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MOLECULAR STUDIES OF SPLICE SITES IN THE CANINE DYSTROPHIN GENE

by

Hayley Durling

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Award of

Bachelor of Science with Honours.

(Human Biology)

At the Faculty of Communications, Health and Science, Edith Cowan University,

Joondalup.

Date of submission: November 22th 1999

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously 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 written by another person except where due reference is made in the text.

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ABSTRACT

The development of an effective therapy for Duchenne Muscular Dystrophy (DMD) is one of the primary goals of all DMD/Becker Muscular Dystrophy (BMD) research. Golden Retriever Muscular Dystrophy (GRMD), an animal model of DMD is a fatal degenerative myopathy. Unlike the *mdx* model, the GRMD dog more accurately reflects the phenotype shown by human DMD patients, making the model better suited for the investigation and assessment of potential therapeutic approaches. The GRMD mutation, a base change from A to G in the 3' splice acceptor site of intron 6, results in exon 7 skipping which disrupts the translational reading frame. As a result, affected dogs have drastically reduced levels of dystrophin and its mRNA transcript.

Recently, genetic therapy with antisense oligonucleotides (AOs) has attracted special interest as a novel therapeutic approach for DMD. AOs may be introduced into myotubes to redirect the splicing of GRMD dystrophin pre-mRNA to restore the reading frame. Ideally, this would increase the quantity of functional dystrophin in affected DMD tissues to levels of therapeutic value.

The objective of this project was to identify intronic sequences from the canine dystrophin gene in the region of the mutation to allow the design AOs to be trialed as a therapy for GRMD. Intronic sequences that needed to be identified for the design of AOs were at the boundaries of intron 5/ exon 6, exon 6/ intron 7, exon 8/ intron 8 and intron 8/ exon 9. It was hoped that the application of the appropriate AOs would induce the processing/splicing of the dystrophin gene to exclude exons 6 and 8 (exon 7 is omitted due to the GRMD mutation) to restore the reading frame.

To determine these intronic sequences, several intronic sequencing strategies were attempted. These included long range PCR amplification, inverse PCR, PCR

screening of a plagemid GR library, construction of a GR cosmid library for hybridization screening and PCR screening of a phage canine genomic library.

This project determined the intronic boundaries of exon6/intron 6, intron 7/exon 8, exon 8/intron 8 and intron 8/exon9. Successful methods that identified intronic sequences included long-range PCR amplification and the PCR screening of both the phagemid and phage genomic DNA libraries. As it eventuated, this thesis also reported the first attempt at AO-induced GR dystrophin exon skipping in a GR primary myoblast tissue culture.

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ABBREVIATIONS

Abbreviation	Term
^{o}C	Degrees Celsius
ABI	Applied Biosystems, Inc.
Advs	Recombinant adenovirus vectors
ANRI	Australian Neuromuscular Research Institute
AO	Antisense Oligonucleotide
BMD	Becker Muscular Dystrophy
β	Beta
BSA	Bovine Serum Albumin
bp	Base pairs
cDNA	Complementary Deoxyribonucleic acid
CEE	Chick Embryo Extract
cfu	Colony forming units
CK	Creatine Kinase
ddH_20	Double Distilled Water
ddNTP	Dideoxynucleotide triphosphate (mix of four bases)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate (mix of four bases)
DMD	Duchenne Muscular Dystrophy
DRP	Dystrophin related protein
EDTA	Ethylene Diamine Tetra-acetic Acid
EST	Expressed Sequence Tag

EtBr	Ethidium Bromide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
5'	Five prime
GFP	Green fluorescent protein
GR	Golden Retriever
GRMD	Golden Retriever Muscular Dystrophy
IM	Intramuscular Injection
kb	Kilobases
KDa	Kilodalton
λ	Lambda
LB	*Luria-Bertani*
LF2000	Lipofectamine 2000
Mb	mega base
µg	microgram
µl	microlitre
MCS	Multiple cloning site
MDA	Muscular Dystrophy Association
mdx	Dystrophin deficient mice
mg	milligram
ml	millilitre
mM	millimolar
mpc	mononucleated precursor cells
mRNA	Messenger ribonucleic acid
nm	nanometres
nNOS	neuronal Nitric Oxide Synthase
OD	Optical Density
OD_{260}	Optical Density read at 260 nanometres

OD_{280}	Optical Desity read at 280 nanometres
OD_{600}	Optical Desity read at 600 nanometres
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pfu	plaque-forming units
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Reverse Transcriptase
SAP	Shrimp Alkaline Phosphatase
TAE	Tris-Acetate EDTA
Taq	*Thermus aquaticus*
T. th	*Thermus thermus*
3'	Three prime
2'	Two prime
U	Units
UV	Ultra-Violet
V	Volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1.0 INTRODUCTION

1.1 Duchenne and Becker Muscular Dystrophy

The identification of the human dystrophin gene (Koenig et al., 1987) and its gene product, dystrophin (Hoffman et al., 1987) was a giant leap in our understanding of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD).

DMD is the most common human X-linked recessive muscular disease, with a world wide incidence of 1 in 3500 live male births (Anderson & Kunkel, 1992). DMD is characterised by progressive muscle wasting and weakness of the skeletal muscles. Affected boys clinically present between the ages of 3 to 5 years with muscle weakness and experiencing difficulty in rising from the floor. This is followed by wheelchair confinement by or before 12 years of age. Death occurs from respiratory or cardiac failure in the early to late 20s (Kakulas, 1997; Matsuo, 1996).

BMD is a milder allelic version of DMD (Becker & Keiner, 1955; Monaco et al., 1988). Although clinically similar to DMD in distribution of proximal limb weakness, BMD's onset and progression is variable and generally less severe (Anderson & Kunkel, 1992). Some BMD patients require a wheelchair from 16 years of age, other BMD patients remain ambulatory into their third or fourth decade and may live full and minimally restricted lives. One BMD patient with a deletion of exons 3 to 10 was not diagnosed until the age of 62 years (Anderson & Kunkel, 1992; Malhotra et al., 1988).

1.2 The Dystrophin Gene and Protein

The dystrophin gene is comprised of 79 exons separated by introns of up to 200 kb spread over approximately 2.5 megabases (Mb) and encodes the major muscle

specific isoform, a 14-kb mRNA which is translated into a dystrophin protein, consisting of 3,685 amino acids (427 kDa) (Anderson & Kunkel, 1992; Roberts et al., 1993). Immunocytochemistry studies revealed that dystrophin is a large cytoskeletal protein expressed predominantly in skeletal muscle, where it is located just beneath the sarcolemma (Koenig et al., 1988; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988).

Immunoblotting with domain specific antibodies, suggests that dystrophin is divided into four distinct structural domains (Ahn & Kunkel, 1993). These are: the actin binding domain (amino N terminus); the internal region of dystrophin consisting of 24 homologous repeats, which, by analogy to β-spectrin, form a rod region; a cysteine rich domain and a carboxyl (C)-terminal domain. The C-terminus is the only region which does not show sequence similarity to other proteins, suggesting that it has dystrophin-specific function (Koenig et al., 1988). The C-terminus is believed to mediate the anchoring of dystrophin to the sarcolemma via a dystrophin-associated complex of proteins and glycoproteins (Campbell & Kahl, 1989; Carpenter et al., 1990).

While the specific function of dystrophin is yet to be elucidated, Samitt and Bonilla (1990) postulate that dystrophin is involved in the connection between the extracellular matrix and the cytoskeleton, which provides strength and flexibility to muscle fibres during contraction and relaxation. Other roles have been postulated, including: an involvement in excitation and contraction coupling (Harris et al., 1992); and regulation of Ca^{2+} movement (Turner et al., 1988; Harris et al., 1992). In addition, dystrophin has recently been shown to anchor the free radical enzyme, nNOS (neuronal nitric oxide synthase). Loss of nNOS localisation in DMD is believed to lead to necrosis and free radical injury (Disatnik et al., 1998).

Western blot analysis of muscle biopsies from patients with DMD indicated that dystrophin was generally either absent or present in undetectable quantities. Dystrophin of altered size and/or abundance was detected in BMD muscle (Hoffman et al., 1987, 1988; Beggs et al., 1991). Thus, the presence of partially functional and/or reduced levels of dystrophin was sufficient to ameliorate the DMD phenotype leading to the milder presentation seen in BMD (Beggs et al., 1991). It may, therefore, be possible to reduce the severity of DMD to that of BMD, if DMD patients can be induced to produce some functional Becker-like dystrophin (Wilton et al., 1999).

DMD and BMD have been shown to be caused by mutations in the dystrophin gene located at Xp21.2 (Hoffman et al., 1987). Approximately 65% of DMD and BMD patients have readily detectable intragenic deletions of one or more exons from the dystrophin gene (Koenig et al., 1987; Forrest et al., 1988), while approximately 6% have duplications of single or multiple exons (Monaco et al., 1987; Hu et al., 1990; Galvagni, et al., 1994). The remaining cases are minor or subtle DNA changes, typically nonsense or splice-site mutations (Hoffman & Kunkel, 1989; Den Dunnen, 1987). The majority of the deletions are not randomly distributed throughout the gene but clustered in hot spots where the introns are particularly large, and including neighbouring exons. For example, in the large intron 7 and in the extremely large intron between exons 44 and 45 (see review Kakulus, 1997; Nobile et al., 1997).

1.3 Dystrophin Mutations and Disease Severity

From early analysis of these partial deletions, it was observed that the size or location of the gene mutation did not appear to correlate with the clinical severity of the patient's disease (e.g. DMD versus BMD). To explain these observations at the molecular level, Monaco et al. (1988) proposed the reading frame hypothesis. This

hypothesised that the severity of the disease was directly correlated with the effect of the genetic lesions on the open reading frame (ORF) in the dystrophin mRNA transcript.

It was proposed that intragenic deletions harboured by severe DMD patients would bring exons together that, when spliced, resulted in a reading frame shift in the mRNA and premature termination of translation. The stop codon generated by the frameshift would lead to the production of truncated dystrophin molecules downstream of the mutation and would lack a carboxyl terminus. This protein is presumed to be non-functional and rapidly degraded.

Conversely, deletions or duplications that brought together exons that maintained the reading frame, resulted in the less severe BMD phenotype. It was presumed that the synthesis of shorter or larger molecular weight protein molecules were semi-functional.

Subsequent analysis of lymphocytes from many hundreds of DMD and BMD patients has proved that Monaco's "frame shift theory" was correct in over 90% of the cases studied. This is now accepted as the explanation for the lack of dystrophin in most DMD patients (Koenig et al., 1989; Den Dunnen et al., 1989). There are some exceptions to the frame shift rule involving patients with a frame shift deletion of exons 3-7 who produce low levels of dystrophin resulting in variable phenotypes, DMD, BMD or intermediate (Malhotra et al., 1988; Koenig et al., 1989; Chelly et al., 1990; Gangopadhyay et al., 1992; Winnard et al., 1993).

