTEM

6.1 Introduction

Amplification refractory mutation system (ARMS), or allele specific amplification, is a method which makes it possible to amplify DNA specific to the mutation or specific for the normal allele. This method uses genomic DNA in polymerase chain reaction (PCR).

Normal and mutant alleles are identified by using two allele specific oligonucleotide primers. The normal and the affected primer mixes have a common reverse primer while the forward primer varies between the normal ("T" at the 3' end) and affected primer mixes ("G" at the 3' end). Fragment amplification occurs only when the allele specific primer matches the nucleotide sequence of the template DNA. The amplified product is detected by agarose gel electrophoresis, followed by staining with ethidium bromide (Wenham et al 1991; Billadeau et al 1991). This method of characterising the point mutation requires sequence information which was derived from the sequencing of exon 19, in order to design forward primers (19FA and 19FC) which are complementary to the 5' end of intron 19. The primers for the normal and affected allele vary only at the 3' end base, which varies from an "T" for the normal primer to a "G" for the affected primer (see Figure 6.1). Once the normal and affected primer mixes were optimised, they would only anneal and allow amplification when their respective alleles were present in genomic DNA. Therefore a carrier female would have the normal and affected primer mixes giving a signal. Affected males would only get a signal from the affected primer mix, while normal males and females would only get a signal from the

77

Figure 6.1

ARMS analysis: a) The BMD primers shown here will only amplify up the genomic DNA if the mutation which causes BMD in this family is present. b) The normal primers will only amplify, genomic DNA, when the normal allele is present. Reactions (a & b) are specific for their respective alleles and only result in amplification when that particular allele is present.

(a)

(19FC)
BMD primer

G ━━━━━

5' ———————————————————————————————— 3'

3' ———————————————————————————————— 5' Genomic DNA

━━━ C

(19R)

(b)

(19FA)
Normal primer

T ━━━━━

5' ———————————————————————————————— 3'

3' ———————————————————————————————— 5' Genomic DNA

━━━ C

(19R)
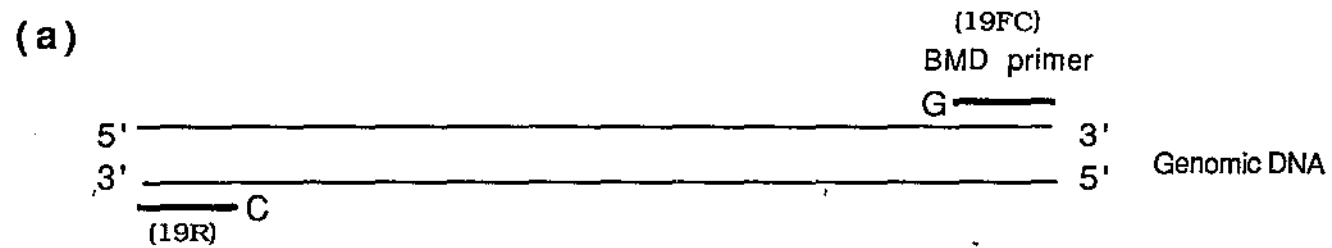
normal primer mix. The size of the signal for the normal and affected primer mixes does not vary as the primers amplify the same region of DNA but are specific for the 3' base change. One of the problems that could be encountered with this method of characterisation is that of getting false negatives. How do you know if the negative result is due to the primer not annealing to the DNA or whether the whole reaction has not worked? Wenham et al, (1991) suggested having a second set of primers in the reaction (internal control) with a different sized product. In this way it is possible to tell if the reaction has worked, reducing the chance of false negatives. The ARMS method of characterising a single base mutation was described by Wenham et al (1991) as "simple, reliable and robust and avoiding the use of radiation or hybridisation with allele specific oligonucleotide probes". This makes for an ideal diagnostic test!

No single protocol is appropriate for all situations, it is therefore necessary to optimise each reaction in order to maximise the yield of the desired product, as opposed to non-specific background bands, primer dimers and mutations or heterogenicity (Innis et al, 1990).