Arahata et al. (1991) used a new monoclonal antibody directed specifically against the C-terminal portion of the dystrophin molecule and confirmed that all BMD patients have dystrophin gene products which include the C-terminal domain. In contrast, the C-terminal was absent in most patients with DMD. This study not only substantiated

the validity of the reading frame hypothesis at the protein level but also emphasized the critical importance of the C-terminal domain. The resulting loss of only the C-terminus is sufficient to destabilize the entire dystrophin protein and prevent proper subcellular localization and function. However, a severe DMD patient with an intact C-terminus was identified by Prior et al. (1993). This patient was shown to have a missense mutation in exon 3 which substituted arginine for Leucine at position 54 in the actin binding domain. This missense mutation did not disrupt the reading frame but resulted in decreased production of correctly localised dystrophin protein to approximately 20% of normal levels of dystrophin. Thus, this study showed that the actin binding domain and the C-terminus are necessary for protein stability and essential for proper dystrophin function.

1.4 Animal Models of DMD

Animal models have contributed significantly to the investigation and understanding of a number of biological and pathological processes. They enable both basic research and clinical trials to be undertaken (Cooper, 1989). For ethical reasons, therapeutic trials cannot be performed on humans until a careful assessment of each therapy in a suitable animal model has been conducted (Kornegay, 1992). The potential advantages of animal models for Duchenne-type muscular dystrophy has lead to the investigation of several models (Cooper, 1989). Defects in the dystrophin gene resulting in animal DMD homologues have been reported in the mouse (Bullfield et al., 1984), dog (Cooper et al., 1988c) and cat (Carpenter et al., 1990).

Of the three animal species in which Duchenne-type muscular dystrophy has been identified, only the *mdx* mouse and the GR dog are available for study (Valentine et al., 1992). Despite offering convenience and economy, the *mdx* mouse model shows little clinical disease, attaining a normal lifespan (Cooper, 1993). Recently,

Partridge et al. (personal communication) found that stress and exercise exacerbated the problem leading to cardiomyopathy. This phenomenon has not been reported in humans. Unlike the *mdx* model, the GRMD dog more accurately reflects the phenotype shown by the human DMD patients, making the model better suited for the investigation and assessment of potential therapeutic approaches. In practical terms, the GR dog best serves as an intermediary between basic research on the *mdx* mouse and the eventual application to human subjects (Partridge, 1991b).

1.5 GRMD as an Animal Model for DMD

A spontaneous GR degenerative myopathy, with clinical and pathologic characteristics bearing close resemblance to DMD, was recognised by a number of investigators (Meier, 1958; de Lanhunta, 1977; Cardinet, 1979; Valentine et al, 1986; Kornegay, 1988).

A similar myopathy had been recorded in many dog breeds including Irish Terriers (Wentink et al., 1972), Samoyeds (Presthus & Nordostoga, 1989), Rottweilers and Dalmatians (Cooper, 1993), Belgian Shepherds (Van Ham et al., 1995) and German short haired pointer (Schatzberg et al., 1999). However, the DMD-like syndrome occurring in the GR is similar to that in man and it is also the most fully characterised (Kornegay, 1992; Cooper, 1993).

Colonies of GRMD dogs have been successfully established in North America using one of the dogs originally studied by Kornegay (1988). Additional colonies have been established in France and in Perth, Western Australia.

Apart from a severe neonatal form where death results in the first few days of life, overt clinical signs of GRMD first become apparent at approximately 6 to 8 weeks.

Before 8 weeks, some affected pups can be tentatively identified from poor weight gain, sucking problems and a tendency to be lethargic (Cooper, 1989; Howell et al., 1997).

Clinical signs include gross muscle weakness, an abnormal stiff-limbed, short strided, shuffling gait (with characteristic bunny hopping of the hind limbs) and limited jaw opening leading to difficulty in eating (Cooper, 1989; Nonaka, 1998). Serum levels of creatine kinase (CK) are consistently and often dramatically elevated in affected dogs (up to 300 times normal). This can be used to identify affected pups within the first few days of life (Cooper, 1992; Cooper, 1993; Valentine et al., 1992). However normal pups can have elevated levels of CK from stress of birth. In advanced stages there is a gradual decrease in serum CK level but it is always sustained above normal levels (Valentine, et al., 1992).

Clinically, the disease onset is earlier and progresses more rapidly in the GR dogs than in man. Again, in contrast to DMD, this rapid early decline is followed by a phase of relative clinical stability, at about 6 months of age, before succumbing to cardiac and respiratory failure months, or years, after birth. Some dogs may survive up to 6 to 7 years (Howell et al., 1997; Nonaka, 1998).

Histological examination shows the disease to be characterised first by the presence of numerous hypercontracted muscle fibres and then progresses to prominent fibre necrosis with infiltration and phagocytosis by macrophages. Concurrent degeneration with mineralisation and regeneration leads to eventual endomysial and perimysial fibrosis with fatty infiltration and cardiomyopathy (Partridge, 1991a; Kornegay et al., 1990; Valentine et al., 1990).

Despite the same descendance from a single affected GR male, considerable phenotypic variation in the severity of clinical signs and disease progression in affected dogs is evident. This suggests that factors other than mutations in the dystrophin gene may play a role in the manifestation of clinical disease. It is important therefore that research is conducted to elucidate these "factors" which may be relevant to an understanding of the clinical heterogeneity reported in human DMD (Valentine et al., 1988; Cooper et al., 1988a).

As all affected dogs were males born to clinically normal parents, it was proposed that GR disease was X-linked (Cooper et al., 1988b). To address this question, breeding trials were conducted in which an affected male GR was mated to unrelated GR female dogs. Putative carrier females from the F1 generation were retained and bred back to the original sire. To test the supposed X-linked pattern of inheritance, two proven carrier bitches were bred to unrelated normal male dogs, a beagle and a GR. From these breedings, three out of seven males were affected, while all nine females were normal. The pattern was consistent with the inheritance of muscular dystrophy as a fully penetrant X-linked recessive trait (Cooper, 1989). Recent breeding trials by Valentine et al. (1992) also obtained results consistent with an X-linked inheritance.

Previously, Selden et al. (1975) established that the normal canine X-chromosome had a size, centromeric location and G-banding pattern that were virtually identical to those of the normal X-chromosome in humans. The cytogenetic studies conducted by Cooper et al. (1988b), analysed the Giemsa-banded (G-banded) karyotypes from affected and non-affected dogs and found no obvious chromosomal abnormalities, the X-chromosome, in particular, being normal.

1.6 GRMD Molecular Genetics

Given that genetic information encoded on the X-chromosome is highly conserved in mammalian species, Ohno (1973) and Cooper et al. (1988c), investigated the possibility that the dystrophin gene might also be defective on the X-chromosome of affected dogs.

Southern blots of genomic DNA from affected dogs were probed with human DMD cDNA in order to detect a deletion in the canine homologue at the Duchenne locus. While no evidence of deletion or other defects in the coding portion of the Duchenne locus was found, informative restriction fragment length polymorphisms (RFLPs) were identified. Preliminary linkage analysis using these RFLPs revealed that the mutation responsible for the canine disease was located on the GR X-chromosome in a region homologous to the region of the human X-chromosome carrying the human DMD locus (Cooper et al., 1988c).

Northern blots of RNA prepared from canine skeletal muscle and hybridised with the same DMD cDNA probes revealed a 13.6 kb transcript in normal dogs similar to that found in human (14 kb) and mouse muscle (12.5 kb). In contrast, affected dogs lacked this mRNA transcript and GRMD appeared to be a animal homologue of DMD (Cooper et al., 1988c).

To establish whether dystrophin was absent in affected dogs, Western blots of skeletal muscle from normal and dystrophin dogs were carried out. Antibodies to dystrophin detected a dystrophin band (400 kDa) from normal dogs, indistinguishable from dystrophin present in human and mouse muscle. No dystrophin was detected in muscle from the affected dogs (Cooper et al., 1988c).

These results were supported by immunohistochemical studies which confirmed that the normal sarcolemmal staining pattern was absent in both skeletal (Cooper et al., 1990) and cardiac muscle (Valentine et al., 1989).

However, a later study conducted by Valentine et al. (1992) used a highly sensitive, chemilluminscent technique to probe Western blots and detected a very faint dystrophin band that corresponded to the dystrophin band in normal dogs. While the origin of this dystrophin is not clear, it was proposed that these very low levels of dystrophin may be the result of revertant fibres which have also been observed in DMD and *mdx* mouse muscle (Hoffman et al., 1990).

Sharp et al. (1992) conducted molecular studies that analysed the canine dystrophin gene in normal and affected dogs. Exon 1 (forward) and exon 11 (reverse) primers detected a dystrophin transcript truncated by 120 nucleotides when cDNA from affected dogs was used as a target for PCR amplification. As this exon was present at the genomic level, it was assumed that there was a splice site mutation inducing the skipping of this exon. This mRNA processing error was subsequently shown to be the result of a point mutation, a base change from A to G in the 3' splice acceptor site of intron 6. The seventh exon is then skipped which disrupts the reading frame and leads to the premature termination of dystrophin translation within exon 8 in affected dogs. This is shown in Figure 1.1.

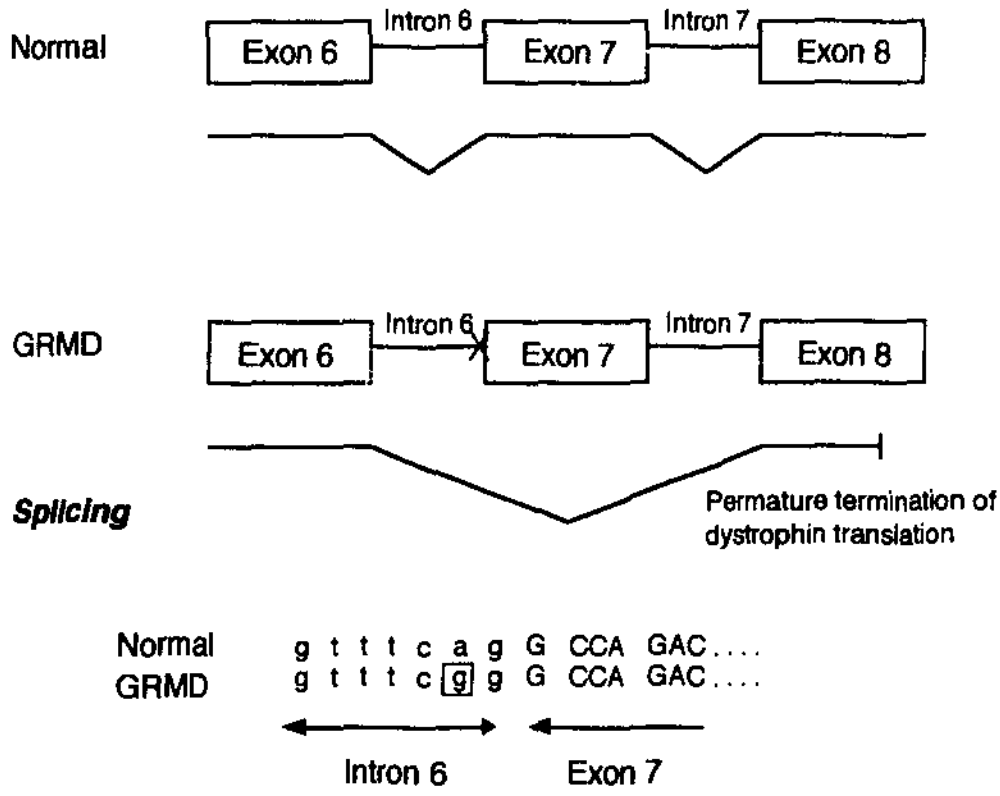


Figure 1.1: Diagrammatic Representation of Genomic DNA from Normal and GRMD dogs during Splicing with the 3' Splice Site Mutation of Intron 6 shown in the pink box.