The magnesium concentration can affect a number of factors within a PCR reaction including primer annealing, product specificity, formation of primer dimers and enzymatic activity. Taq polymerase requires free magnesium on top of that bound by template DNA, primers and dNTP's. Therefore PCR reactions should contain between 0.5 and 2.5 mM $MgCl_2$. Decreasing the magnesium concentration leads to an increase in specificity (Innes et al, 1990).

The temperature and length of time required for primer annealing depends upon the base composition as well as the length and concentration of the amplification primers. Annealing temperatures between 55°C and 72°C generally yield the best results, with increased annealing temperature leading to greater specificity of amplification (Innis et al, 1990).

The number of cycles depends on the concentration of target DNA. Too many cycles can lead to non-specific background products, while too few cycles will give a low product yield. As a rule, however, to go over 40 cycles without getting a signal from a reaction indicates that something is wrong (Innis et al, 1990).

Primer concentrations 0.1 to 0.5uM provide optimal conditions for PCR reactions. Primer concentrations above 0.5uM can lead to non-specific products being produced and also primer dimers annealing to each other. Primer concentrations below 0.1uM may lead to insufficient amplified product being produced (Innis et al, 1990) .

Efficient primers should be 18 to 28 nucleotides long with a 50 to 60% "G" and "C" composition. The 3' end of primer pairs should not be complementary, this helps to reduce the formation of primer dimer artifacts. It is also important to avoid long stretches of "C" and "G" at the 3' end of primers as this may promote mispriming at "G" and "C" rich sequences and palindromic sequences within primers (a sequence that reads the same in both directions). In some cases a primer may not work, due to the secondary structures within the template DNA. In such cases it may be necessary to design another set of primers for that region (Innis et al, 1990).

6.2   Materials and Methods

Primers specific to the mutation in exon 19 and primers for the normal allele were produced from the sequence data, obtained in chapter 4 (see Table 6.1). It was then necessary to optimise the conditions for each set of primers.

6.2.1   Optimised normal primer

PCR reaction consisted of 5ul of 5x buffer (no $MgCl_2$ in this buffer), 2ul of primer mix 80ng/ul, 0.5 units of Taq Polymerase, 1.7mM of $MgCl_2$, 50ng of genomic DNA and then brought to the volume with DDW and overlaid with one drop of paraffin. Thermal cycling of samples involved 33 cycles of $94^{o}C$ for 3 seconds, $56^{o}C$ for 1 minute and $72^{o}C$ for 2 minutes (the initial denaturation step at $94^{o}C$ was carried out for 5 minutes).

6.2.2   Optimised affected primer

PCR reaction was carried out as per normal primer, except the normal 5x buffer was used giving the reaction a $MgCl_2$ concentration of 2mM and the annealing temperature was changed from $56^{o}C$ to $58^{o}C$.

6.2.3   Running ARMS products

The products produced by the ARMS amplification were run on a 3% agarose gel at 75 volts for 90 minutes, the gel was then stained with ethidium bromide and observed under UV light. The product size will not vary from "normal" to the "affected" allele. Instead, a signal will only occur when the affected and normal primers anneal to their exact

Table 6.1

Primers used in ARMS analysis

Sequence (5' to 3')

<u>PRIMERS USED TO AMPLIFY NORMAL ALLELE</u>

<u>PRIMER "A"</u>

19R   GATGGCAAAAGTGTTGAGAAAAAGTC
19FA   TATCTAAATCAACTCGTGTAATT

<u>PRIMERS USED TO AMPLIFY AFFECTED ALLELE</u>

<u>PRIMER "C"</u>

19R   GATGGCAAAAGTGTTGAGAAAAAGTC
19FC   TATCTAAATCAACTCGTGTAATG

Primer 19R was specified by Abbs et al (1991).

complementary sequence on the template DNA. This means that the identification of the disease and normal allele is done simply by the presence or absence of a signal, when doing an amplification, dependent on which set of primers is being used.