1.7 Therapies for DMD

The development of an effective therapy for DMD is one of the primary goals of all DMD/BMD research. Following the discovery of the central importance of the mutated dystrophin gene to DMD, myoblast transplantation, gene therapy and genetic therapies were pursued. The objective of all of these therapeutic approaches was to

increase the quantity of functional dystrophin in affected DMD tissues to levels of therapeutic value (Anderson & Kunkel, 1992).

1.7.1 Myoblast Transplantation

During growth and development, multinucleated skeletal muscle fibres are formed by the fusion of mononucleated precursor cells (mpc) (See review Morgan, 1994). Experiments with mice revealed that implanted mpc can fuse with growing or regenerating muscle fibres to form mosaic fibres in which myonuclei of both host and donor origin share a common cytoplasm (Watt et al., 1982, 1984). Mpcs have been isolated from muscle, enriched, expanded in numbers and genetically engineered with retroviruses *in vitro* before being injected into muscle (Blau & Springer, 1995). Recently Gussoni et al. (1999) have demonstated the partial restoration of dystrophin expression in DMD muscle by the intravenous injection of either normal haematopoietic stem cells or a novel population of muscle-derived stem cells into irradiated animals.

The results from these preliminary studies indicate that the transplantation of normal mpc (with normal genetic constitution) could become incorporated into the myopathic muscle fibres where they would grow and repair the muscle. Therefore, the gene products derived from the normal donor myonuclei would compensate for products which are deficient or abnormal within the myopathic host muscle fibre (Morgan & Watt, 1993).

Despite some considerable success in the *mdx* mouse, similar experiments on DMD patients have been disappointing (Morgan, 1994, Karpati et al., 1993). While the clinical trials with DMD patients have reported no adverse effects from cell injections, patients failed to show any significant improvement in muscle strength. The estimated

number of surviving donor cells able to express dystrophin was very low (~1%) despite the delivery of millions of donor myoblasts (Gussoni et al., 1997).

In addition, mpc implantation in humans have been shown to remain relatively localised at the site of injection with only limited migration to damaged sites. As a result, the large number of injections of myoblasts necessary to treat all of the affected muscle reduces the likelihood that this method of treatment for DMD will succeed in the near future (Blau & Springer, 1995).

1.7.2 Gene Therapy

The object of gene therapy involves reconstituting a biological function by introducing a gene into somatic cells, which are genetically deficient in that gene product (Dickson & Dunckley, 1993). The large size of the dystrophin gene makes it impossible to transfer it in its entirety to dystrophin deficient somatic cells. Instead, the main aim of DMD gene therapy has been to supply dystrophin cDNAs, in particular the muscle specific isoform consisting of a partial or full length cDNA driven by an appropriate promotor (Matsuo, 1996).

While somatic gene therapy has been recognised as a possible approach leading to effective therapy for DMD, it has only been in the last few years that rapid scientific advances have been made with the development of novel plasmid and viral vectors (Marshall & Leiden, 1998).

As skeletal myofibres have a relatively long half-life *in vivo*, they provide a stable platform in which to express recombinant genes (Marshall & Leiden, 1998). Several gene transfer approaches have been tried to transduce skeletal myocytes *in vivo*. These approaches include intramuscular injection (IM) with a variety of vectors including

naked plasmid DNA, liposome complexed DNA, adenoviruses and adeno-associated viruses (Morgan, 1994; Marshall & Leiden, 1998).

Dystrophin cDNA has been introduced into skeletal muscle fibres of dystrophin deficient mice (*mdx*) through direct injection in plasmid expression vectors (Wolff et al., 1990) and by replication-defective recombinant adenovirus vectors (Advs) (Karpati & Acsadi, 1993). The introduced genes appear to protect transduced muscle fibres from necrosis. By direct injection of dystrophin cDNA as plasmids, expression has only been achieved in about 1-2% of the adult *mdx* muscle fibres adjacent to the site of injection (Wolf et al., 1990). Greater efficiency has been reported for recombinant adenovirus injection into young *mdx* muscle (Karpati & Acsadi, 1993).

Although DMD gene therapy by the introduction of dystrophin constructs via retroviral or adenoviral vectors has been shown to be possible in the *mdx* mouse (Morgan, 1994), there is an urgent need for the use of the GRMD dog in gene therapy trails. The GRMD dog muscle bulk reflects the proportions and distributions seen in humans much more so than the tiny muscles of the *mdx* mouse. It is obviously a more relevant animal model in which to trial potential DMD therapies before their use in humans (Dickson & Dunckley, 1993).

Howell et al. (1998a) have recently demonstrated human dystrophin expression in dog muscle following the direct injection of a plasmid containing a dystrophin mini-gene. In a further study Howell et al. (1998b) administered modified adenoviruses which carried the dystrophin mini-gene. This was rapidly followed by expression of human dystrophin in over 50% of the fibres. This was a much greater degree of expression than had been previously obtained with plasmids. Immunosuppression, premitted dystrophin expression has been maintained for periods up to 60 days in mdx mice and GRMD dogs (Howell, personal communication, 1999).

Host immune responses to foreign cells or to adenoviral vectors have been reported (Gussoni et al., 1997). The problem of immune rejection poses serious limitations to the success of skeletal muscle based gene therapy. Strategies to minimise potential pathological consequences, particularly if continual re-injection of the vector is necessary, need to be developed in animal models. Trials should be conducted in both immunocompetent and immunocompromised hosts (Morgan, 1994; Marshall & Leiden, 1998).

The first human trials of muscle-directed gene therapy are planed in the near future. The timing of these trials depends on the Food and Drug Administration (FDA) response to Muscular Diseases Association (MDA) proposals to sponsor these trials (Wahl, 1998). It must be emphasised, however, that even if successful therapy were to be accomplished in skeletal muscle, the treatment of heart and brain for cardiomyopathy and borderline mental retardation still remains an outstanding problem (Karpati & Acasdi, 1993).

1.7.3 Genetic Therapy

As the injection of dystrophin minigenes into muscle is approaching human trials, alternative genetic therapy strategies for DMD treatment are under development. These aim to retard progression of the clinical symptoms by converting DMD phenotypes to BMD phenotypes (Matsuo, 1996). Recently, revertant fibres (Wilton et al., 1997a,b) and the antisense oligonucleotide (AO) alteration of splicing patterns have been investigated (Dominski & Kole, 1993; Dunkley et al., 1995; Wilton et al., 1999).

1.7.3.1 Revertant Fibres

Despite the absence of dystrophin as detected initially by Western blot analysis, subsequent immunohistochemical detection with anti-dystrophin antibodies revealed the presence of dystrophin-positive fibres and clusters in approximately 50% of DMD patients (Nicholson et al., 1989). These findings were also confirmed in *mdx* mice (Miike et al., 1989; Miyatake et al., 1989, 1991; Hoffman et al., 1990). Dystrophin-positive fibres and in-frame transcripts have also been detected in the GRMD muscle tissue indicating that this particular "reversion" phenomena might be widespread across several species (Valentine et al., 1992; Wilton et al., 1997b).

Dystrophin-positive fibres in DMD patients, GRMD dogs and *mdx* mice are termed "revertants" or "revertant fibres" and appear to result from a rare but natural phenomena. Revertant fibres represent less than 10% of the total muscle fibre population (Sheratt et al., 1993). Dystrophin-positive fibres have been shown to express true dystrophin and do not cross react with other related peptides (Hoffman et al., 1990). Utrophin or dystrophin related protein (DRP) (397 kDa), shares similarity to the protein domains of dystrophin and therefore can react with some anti-dystrophin antibodies (Pearce, 1993).

While precise aetiology of the revertant fibre dystrophin has yet to be determined (Klein et al., 1992; Uchino et al., 1995), postulated mechanisms for dystrophin-positive fibres include: (i) normal, developmentally regulated alternative splicing (ii) low-level illegitimate splicing (iii) somatic reversions (iv) somatic suppression and (v) somatic mosaicism (Hoffman et al., 1990, Klein et al., 1992).

Evidence has accumulated indicating that the first two mechanisms are unlikely (Hoffman et al., 1990). There is now growing support for dystrophin positive fibres as

true genetic revertants generated from a somatic reversion-mutation mechanism in the dystrophin gene. The primary disease causing mutation (nonsense/frameshift) appears to be bypassed by a second site in-frame mutation which restores the translational reading frame. This provides a template for functional Becker-like proteins (Hoffman et al., 1990; Klein et al., 1992; Zhao et al., 1993).

Mdx mouse dystrophin-positive fibres increase in frequency with age and X-irradiation treatment (Hoffman et al., 1990). This may suggest that these fibres persist longer because they have a selective advantage over dystrophin-negative fibres (Uchino et al., 1995; Wilton et al., 1997b).

Evidence by Fanin et al. (1995) have revealed no correlation between dystrophin-positive fibres and the clinical severity of DMD, suggesting that these dystrophin-positive fibres are unable to improve the clinical phenotype of DMD, primarily due to their low abundance. However Nicholoson et al. (1993) has found some correlation between low levels of dystrophin and delayed restriction to wheelchair for some DMD patients.

Characterisation of dystrophin-positive fibres, has raised the possibility of "genetic therapy" for DMD. The dystrophin-positive fibres could be exploited by increasing their numbers to biologically significant levels in DMD patients (Wilton et al., 1997a).

1.7.3.2 Antisense Oligonucleotide (AO) Technology

Over the past few years antisense oligonucleotides (AOs) have attracted special interest as a novel class of therapeutic agents for treatment of genetic disorders, emerging as a powerful tool in genetic therapy with a large and growing number of applications (Temsamani & Guinot, 1997; Lavrosky et al., 1997). AOs are short single

stranded molecules of RNA or typically DNA that hybridize target DNA or RNA molecules in a sequence specific manner (Dunckley et al, 1995). AOs have previously been used to down-regulate gene expression, and has been achieved by blocking translation (Agrawal, 1992) or by targeting an mRNA for RNaseH degradation (Ghosh & Cohen, 1992). AOs have also been used to target specific regions of DNA to inhibit transcription by RNA polymerase II (Maher et al., 1991; Hanvey et al., 1992) and used to alter RNA processing (Dominski & Kole, 1993).

Dominski and Kole (1993) conducted an experiment which used AOs to manipulate splice site selection in human β-globin thalassemic pre-mRNA. In β110 thalassemia, an A to G mutation at nucleotide 110 in the first intron of the human β-globin gene creates an additional aberrant 3' splice site. In spite of the presence of a normal splice site, the aberrant splice site is preferentially used by the splicing machinery resulting in an incorrectly spliced mRNA that contains 19 nucleotides of intronic sequence. A 14 mer antisense 2'-O-methylribonucleotide was designed to bind to the aberrant splice site in intron 1 of the pre-mRNA. This type of modified AO was selected as the 2'-O-methylation (in which the non-binding oxygen atom is replaced by a methyl group) renders the molecule resistant to nucleases. Thus stable hybrids are formed and are not degraded by RNase H which would destroy the hetero duplex and prevent This AO effectively reversed the aberrant splice site and restored correct splicing *in vitro*. The results of Domonski and Kole (1993) raised the possibility of a new therapeutic approach for DMD if normal splice sites could be blocked.

Dunckley et al. (1995) has shown that AOs can be used to manipulate splice site selection in *mdx* mouse dystrophin pre-mRNA. The *mdx* mouse carries a nonsense mutation at position 3185 in exon 23 of the dystrophin gene. This mutation inactivates the dystrophin gene of the *mdx* mouse resulting in termination of translation within exon 23. A 2'-O-methyl oligonucleotides complementary to the 3' splice site of

dystrophin intron 22 was delivered to the nuclei of primary *mdx* myoblast cultures. Direct sequencing of RT-PCR products from these cells revealed the removal of exons 22 to 30, thus skipping the mutant exon 23 and creating a novel in-frame dystrophin transcript.

In a parallel study by Wilton et al. (1999) *mdx* myoblast cultures were incubated with an antisense oligonucleotide directed to the 3' and 5' splice site of intron 22 and intron 23 respectively in the *mdx* pre-mRNA. The 5' AO appeared to efficiently displace factors normally involved in the removal of intron 23 so that exon 23 was also removed during the splicing of the dystrophin pre-mRNA. The identity of the shorter transcript, was confirmed by direct sequencing as an inframe dystrophin transcript skipping exon 23, that is exon 22 joined to exon 24. This is shown in Figure 1.2.

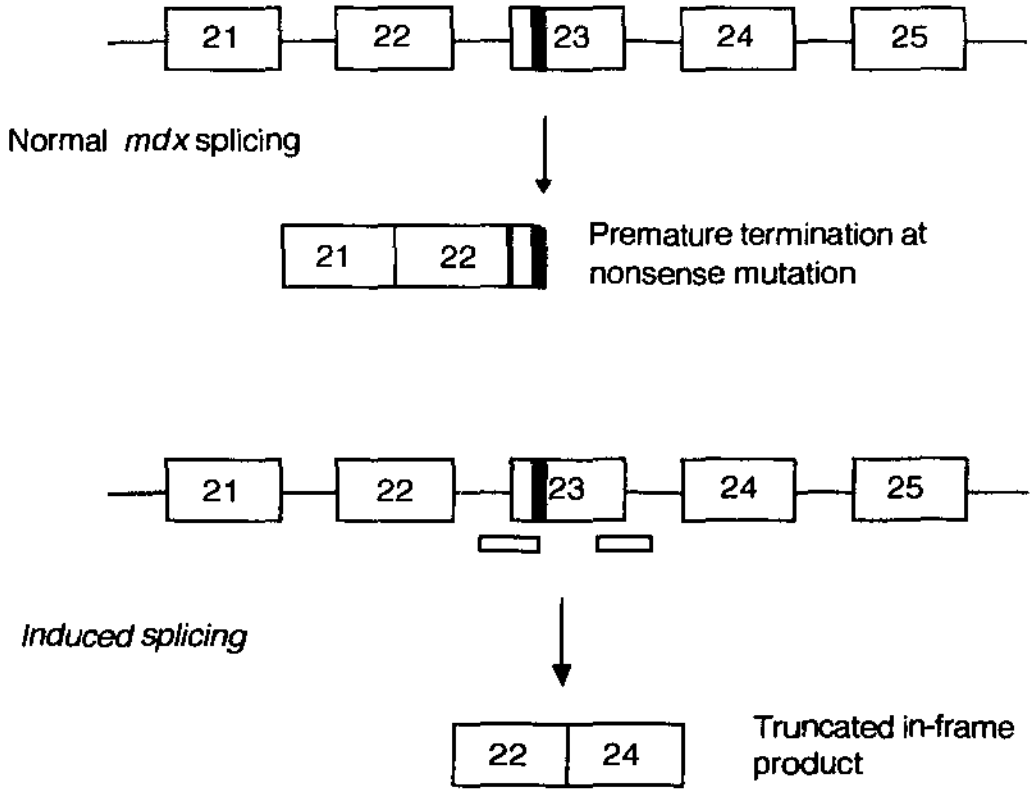


Figure 1.2: Diagrammatic Overview of Induced Exon Skipping, by the Wilton et al (1999) study, to remove the Nonsense Mutation from the *mdx* Dystrophin Gene. (not drawn to scale)

Redirected splicing of pre-mRNA induced by AOs has the potential to minimise the consequences of the gene mutation that causes DMD by restoring the reading frame. Although this new approach is unlikely to herald a complete cure, it could significantly reduce the severity of this devastating disease. Of particular importance is that all tissue specific isoforms would be expressed under endogenous control

1.8 Objectives of the Project

This project aims to provide the basic research necessary for the evaluation of AO technology as a method of reducing the severity of DMD in another model of this disease. The project focuses on the GRMD model of muscular dystrophy. The mutation that causes this disease falls in a region that is a known "hot spot" for DMD deletions and therefore is very significant to human genetic therapy.

The objective of this project is to identify intronic sequences from the canine dystrophin gene in the region of the mutation to allow the design of antisense oligonucleotides to be trialled as a therapy for GRMD.

Intronic sequences that need to be identified for the design of AOs are at the boundaries of intron 5/ exon 6, exon 6/ intron 7, exon 8/ intron 8 and intron 8/ exon 9. Once characterized, the appropriate AOs will be applied to induce the processing/splicing of the dystrophin gene to "cut out" exons 6, 8 and 9 (exon 7 is omitted due to the GRMD mutation). This is the minimum change required to restore the reading frame. Ideally, the dystrophin molecule translated from this novel in-frame dystrophin transcript will be functional (Figure 1.3). At present, it is not certain whether these exons to be skipped encode significant amino acids necessary for the function of dystrophin. However human BMD patients missing exons 2-11, 2-8 and 3-16 (Bushby et al., 1993) have mild to moderate phenotypes, suggesting that the dystrophin produced by the skipping of exons 6 to 8 will be at least partially functional in Golden Retriever.

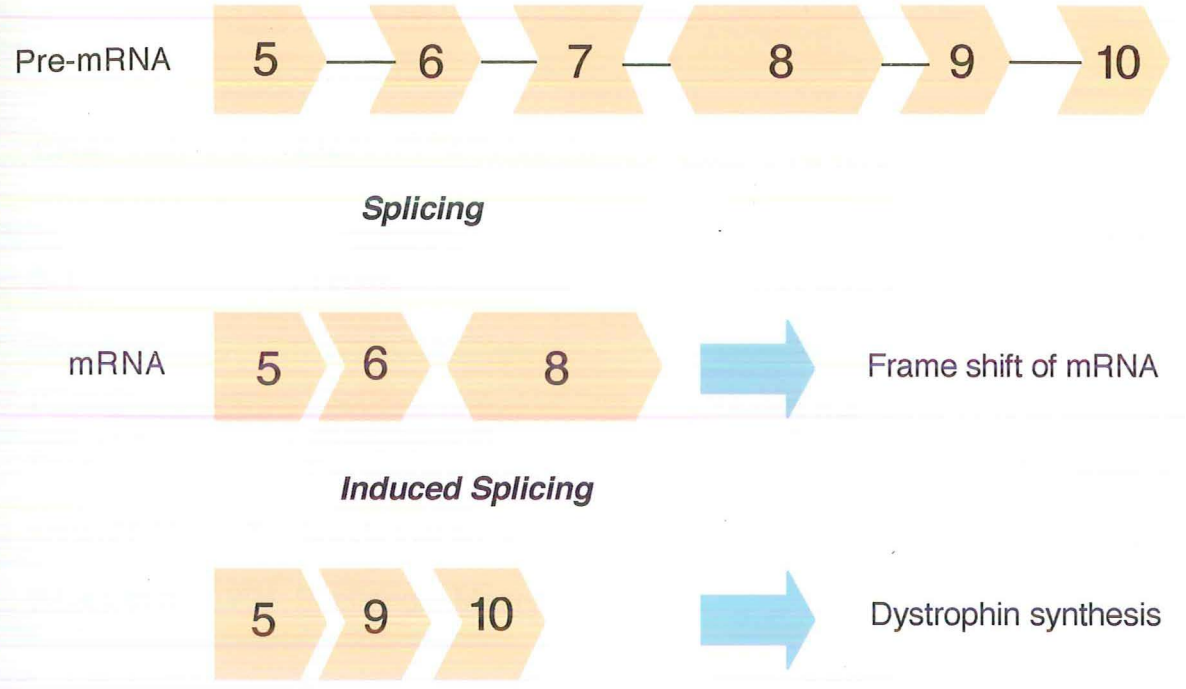


Figure 1.3 Diagrammatic Representation of the Aims of this Project

1.9 Experimental Approach

Since only the intronic sequence at the intron 6/exon 7 junction of the GR is available (Sharp et al., 1992), several intronic sequencing strategies will be attempted. If selected introns are small, then it should be possible to amplify across individual introns by conventional or long range PCR and then undertake direct DNA sequencing. In the event that the introns are too large for the DNA amplification, other approaches to find and identify selected splice sites will be undertaken. These include phagemid library construction, Cosmid genomic DNA library and Lambda (λ) genomic DNA library screening.

CHAPTER 2.0 AMPLIFICATION OF CANINE DYSTROPHIN INTRONS

2.1 INTRODUCTION

PCR is an *in vitro* method for enzymatically synthesising millions of copies of defined DNA sequences. The reaction involves two synthetic oligonucleotide primers that anneal to opposite strands that flank the target DNA sequence which is to be amplified (Mullis et al., 1994; Boehringer Mannheim, 1995).

Extension of the primers is catalysed by a thermostable DNA polymerase, derived from thermophilic eubacteria like *Thermus aquaticus.* This is known as *Thermus aquaticus* DNA polymerase, frequently abbreviated to *Taq* DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing and primer extension by *Taq* DNA polymerase, results in an exponential accumulation of a specific DNA fragment. With each cycle, theoretically the amount of target DNA doubles, therefore after n cycles there is a 2^n increase in the amount of double-stranded target DNA (Erlich, 1989; Mullis et al.,1994; Cummings, 1994; Boehringer Mannheim, 1995).

Conventional PCR is capable of amplifying up to 3 kb from genomic DNA targets (Cheng, 1995). The lack of a 3' to 5' exonuclease or proofreading activity is believed to account for this limitation, due to the inability of *Taq* DNA polymerase to correct nucleotide misincorporations and continue primer elongation (Barnes 1994).

As the intron sizes for the canine dystrophin gene are unknown, the 'conventional' PCR approach may be unsuccessful if the introns under investigation are larger than 3 kb. To overcome this limitation of conventional PCR, a modification allowing for the amplification of long DNA fragments has been developed (Barnes, 1994; Cheng,

1995). One such long-range PCR system has been developed by GibcoBRL, the ELONGASE™ enzyme kit. This kit uses a combination of two thermostable DNA polymerases, *Taq* DNA polymerase and a potent proofreading *Pyrococcus sp.* thermostable polymerase present at a reduced quantity. The presence of a proofreading enzyme serves to remove nucleotides misincorporated by *Taq* DNA polymerase, driving the extension of the target strands to between 12 to 20 kb (GibcoBRL, 1999a).