6.3   Results

Optimisation of the ARMS primer was achieved by varying a number of the PCR conditions. This included varying the number of cycles, the annealing temperature (data not shown) and the $MgCl_2$ concentration (see Figure 6.2). In Figure 6.2 the annealing temperature and the number of cycles (PCR) remained constant for both the "normal A" primer and the "affected C" primer mixes, only the $MgCl_2$ concentration was varied. Figure 6.2 shows that the "affected C" primer worked well at both 2mM $MgCl_2$ and 1mM $MgCl_2$ ie. only the BMD male and carrier female have the affected allele, as expected. The "normal A" primer in figure 6.2 indicated at a 2mM $MgCl_2$ concentration that the BMD male had a normal allele when using "normal A" primer. The BMD male should not have the normal allele. Reducing the $MgCl_2$ concentration to 1mM $MgCl_2$ when using the "normal A" primer resulted in no signal in any of the four samples (see Figure 6.2). The expected result using "normal A" primer is that only Normal Male/Female and Carrier Female would have this allele. In order to optimise the "normal A" primer the $MgCl_2$ was then varied between 1 and 2mM, along with the PCR conditions, until the expected result was obtained as in Figure 6.4.

Polymerase chain reaction provides a very effective way of analysing a sequence of DNA. The amplification of a sequence of DNA many millions of times can lead to contamination of subsequent PCR reactions therefore making it necessary to have a negative control in

84

Figure 6.2

Optimising ARMS reaction: Varying the $MgCl_2$ concentration from 1 to 2mM, while maintaining all other PCR conditions. Primer "A" should only amplify the normal allele, while primer "C" should only amplify the affected allele. Lambda marker digested with Pst I.

Primer "A"

Primer "C"
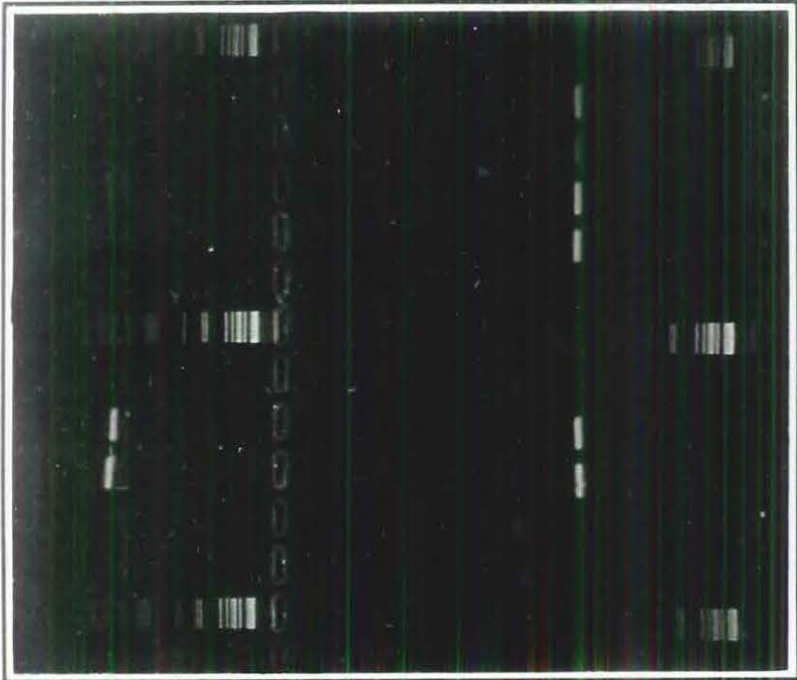
Lambda Marker
Normal Male
BMD Male
Carrier Female
Normal Female
-ve Control
Lambda Marker
Normal Male
BMD Male
Carrier Female
Normal Female
-ve Control
Lambda Marker

1mM MgCl$_2$

2mM MgCl$_2$

all PCR reactions and special lab procedures to minimise contamination. In Figure 6.3, while attempting to optimise the conditions for both the "affected C" primer and the "normal A" primer, the reaction was contaminated resulting in a signal in the "normal A" primer negative control. It was then necessary to do this amplification again, making sure not to contaminate the reaction (data not shown).

Once both "normal A" and "affected C" primers were optimised (see Figure 6.4) it was possible to distinguish a BMD male who only had the affected allele and a carrier female who had both the affected allele and the normal allele. Normal males/females only had the normal allele.

Once the conditions had been optimised, all the family members of patient "G", for which genomic DNA was available were tested in a double blind test. This test involved getting genomic DNA from as many members of patient "G"s family as possible. These samples were only numbered and by using the ARMS test it was possible to determine whether the samples came from normal individuals, carrier females or affected males (data noy shown).