2.2 METHODS

2.2.1 Genomic DNA Extractions

To provide target for long range amplification of dystrophin introns, genomic DNA was extracted from three normal GRs. These animals were members of the GR colony at Murdoch University. Thymic tissue samples from two normal male GR (Chip numbers, 19C5 and 6B00) and one normal female GR (Chip number 1263) were acquired from frozen (-80^{0}C) archival storage. Thymic tissue was used for the genomic DNA extractions due to its ready availability and its high DNA content.

2.2.1.2 The DNA Isolation Kit for Cells and Tissues

The DNA Isolation Kit for Cells and Tissues supplied by Roche Diagnostics, Australia, was used to extract genomic DNA. This product claims to permit the rapid, large scale isolation of genomic DNA ranging in size from 50 to 150 kb, free of contaminating RNA and proteins. All components of the kit were stored at 4^{0}C.

2.2.1.3 Genomic DNA Extraction Protocol

DNA extractions were performed using 150-250 mg of thymic tissue. Due to the high DNA content of this tissue, the volumes of each solution required for the extraction were based on specifications provided by the manufacturer for a starting tissue sample of 400 mg. Throughout the DNA extraction, the samples and solutions were kept at room temperature unless otherwise stated.

The tissue was frozen in liquid nitrogen, homogenised with a mortar and pestle, and resuspended in 5 ml of cellular lysis buffer in a sterile 30ml centrifuge tube. To this mixture, 6.1 µl of Proteinase K solution was added, followed by another 5 ml of cellular lysis buffer. The sample was gently inverted 2 to 3 times to ensure that the Proteinase K solution was mixed into the suspension. In the presence of the Proteinase K, the DNA and RNA were released into the solution. The sample was then placed at 65°C for 1 hour. A 400 µl aliquot of RNAase A solution was added to remove the RNA from the sample. The sample was then placed at 37°C for 15 minutes.

4.2 ml of protein precipitation solution was added to the sample and gently mixed for 5 to 10 seconds. The sample was then placed on ice for 5 minutes followed by centrifugation at 13 000 rpm at room temperature for 20 minutes to remove the protein from the suspension. Upon centrifugation a protein pellet (and cell debris) was formed. The supernatant containing the DNA was carefully withdrawn using a wide bore tip and transferred into a sterile 50 ml centrifuge tube.

0.7 volumes of isopropanol was added to precipitate the DNA. The tube was gently inverted until the upper and lower phases mixed. The visible DNA "strands" were removed from the isopropanol with a plastic tip and placed into a sterile tube

containing cold 70% ethanol. The mixture was swirled until the DNA strands were released into the 70% ethanol.

The sample was then centrifuged for 5 minutes at 13 000 rpm. The supernatant was discarded and the DNA pellet was left to air dry for 10 minutes. The DNA pellet was resuspended in 300 µl of nuclease free water and placed at 4°C overnight to dissolve.

2.2.1.4 Spectrophotometry

Spectrophotometery of the purified genomic DNA was performed using a Beckman DU 650 (UV) spectrophotometer. Nucleic acids absorb UV light predominantly at 260 nm whereas proteins absorb UV light predominantly at 280 nm. By measuring the samples optical density (OD) at these two wavelengths, the concentration and relative purity of the DNA was calculated.

To ensure accurate and consistent results, the UV light on the spectrophotometer was switched on at least 15 minutes prior to measuring. The spectrophotometer was calibrated against the same water used to dilute the samples. An OD_{260} value of 1.0 was considered to be equivalent to 50 µg/ml of DNA (Sambrook et al., 1989). The concentration of DNA samples was calculated as follows:

$$\text{Concentration of DNA (µg/ml)} = OD_{260} \times \text{dilution factor} \times 50$$

Nucleic acid purity was determined by comparing the ratio between reading at 260 nm and 280 nm. The purity of the DNA sample was measured for protein contaminants from the ratio OD_{260}/OD_{280}. An OD_{260}/OD_{280} of 1.8 represents high quality, pure DNA. Values less than these figures are indicative of protein and/or phenol contamination. To check the sample for possible RNA contamination, an aliquot of the extracted DNA

was fractionated on an agarose gel. Any RNA contamination would appear as a smear towards the bottom of the lane.

2.2.1.5 Agarose Gel Electrophoresis

The agarose concentration used to make the gel was determined by the anticipated sizes of DNA fragments to be fractionated. This shown in Table 2.1.

Table 2.1: ***Agarose Concentrations Required to Separate DNA of Various Sizes***

Amount of Agarose (%) in gel (w/v)	Linear DNA Size (kb)
0.5	5 to 60
1.0	6 to 0.4
2.0	4 to 0.2 kb
3.0	2 to 0.1kb

(Sambrook et al., 1989)

As the extracted genomic DNA products were expected to be in the 50 to 150kb range, a 0.5% agarose gel was prepared by pouring a 1% agarose in TAE base, allowing this to set and then overlaying with 3 to 5 mm of 0.5% agarose. An appropriate amount of agarose powder was weighed out and added to 1xTAE in a bottle. With the lid on loosely, the solution was heated in a microwave oven until the agarose had completely dissolved. It was then stored in a dry oven at 65°C until required.

The molten 1% agarose solution was poured onto a perspex casting tray (Biorad) to a depth of 3 to 5 mm. The gel was left to set for 15 to 30 minutes at room temperature. A well forming comb was placed approximately 1 cm from one end of the casting tray

above the 1% agarose base. The 1% agarose gel was then overlayed with 2 mm to 4 mm of 0.5% agarose and allowed to set.

The gel was then placed into an electrophoresis tank (Biorad electrophoresis sub cell) and sufficient 1xTAE buffer was added to just cover the surface of the gel to a depth of 1 mm. The comb was removed. 5 µl aliquots of each sample were mixed with 1 µl of 6x GLB loading buffer and loaded into the wells of the agarose gel. A 5 µl aliquot of a DNA molecular marker (50 ng/µl) was also loaded into the wells on the far left and right sides of the gel (Figure 2.1).

Electrophoresis of the samples was performed between 70-100 volts for approximately 1 hour. After electrophoresis was complete, the gel was stained in ethidium bromide (0.5 µg/ml) at room temperature with gentle agitation for 30 minutes.

After staining, the gel was visualised using a Fotodyne UV-transilluminator and black and white images were recorded using the Kodak® Digital Science™ DC120 camera.

2.2.2 Long Range Amplification

Long range PCR amplification of intronic targets was performed using Elongase enzyme kit (GibcoBRL, 1999a) according to the recommendations for genomic targets provided by the manufacturer. The PCR included a 'hot start' and was composed of two separate mixes, one containing the template, primers and nucleotides (Mix 1, Table 2.2) and another that contained the buffer and Elongase enzyme (Mix 2, Table 2.3). Both mixes were prepared on ice in 0.2 ml thin walled tubes. Mix 1 was constructed individually for each intronic primer set. Primers used to amplify across the introns are illustrated in Table 2.4.

SPP-1 digested with *EcoRI*
(Geneworks, Australia)

8500 bp
7350 bp
6100 bp
4840 bp
3590 bp
2810 bp
1950 bp
1860 bp
1510 bp
1390 bp
1160 bp
980 bp
720 bp
480 bp
360 bp

pUC19 digested with Hpa II
(Geneworks, Australia)

501 bp
489 bp
404 bp
331 bp
242 bp
190 bp
147 bp
111 bp
110 bp
67 bp
34 bp
34 bp
26 bp

DMW-100M
(Geneworks, Australia)

3000 bp
2000 bp
1000bp

Figure 2.1: **DNA Molecular Weight Markers used in Agarose Gel Electrophoresis.** The markers were diluted in loading buffer to a final concentration of 50 ng/µl before use.

Table 2.2: ***Components of Mix 1***

Components of Mix 1	Volume (µl)	Final Concentration
5 mM dNTP mix	2	100µM
Forward Primer	2	200nM
Reverse Primer	2	200nM
Genomic DNA (752) 1µg/µl	1	1µg
Sterile water	to 20 µl	
Total	**20**	

Table 2.3: ***Components of Mix 2***

Components of Mix 2	Volume (µl)	Final Concentration
5X Buffer A	5	60 mM Tris SO_4 (pH 9.1), 18 mM $(NH_4)_2$ 1.5 mM Mg SO_4
5X Buffer B	5	
Elongase Enzyme Mix (1 unit (U)/µl)	1	1 U
Sterile water	to 30 µl	
Total	**30**	

Table 2.4: ***Primers used to Amplify Canine Dystrophin Introns***

Canine Intron	Primer Set	Sequence 5' to 3'
5	CanDys5F	GGAAATCA(C/T)AAACTGACTCTTGG
	Can6R	CTGTGACTATGGATAAGAGCATTCA
6	Can6F	GTCAAAAATGTAATGAAAAATATCAT
	CanDys7R	TTGTGTGGCTGACTGCTGGC
7	Can7F	GAACATGCATTCAACATTGCC
	Can8OR	GATGGCCTTGGCAACATTTCC
8	ConDys8F	CAACAAGTGAGCATTGAAGCGAT
	ConDys9R	CATAAGCAGCCTGTGTG(A/T)AGGCAT
9	DysEX9F	CCTAAGCCTGGATTCAAGAG
	Can10OR	AATCTCTCCTTGGGCTTGGAG
Positive control	Can 79F	CAATGTAGGAAGCCT
	UTR 13643R	TAAAACGTAGCAATAAAGCTC

All reaction components of the PCR master mixes, with the exception of the DNA template, were added in a pre-PCR room with micropipettes that had never been exposed to PCR products. A negative control consisting of all components of the reaction mix except the template was also prepared for each primer set in order to check for possible reagent contamination.

The DNA template was added using aerosol-resistant tips (to minimise contamination) to Mix 1. The reaction was overlayed with one drop of paraffin oil to prevent evaporation and condensation during thermocycling. The MJ tube control facility minicycler was used to monitor reaction temperatures. The individual Mix 1 tubes were placed in the thermocycler. The reaction was incubated at 94°C for 30 seconds after which the Mix 2 containing the Elongase was added, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 50°C for 30 seconds and primer extension at 68°C for 10 minutes.

On completing the PCR, a 5 µl aliquot of the products was electrophoresed on an agarose gel and the remaining products were stored at -20°C until further use.

2.2.2.1 Gel Purification of Long Range PCR Products

The long range amplification PCR products were gel purified on a 1.5% low melt agarose gel. 20 to 30 µl of the PCR product was mixed with 5 µl of loading 6x GLB buffer and loaded into a wide well created by taping two comb teeth together. After fractionation, the gel was stained with freshly prepared ethidium bromide and visualised briefly by UV transillumination. The band of interest was excised from the gel with a clean sterile scalpel blade as quickly as possible to minimise damage to the DNA by UV light and then transferred into a 1.5 ml eppendorf tube.

The QIAquick PCR purification kit (supplied by QIAGEN, Australia) was used to purify gel fractionalised PCR products from contaminants such as primer dimers and amplification primers, unincorporated nucleotides, and enzymes (Qiagen, 1997). All solutions and spin columns were provided with the kit. Purification was performed according to the protocol provided by the manufacturer.