6.4　Discussion

The ARMS method of characterising this "A" to "C" point mutation in the family of patient "G" proved to be very simple and effective once the conditions had been optimised. The nature of PCR reactions make them susceptible to contamination. It is therefore important to both follow protocols which minimise the likelihood of contamination (Erlich, 1989) and also to always have a negative control. The double

Figure 6.3

Contamination of ARMS reaction: The -ve control Primer "A" has given a signal. pUC marker digested with Msp I.

Primer "C"

Primer "A"
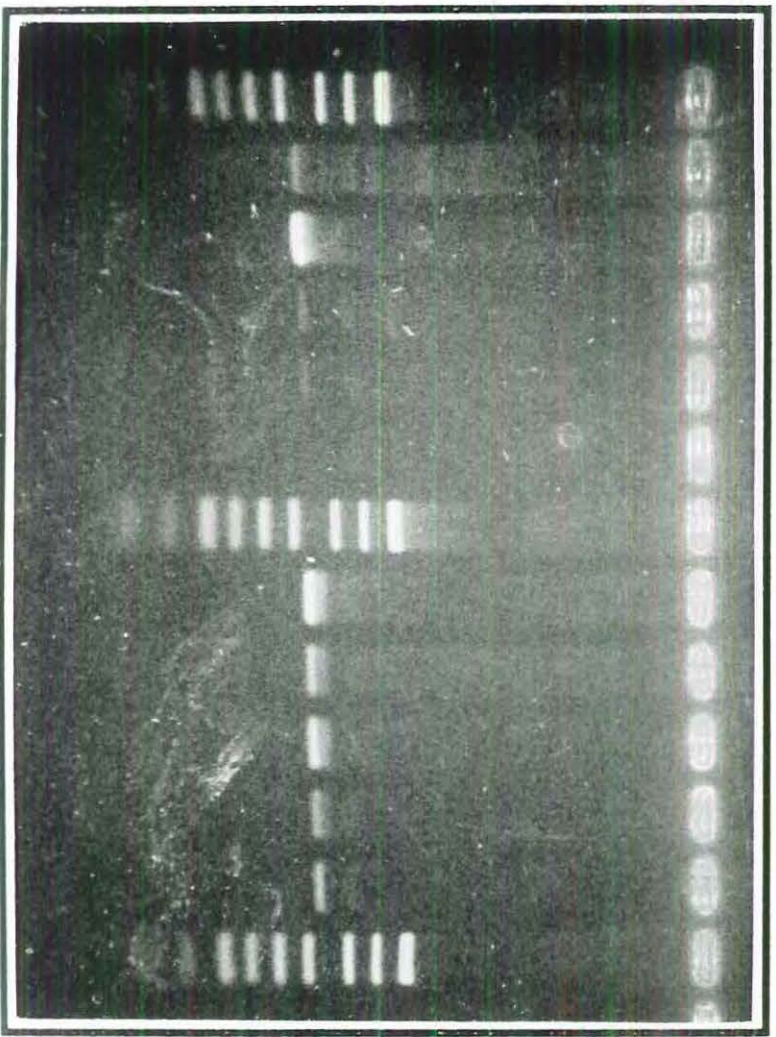
pUC Marker
BMD Male
Carrier Female
Normal Male
Normal Female
-ve Control
pUC Marker
BMD Male
Carrier Female
Normal Male
Normal Female
-ve Control
pUC Marker

Figure, 6.4

ARMS reaction optimised for BMD family: Primer "A" amplifies
only when normal allele is present, while primer "C" only
amplifies when the affected allele is present. pUC. marker
digested with Msp I.

Primer "A"

Primer "C"
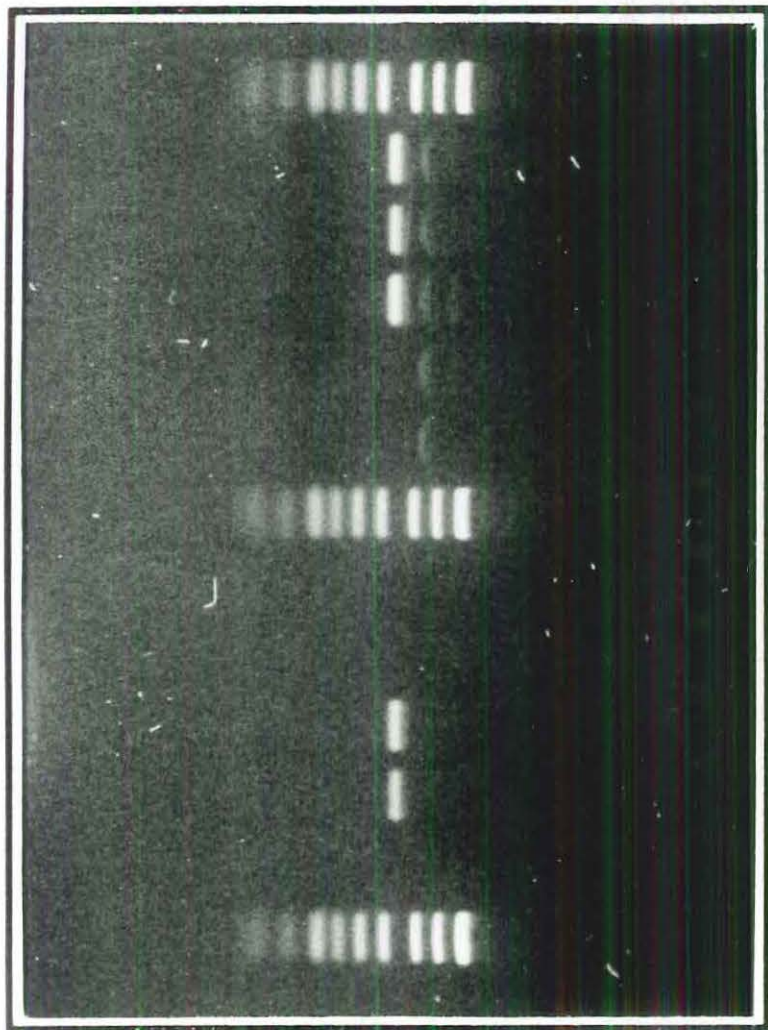
pUC Marker
Normal Male
Normal Female
Carrier Female
BMD Male
-ve Control
pUC Marker
Normal Male
Normal Female
Carrier Female
BMD Male
-ve Control
pUC Marker

blind ARMS test that was carried out supported previous linkage analysis done on all family members at this laboratory. These allele specific oligonucleotide tests therefore provided an extremely easy and specific method for detecting this mutation.

Chapter 7:   GENERAL DISCUSSION

Point or minor mutations in the dystrophin gene account for 30% of DMD and BMD patients (Laing, 1993) In Western Australia this amounts to twenty families. These families are offered prenatal diagnostic testing based on linkage analysis. In linkage analysis the probability of the disease causing allele travelling with a polymorphic marker is determined. The closer the marker to the mutation, the more likely it is to travel with the mutation. It is then possible with an informative marker and an extended family, to determine the probability of the marker travelling with the mutant allele. Linkage analysis provides a probability based test which results in a LOD score, where the LOD score is the logarithm to the base 10 of the odds of linkage between a marker and the disease gene (Gelehrter and Collins, 1990, p. 206). A LOD score of +3 means odds of 1000:1 in favour of linkage, whereas a LOD score of -2 means odds of 100:1 against linkage. In practice LOD scores are calculated for different distances/number of nucleotides (θ) between the marker and the disease gene, from 0 to 50 centiMorgans. At 50 centiMorgans genes are considered unlinked.

$$\text{Likelihood ratio } (\theta) = \frac{\text{likelihood (data}\backslash\theta)}{\text{likelihood (data}\backslash\text{no linkage)}}$$

(Gelehrter and Collins, 1990, p. 205)

The higher/more positive the LOD score the more likely the marker and the disease gene are linked. Of course if a high LOD score occurs at a low (θ) value the genes are closely linked (Gelehrter and Collins, 1990, p. 206). It has been estimated that in 33% of dystrophic patients, the mutation first occurs in the affected male. In another 33% of

dystrophic males the mutation first occurred in their mother and in the final 33% of dystrophic males the mutation first occurred with their grandmother (Laing, 1993). Linkage analysis therefore can not always determine the status of the family members who have a relative suffering DMD or BMD as the mutation may be *de-novo* or the pedigree not large enough to provide accurate linkage analysis for that family (Laing, 1993). It is important in these families to locate the actual mutation to provide them with an accurate prenatal diagnostic test. This test could also eventually result in the eradication of the mutant allele in future generations, in these families.