Five volumes of buffer PB was added to the excised DNA agarose band in the 1.5 ml eppendorf tube. The sample was then placed at 65°C to melt the agarose (~1 minute). The sample was then applied to a QIAquick spin column placed in a 2 ml collection tube. The sample was then centrifuged for 1 minute at 14 000 rpm and the flow-through discarded. The QIAquick column was placed back into the same collection tube and the sample washed with 750 µl of buffer PE which was subsequently centrifuged for a further 1 minute. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at maximum speed to remove any residual buffer PE. The QIAquick column was then placed into a sterile 1.5 ml eppendorf tube. The DNA was eluted by adding 30-50 µl of nuclease free water to the centre of the QIAquick column and centrifuging for 1 minute.

An aliquot of the purified DNA products sample was electrophoresed on an 2% agarose gel to estimate the concentration and the integrity of the DNA for the purpose of DNA sequencing.

2.2.2.2 DNA Sequencing

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit supplied by Applied Biosystems is a modification of the Sanger dideoxy sequencing method (Sanger et al., 1977). Cycle sequencing is based on the ability of a DNA polymerase to extend a primer, annealed to the sequencing template, until a chain

terminating dideoxy nucleotide is incorporated. Each sequencing reaction includes the four types of deoxyribonucleotide triphosphates (dNTPs: dATP, dCTP, dTTP and dGTP), four different dideoxyribonucleotide triphosphate (ddNTPs) each labelled with a different fluororescent dye (ddATP-green, ddCTP-blue, ddTTP-red and ddGTP-yellow), one primer, buffer, magnesium and a thermostable DNA polymerase. Chain termination occurs when a ddNTP is incorporated in to the DNA strand as the ddNTPs lack the 3'-OH group necessary for chain extension. As the concentration of ddNTPs is much lower than that of the dNTPs, the chain termination occurs randomly at G, A,T or C, depending on the respective dideoxy analogue in the reaction. The resulting fragments, each with a common origin but a different termination point, are separated by electrophoresis allowing the nature of the fluorescence and hence the chain terminating nucleotide to be identified. Data is recorded electronically and presented as a chromatographic intensity profile with ATGC translation (Brown, 1994).

The BigDye™ terminator cycle sequencing reaction contained 4 µl of the terminator premix, 1 µl primer (~50ng) and the appropriate amount of DNA as template 1-5 µl made up to a final volume of 10 µl with nuclease free water.

All reactions were placed into a MJ mini cycler with a Hot Bonnet (does not require paraffin overlay) and amplified as follows: 94^{0}C for 30 seconds, 50^{0}C for 30 seconds and 60^{0}C for 4 minutes and 15 seconds. The cycle sequencing was conducted for 30 cycles and left at 4^{0}C until purification.

On completion of the thermal cycling, it was necessary to separate the sequencing reaction products from excess nucleotides and primers from the sequencing reaction, prior to the products being analysed on an ABI 373A DNA sequencer.

Ten microlitres of nuclease free water, 50 µl of 95% ethanol and 2 µl of 3M sodium acetate (pH 5.2) was added to the sample which was vortexed and left to stand at room temperature for 15 minutes. The precipitate was then centrifuged at 14 000 rpm for 30 minutes. The supernatant was removed with a pipette and the remaining pellet was washed with 250 µl of 75% ethanol and centrifuged for 15 minutes. The ethanol wash was removed and the sample air-dried for approximately 1 hour.

The SeqEd 675 DNA sequence editor was used to visulise text and chromatograms of the sequence template. Chromatograms were also manually examined to confirm the computer assigned calling of bases in the sequence.

2.3 RESULTS

2.3.1 Genomic DNA Extractions

The concentration and relative purity of the extracted DNA was assessed after spectrophotometry and agarose gel electrophoresis.

The DNA extracted from the three GR samples ranged in concentration and purity. There was no evidence of RNA contamination after agarose gel electrophoresis as illustrated in Figure 2.2 (p. 35). Protein contamination was present in the DNA extraction from dog 1263 sample, as indicated by an $OD_{260/280}$ nm ratio of less than 1.8 (Table 2.5). This sample was disregarded and not used in further applications.

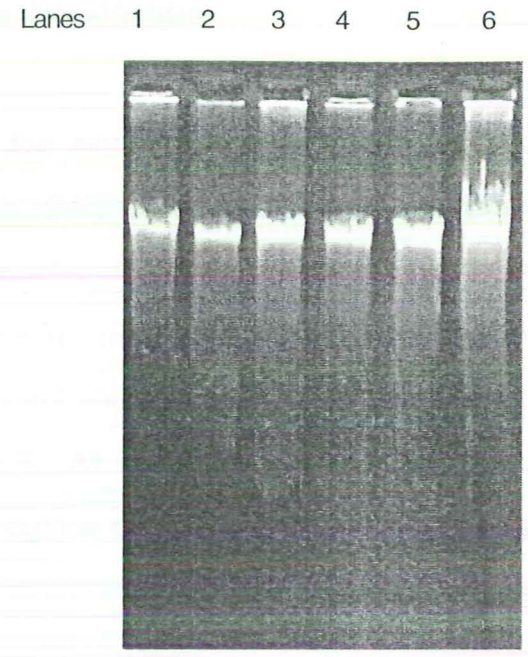


Figure 2.2: Genomic DNA Extractions. Agarose gel electrophoresis of genomic DNA extractions from three golden retriever thymic samples. Extractions and electrophoresis was performed as described in methods sections 2.2.1.3 and 2.2.2.5. Lane 1: Lambda (λ) Ladder one (~ 100 kb). Lane 2: λ Ladder two (~ 50 kb). Lane 3: Sample 19C5. Lane 4: Sample 6B00. Lane 5: Sample 1263. Lane 6: Undigested λ.

Table 2.5: ***Concentration and Purity of the Extracted Genomic DNA***

Sample (chip numbers)	$OD_{260/280}$ ratio	Concentration (μg/ml)
19C5	1.8	1088.3
6B00	1.8	768.3
1263	1.6	1562.5

The genomic DNA samples were approximately of 50 kb or greater. This was determined by comparison to the undigested λ cloning vector which is approximately 48.5 kb and to the two λ ladders created through concatemerisation of the λ vector.

2.3.2 Long Range Amplification

Figure 2.3 shows the long range amplification of introns 5 to 9 by Elongase (GibcoBRL, 1999a). Only the amplification with the intron 8 and intron 9 primer sets yielded strong product bands that were observed at approximately 1.4 kb and 1.1 kb respectively (Figure 2.3). In contrast, the amplification with primer sets for introns 5, 7 and the positive control showed non-specific smearing, while multiple bands were observed for intron 6. All reaction negative controls were free from contamination, except the intron 6 negative control in which a faint but distinctive band was observed at ~ 2.0 kb.

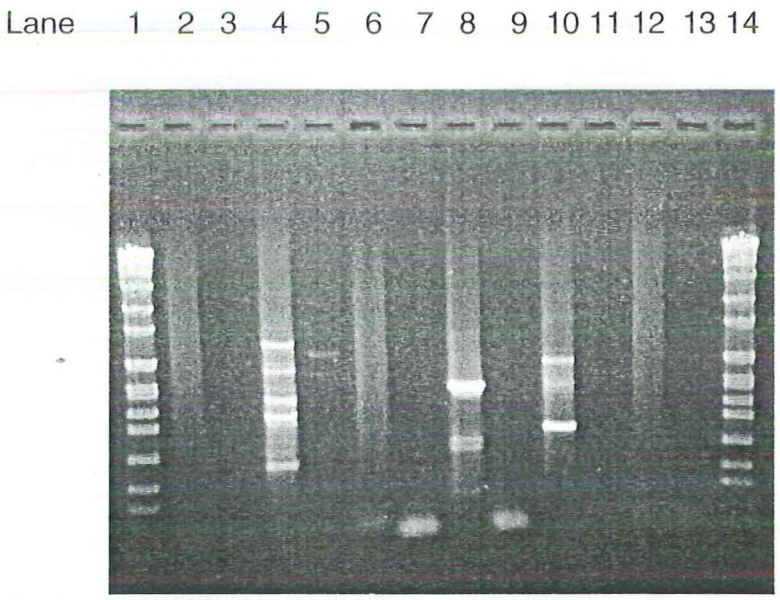


Figure 2.3: Long Range Amplification of Canine Dystrophin Introns. Lane 1: SPP-1 molecular weight marker. Lane 2: Intron 5. Lane 3: Intron 5 negative control. Lane 4: Intron 6. Lane 5: Intron 6 negative control. Lane 6: Intron 7. Lane 7: Intron 7 negative control. Lane 8: Intron 8. Lane 9: Intron 8 negative control. Lane 10: Intron 9. Lane 11: Intron 9 negative control. Lane 12: Exon 79-UTR. Lane 13: Exon 79-UTR negative control. Lane 14: SPP-1 molecular weight marker.

Forty microlitres of the intron 8 and 9 PCR reaction were separated on a 1.5% LMPAG. The bands were excised and purified using Qiaquick purification system (Qiagen, 1997). Five microlitres of the purified DNA was electrophoresed on a 1% agarose gel at 100 V for 50 minutes to estimate the concentration of the PCR product after purification. Discreet bands were observed at 1.4 kb and 1.1 kb for intron 8 and 9 respectively.

2.3.3 Sequencing

Two microlitres of the purified intron 8 and 9 products were used as templates in the sequencing reactions. The original amplification primers were used to sequence intron 8 and 9 in both directions to establish the sequence of the 5' and 3' splice sites. Intronic sequences were obtained for intron 8 in both directions (Figure 2.4 and 2.5). The sequencing of intron 9 revealed that this PCR product was in fact a PCR reaction artefact. An 2'-0-methyl antisense oligonucleotide was designed at the 5' splice site of intron 8 to induce skipping of exon 8 and ordered through Geneworks, Australia (Figure 2.4).

Further attempts were made to optimise the PCR for the amplification of introns 5, 6, and 7. PCR strategies that were attempted included varying the amount of DNA to decrease background smearing, increasing the annealing temperature to 55^{0}C to increase the specificity of the primers and lastly, increasing the extension times to 15 minutes. Temperatures could not be raised above 55^{0}C as the GC content of the primers was less than 50%. Despite these further attempts to optimize the PCR, no specific PCR products were obtained.