The procedure for locating and characterising point mutations takes advantage of a number of molecular techniques. PCR provides the basis for detection and analysis of these point mutations. This technique, although very powerful, is subject to contamination by previously amplified products (see Figure 6.3). It is very important to use procedures that limit cross contamination of PCR products and the most effective way of accomplishing this is to use a PCR dedicated micropipette to load all amplified products. The use of sterile technique and a PCR dedicated micropipette resulted in only one instance of contamination.

A point mutation was identified in patient 'G'. This mutation was in the consensus sequence at the 5' end of intron 19. The base after the universally conserved donor splice site had changed from "A" to "C". This point mutation was subsequently characterised by SSCP and ARMS analysis, providing this family with accurate diagnostic tests.

Patient 'G' has a splice site mutation which results in exon 19 being spliced out. This is a frameshift mutation and would generally indicate

94

DMD phenotype (Monaco et al, 1988). However patient 'G' has BMD which could be explained by alternate splicing resulting in some full length mRNA transcript which maintains the reading frame. This alternate splicing is demonstrated by the two PCR products for patient 'G', exons 17-25 in figure 3.4, one the normal size and the other smaller as a result of exon 19 being deleted. Chelly et al (1990) describes two cases in which BMD patients, had apparent out of frame gene deletions, which according to the frameshift hypothesis of Monaco et al (1988), should result in DMD. It was noted, though, that in these patients alternate splicing had occurred and this restored the reading frame. Roberts et al (1991) also describes a patients with a frameshift mutation caused by a deletion of exons 42 and 43 of the dystrophin gene. Subsequent analysis showed alternate splicing which resulted in two mRNA transcripts. The minor product results in exon 44 also being excluded which resulted in the restoration of the reading frame. This could explain the mild phenotype that was observed in this patient.

The point mutation found in patient 'G' and the subsequent characterisation of this point mutation by sequencing, SSCP and allele specific amplification will provide this family with a diagnostic test. Each of these methods have their own limitations. For example sequencing gels can be hard to read (cross-banding) and SSCP and allele specific amplifications can be hard to optimise. A diagnostic test would be of considerable importance should a carrier female ever decide to have a child. The allele specific amplification test devised for this family was the easiest test to perform and interpret, once the conditions had been optimised. Yet SSCP analysis may be used as a confirmatory test in prenatal diagnosis. Prenatal diagnostic tests on

family members of patient 'G' previously performed by this laboratory consisted of linkage analysis. The limitations of this procedure have already been noted. The allele specific amplification test detects the actual point mutation reducing the chance of mis-diagnosis.

The identification and characterisation of this one point mutation in patient 'G' is of no direct consequence to the other families throughout Western Australia who also have a small disease causing mutation in the dystrophin gene. The successful identification of this point mutation could hold some hope for them that, in the not to distant future, a similar feat may be achieved for them. However, in the meantime these families still rely on linkage analysis for prenatal diagnosis, which in many cases is uninformative and until such time that their specific mutations are characterised, the accuracy of their diagnoses can not be assured.

The mutation in patient 'G' resulted in a complete exon being skipped most of the time. This mutation only becomes obvious when mRNA was converted to cDNA and then the cDNA amplified. The major problem with this technique was the mRNA for dystrophin is mainly present in muscle, a tissue that was/is often hard to obtain from patients with BMD or DMD (Chelly et al, 1991). When it is obtained it is often fibrous and the muscle cells have degenerated (Kaido et al, 1991). Chelly et al (1991) noted that illegitimate transcription occurs in all cell types and that the illegitimate transcripts are a bona fide version of tissue specific mRNA. Chelly et al (1991) also showed it was possible to extract from non-specific cell types (lymphocytes), illegitimate mRNA which is tissue specific for, say, muscle cells. Although the presence of this illegitimate transcript can be up to 25 000 times less in such non-specific cells (Chelly et al 1988), this

method of mRNA extraction from non-specific cells, should provide a much more practical and less traumatic means of obtaining mRNA from dystrophic patients in the future.