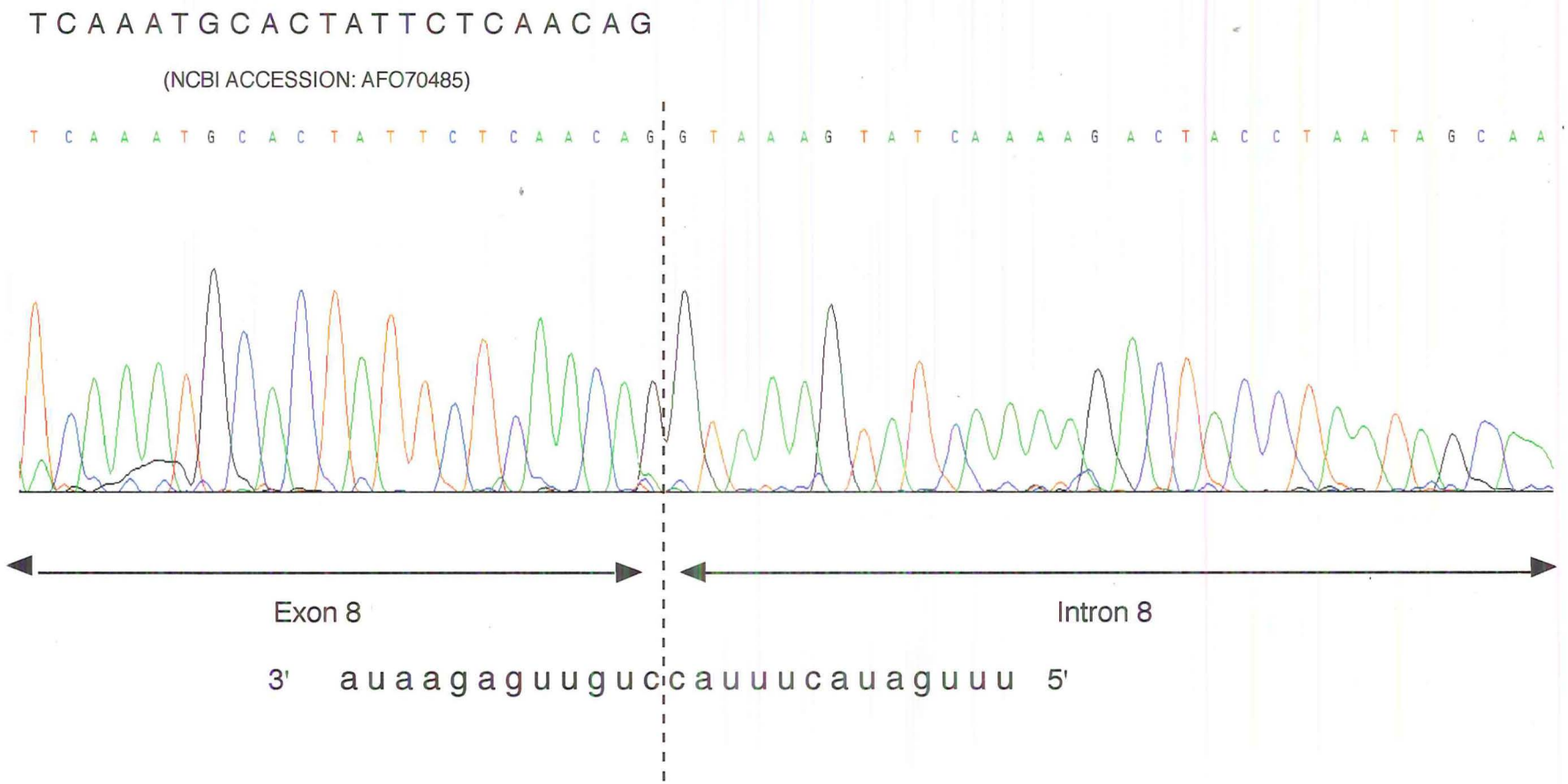


Figure 2.4: **Chromatogram of the 5' Splice Site of Intron 8 obtained from Long Range Amplification of Canine Dystrophin Introns and below the AO Designed to Block this Stite**

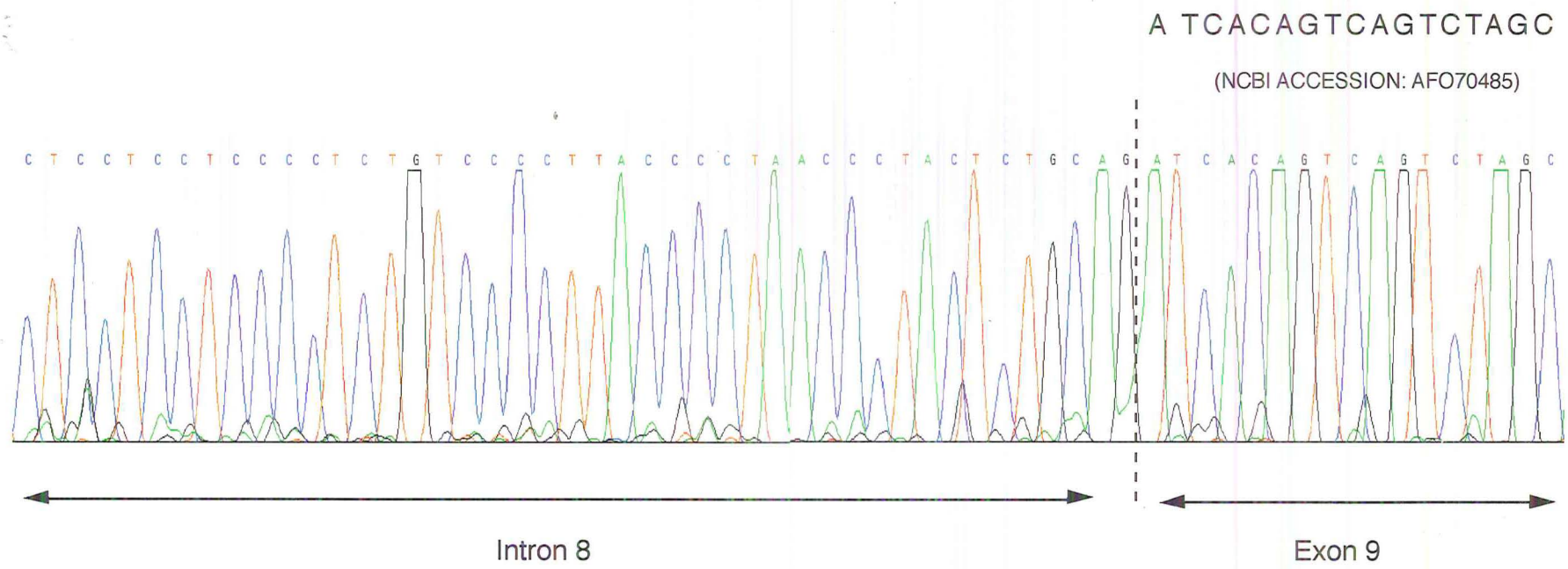


Figure 2.5: **Chromatogram of the 3' Splice Site of Intron 8 obtained from Long Range Amplification of Canine Dystrophin Introns**

2.4 CONCLUSIONS

Both spectrophotometric and agarose electrophoresis analysis indicated that the genomic DNA extracted using the DNA isolation kit for cells and tissues (Roche Diagnostics, Australia) was of sufficient quantity and quality to be suitable for long range amplification and for further applications in this project.

Long range amplification of intron 8 from canine genomic DNA was successful in amplifying a 1.4 kb PCR product.

Sequencing of this PCR product with both the 8 forward and 9 reverse primers identified the 5' and 3' splice sites of intron 8.

CHAPTER 3.0 PCR SCREENING OF A CANINE PHAGEMID LIBRARY

3.1 INTRODUCTION

The next experimental approach was to construct a canine (GR) genomic DNA phagemid library. This was to be used to PCR screen the remaining dystrophin intron/exon boundaries.

As conventional PCR can only amplify DNA targets less than 3 kb, the basic strategy of this experiment was to construct a phagemid library in which the genomic DNA inserts were less than 1.0 kb. Due to the small sizes of the DNA inserts, the library was to be screened by conventional PCR using exon specific primers and the M13 primer sites flanking the multiple cloning site (MSC).

3.2 METHODS

3.2.1 The Plagemid Vector

Phagemid vectors are plasmids which have been artificially manipulated so as to contain a small segment of filamentous phage genome such as M13, fd or f1. Phagemids permit the successful cloning of inserts several kilobases long. The pBluescript II SK' phagemid vector supplied by Stratagene was selected for the construction of the canine phagemid library, because it was a suitable vector and it was available in the laboratory. The pBluescript II SK' phagemid vector is a 2961 bp phagemid derived from pUC19 and contains a MCS of 657-759bp.

3.2.2 Preparation of Vector DNA

15 µg of the pBluescript II SK' vector DNA was digested with 100 U of *Sma*1 (recognition sequence: CCC↓GGG) in a total volume of 200 µl (20 µl of 10x multicore buffer, 165 µl water) for 2 hours at 37°C. Enzyme activity was terminated by heat inactivation for 15 minutes at 65°C. The sample was then ethanol precipitated by the addition of 2.5 volumes of 100% ethanol and 1/10 volume 0.3 M sodium acetate, followed by centrifugation at 13 000 rpm for 10 minutes at 4°C. The supernatant was removed and 100 µl of 70% (w/v) ethanol was added. The sample was again centrifuged at 13 000 rpm for 10 minutes at 4°C. The ethanol wash was removed and the DNA pellet left to air dry at room temperature for 15 minutes. The DNA pellet was resuspended in 30 µl of water to a final concentration of 0.5 µg/µl.

3.2.3 Preparation of Genomic DNA: Blunt End Digestion

Using the canine genomic DNA extracted from GR dog 6B00 (Section 2.2.1., p. 24), two complete digests of genomic DNA were constructed. One digest using *Sma*1, a six-base cutter, with the other using *Rsa*1, a four-base cutter (recognition sequence: GT↓AC).

3.2.3.1 *Sma*1 Complete Digestion of Genomic DNA

10 µg (1.0 µg/µl) of canine genomic DNA was digested with 80 U of *Sma*1 and dephosphorylated with 0.8 µl of Shrimp Alkaline Phosphatase (SAP) (U/µl) in a final volume of 200 µl at standard buffering conditions (20 µl of 10 X multicore buffer and 160 µl of water) for 2 hours at 37°C. The sample was ethanol precipitated as

described in Section 3.2.2 (p. 42). The DNA pellet was resuspended in 20 µl of nuclease free water to a final concentration of 0.5 µg/µl.

3.2.3.2 *Rsa*1 Complete Digestion of Genomic DNA

10 µg (1.0 µg/µl) of canine genomic DNA was digested with 80 U of *Rsa*1 and dephosphorylated with 0.8 µl of SAP (U/µl) to a final volume of 200 µl at standard buffering conditions (20 µl of 10 X multicore buffer and 160 µl of water) for 2 hours at 37°C. The sample was ethanol precipitated as in Section 3.2.2 (p. 42). The DNA pellet was resuspended in 20 µl of nuclease free water to a final concentration of 0.5 µg/µl.

3.2.3.3 Agarose Gel Electrophoresis

5µl aliquots of complete digestions of both vector and insert DNA were electrophoresised on a 2% agarose gel, stained with ethidium bromide and photographed for analysis.

3.2.4 Ligation of Phagemid Vector and Insert DNA

Ligation was performed using a 1:3 molar ratio of vector to insert DNA as per the recommendations made by the Promega (1996), "Protocol and Application Guide" (p. 45) for a 3.0 kb vector and a 0.5 kb insert. The ligation was constructed according to the manufacturers directions (see, Table 3.1). Incubation was overnight at 14°C.

Table 3.1: ***Ligation Reaction***

Component	Volume (µl)	Final Concentration
Vector DNA (0.5µg/µl)	1	500 ng
Insert DNA (0.5µg/µl)	3	1500 ng
T4 DNA Ligase (GibcoBRL)	0.3	1 U (Weiss units)
Ligase 10x Buffer	5	1x
Nuclease Free Water	41	-
Total	**50**	

Three 10 µl ligation controls were also constructed for the vector DNA only and for each of the digested insert DNA.