SSCP provides a means of characterising a mutation (which was done with patient 'G') as well as a means of locating point mutations (Orita et al, 1989b). Another method, mis-match analysis, encourages heteroduplexes to be formed between normal and affected DNA. The resultant mis-matches are incubated with osmium tetroxide and then incubated with piperidine to cleave the DNA at the mis-match. End labelling of one primer will indicate where the mutation lies. (Cotton et al, 1988; Roberts et al, 1989). This may provide a much more effective way of looking at the amplified cDNA transcript of the dystrophic patients with point mutations. Roberts et al (1989), indicates that all types of mutations may be identified using the mis-match cleavage method. However the mis-match cleavage method does use highly toxic chemicals and this is a major disadvantage of the technique.

Future research in identifying point mutations in the dystrophin gene and any other localised gene would need to consider the techniques of extracting mRNA from non-specific cells and the mis-match cleavage technique. It is hoped that by using these new techniques, the trauma to the patient will be reduced as will the time needed to characterise each point or minor mutation. These procedures of locating and then working up a diagnostic test specific for the mutation, will become common laboratory practices in the future. Although time consuming at present, locating these point mutations provides these families with the confidence to make an informed decision about whether or not to have more children.

TABLE A)   BUFFERS AND ETHIDIUM BROMIDE

ELECTROPHORESIS BUFFERS

BUFFER	WORKING SOLUTION	CONCENTRATED STOCK SOLUTION (per Litre)
Tris-acetate (TAE)	1x: 0.04M Tris-acetate 0.001M EDTA	50x: 242g Tris base 57.1ml glacial acetic acid 100ml 0.5M EDTA (pH 8.0)
Tris-borate (TBE)	0.5x: 0.045M Tris-borate 0.001M EDTA	5x: 54g Tris base 27.5g boric acid 20ml 0.5M EDTA (pH 8.0)

GEL LOADING BUFFERS

BUFFER TYPE	BUFFER	STORAGE TEMPERATURE
Ficoll (6x)	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400; Pharmacia) in water	room temp
Formamide (1x)	98% deionized formamide 10mM EDTA (pH 8.0) 0.025% xylene cyanol FF 0.025% bromophenol blue	$4^{o}C$

ETHIDIUM BROMIDE

STOCK CONCENTRATION	STAINING CONCENTRATION
10mg/ml (1g of Ethidium Bromide in 100ml of H_2O)	0.5ug/ml

(Sambrook et al, 1989)

TABLE B) ABBREVIATIONS

AMV-RTase Avian myeloblastosis virus reverse transcriptase

ARMS Amplification refractory mutation system

APS Ammonium persulphate

BMD Becker muscular dystrophy

bp Base pairs

cDNA Complementary deoxyribonucleic acid

ddATP Dideoxyadenine triphosphate

ddCTP Dideoxycytosine triphosphate

ddGTP Dideoxyguanine triphosphate

ddNTP Dideoxynucleotide triphosphate

ddTTP Dideoxythymine triphosphate

DDW Double distilled water

DMD Duchenne muscular dystrophy

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphate

dsDNA Double stranded deoxyribonucleic acid

kb Kilobases

mRNA Messenger ribonucleic acid

PCR Polymerase chain reaction

rRNA Ribosomal ribonucleic acid

RNA Ribonucleic acid

snRNA Small nuclear ribonucleic acid

SSCP Single strand conformation polymorphism

ssDNA Single stranded deoxyribonucleic acid

TAE Tris-acetate

TBE Tris-borate

TEMED N,N,N',N',-Tetramethylethylenediamine

tRNA Transfer ribonucleic acid

UV Ultra-violet

V Volts

SDS Sodium dodecyl sulfate

EDTA Disodium ethylenediaminetetraacetate

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