3.2.5 Screening by PCR Amplification

3.2.5.1 Primary Amplification

As only a few copies of the exons of interest existed in the phagemid library, an asymmetric PCR approach was adopted for primary amplification. Components of the primary amplification are outlined in Table 3.2. In this method, the conventional PCR was set up but using only the exonic primer for the first 40 cycles of amplification. On completion of thermocycling, the M13 primer was added and thermocycling conditions recommenced for a further 20 cycles. In this way the reaction was enriched with linear fragments that contained only the exon of interest before the M13 primer was added. Primers used for primary amplification are illustrated in Table 3.3.

Table 3.2: ***Components of the Primary Amplification PCR***

Component	Volume (µl)	Final Concentration
Sterile water	12.9	-
5.2^{100}	5	1x PCR buffer, 2mM $MgCl_2$ + 100µM dNTP's
Primers (50ng/µl each)	2	4 ng/µl
Thermus thermus (*Tth*) DNA polymerase (5.5U/µl)	0.1	0.05 U/µl
Target DNA	5	-
Total	**25**	-

Table 3.3: ***Primers used for Primary Amplification***

Canine Exon	Primer Direction	Sequence 5' to 3'
6	F	GTCAAAAATGTAATGAAAAATATCAT
	R	CTATGACTATGGATGAGAGCATTCA
8	R	GATGGCCTTGGCAACATTTCC
9	F	CCTAAGCCTGGATTCAAGAG
M13F	F	CGCCAGGGTTTTCCCAGTCACGAC
M13R	R	AGCGGATTTCAATTTCACACAGGA

Thermal cycling was performed under the following conditions: 94°C for 2 minutes followed by 39 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes. On completion of the thermocyling conditions the vector primer was added and the thermocycling conditions recommended for a further 20 cycles. The PCR reaction products were then electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed for analysis.

3.3.5.2 Secondary Amplification

Fragments generated from primary amplifications were isolated from other products in the sample through secondary amplification using a semi-nested PCR technique. The

semi-nested approach involves using exonic primers internal to the exonic primers used in the primary amplification. This acts to increase the specificity and sensitivity of the amplification reaction. The components of the semi-nested PCR reaction are shown in Table 3.3. Primers used for semi-nested PCR are illustrated in Table 3.4.

Table 3.4: ***Components of Secondary PCR Reaction Mix***

Component	Volume (µl)	Final Concentration
Sterile water	34	
5.1^{50}	10	1X PCR Reaction Buffer, 50 mM dNTP's and 1 mM $MgCl_2$
Primers (50ng/ml)	4	4 ng/µl
T. th DNA polymerase (5.5U/µl)	0.2	0.11 U/µl
Target DNA	1	
TOTAL	50	

Thermal cycling was performed under the following conditions: 94°C for 2 minutes followed by 39 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes.

Table 3.5: ***Primers used for Secondary Amplification***

Canine Exon	Primer Direction	Sequence 5' to 3'
Exon6IR*	Nested R	CG(A/G)ACCCAGCTCAG(A/G)AGAATC
Can8R	Nested R	CATTAAGATGGACTTCTTATCTGG
DMD2a	Nested F	CGATTCAAGAGCTATGCCTAC

3.3.5.3 Bandstab PCR

Bandstab PCR (Wilton et al., 1997c) was another technique used for secondary amplification of PCR products. The bandstab mixtures were prepared using the

same reaction conditions as the semi-nested secondary PCR (Section 3.3.5.2), however template DNA was taken directly from a band in an agarose gel. After gel fractionation and ethidium bromide staining of the PCR products, the gel was rinsed briefly with distilled water. The band of interest was stabbed with a sterile yellow pipette tip and the tip placed into the reaction mix for one minute. The contents of the pipette tip were expelled into the PCR reaction. The reaction was overlayed with paraffin oil before thermocycling. The cycling conditions are outlined in section 3.3.5.2.

3.2.6 DNA Sequencing

Products to be sequenced were purified using the Qiaquick purification system as previously described in Section 2.2.2.1. (p. 31). DNA Sequencing was conducted as outlined in Section 2.2.2.2 (p. 32) using the nested exonic primers to generate the product.

3.3 RESULTS

3.3.1 Digestion of Vector and Insert DNA

Figure 3.1 (p. 48) shows the digested products of the phagemid vector DNA and the canine genomic DNA. The digestion of the vector was successful and observed as a 2.81 kb fragment. The *Sma*1 digest of the dog genomic DNA was not successful. The genomic DNA appeared to have changed little in regard to size. In contrast, the complete *Rsa*1 digestion was successful and observed as a smear of DNA extending from ~ 3.5 kb to less then 300 bp.

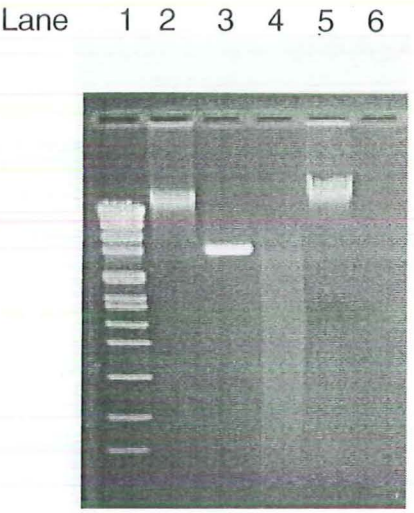


Figure 3.1: Digestion of Phagemid Vector and Canine Genomic DNA. Lane 1: SPP-1 molecular weight marker. Lane 2: *Sma*1 digested canine genomic DNA. Lane 3: *Sma*1 bluescript vector DNA. Lane 4: *Rsa*1 digested canine genomic DNA. Lane 5: undigested λ. Lane 6: Blank.

3.3.2 Ligation of Vector and Insert DNA

The ligation of the vector and insert DNA is shown in Figure 3.2 (p. 49). While smeary, a distinct upward shift of DNA above the digested vector was observed for the *Rsa*1 digested genomic DNA inserts and the phagemid vector, indicating that ligation had taken place. The *Sma*1 digest did not appear to have worked as successfully. Out of the three controls only the *Sma*1 digested DNA was not detected on the agarose gel suggesting the presence of an inhibitory substance contained with the digested DNA sample.

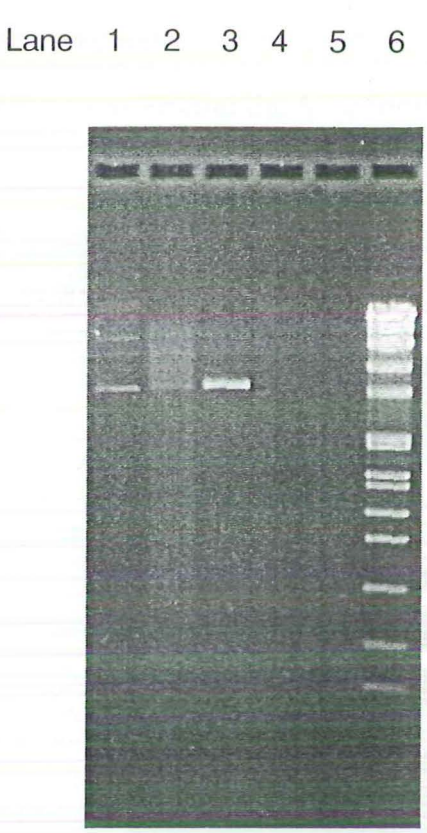


Figure 3.2: Ligation of Vector and Insert DNA. Lane 1: Ligation of *Sma*1 digested canine genomic DNA with the digested bluescript vector. Lane 2: Ligation of *Rsa*1 digested canine genomic DNA with the digested bluescript vector. Lane 3: Ligation of digested bluescript vector. Lane 4: Ligation of *Sma*1 digested genomic DNA. Lane 5: Ligation of *Rsa*1 digested canine genomic DNA. Lane 6: SPP-1 molecular weight marker.

3.3.3 PCR Screening

Figure 3.3 (p. 50) shows the primary amplification of exons 8 and 9. Two product bands were observed at approximately 720 bp and 350 bp for the exon 8 and

M13-120 forward primer combination. For exon 9, only one product band was observed at ~720 bp for the exon 9F and the M13 forward primer.

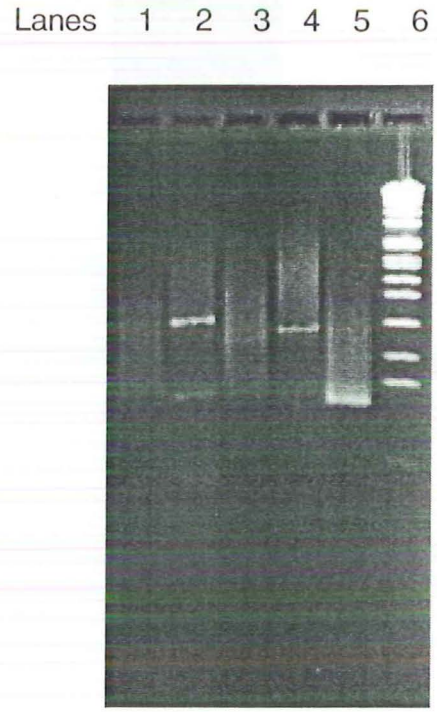


Figure 3.3: Primary Amplification of the Canine Genomic DNA Phagemid Library for Exons 8 and exon 9. Lane 1: Exon 8 reverse and M13R. Lane 2: Exon 8 reverse primer and M13F. Lane 3: Exon 9 forward and M13R. Lane 4: Exon 9 forward and M13F. Lane 5: Positive control; M13 F and R. Lane 6: SPP-1 molecular weight marker.

Secondary amplification of exon 8 using nested primers amplified both the small and large PCR fragments (Figure 3.4, p. 51). These fragments were separated through gel purification on a 1.5% LMPAG (as described in Section 2.2.2.1, p. 31). The sequencing of the only, small ~ 350 bp product obtained the intronic sequence for the 3' splice site of intron 7 as illustrated in Figure 3.5.

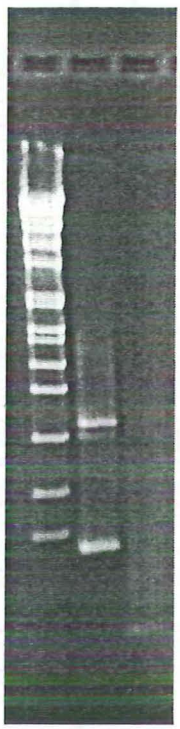


Figure 3.4: Secondary Amplification of Primary Amplification Products from Exon 8 and 9. Lane 1: SPP-1 molecular weight marker. Lane 2: Semi-nested amplification of exon 8 and M13F. Lane 3: Semi-nested amplification of exon 9 and M13F.

Secondary amplification of exon 9 failed to produce any significant product. Further optimisation by decreasing the annealing temperatures to 55 °C and 58°C failed to produce any significant products.

Amplification of exon 6 (Figure 3.6) was conducted at 55°C as a result of the low G C content of the exon 6 primers. Figure 3.6 shows the primary amplification of exon 6 from the phagemid library. A ~350 bp PCR product was produced for both the 6F

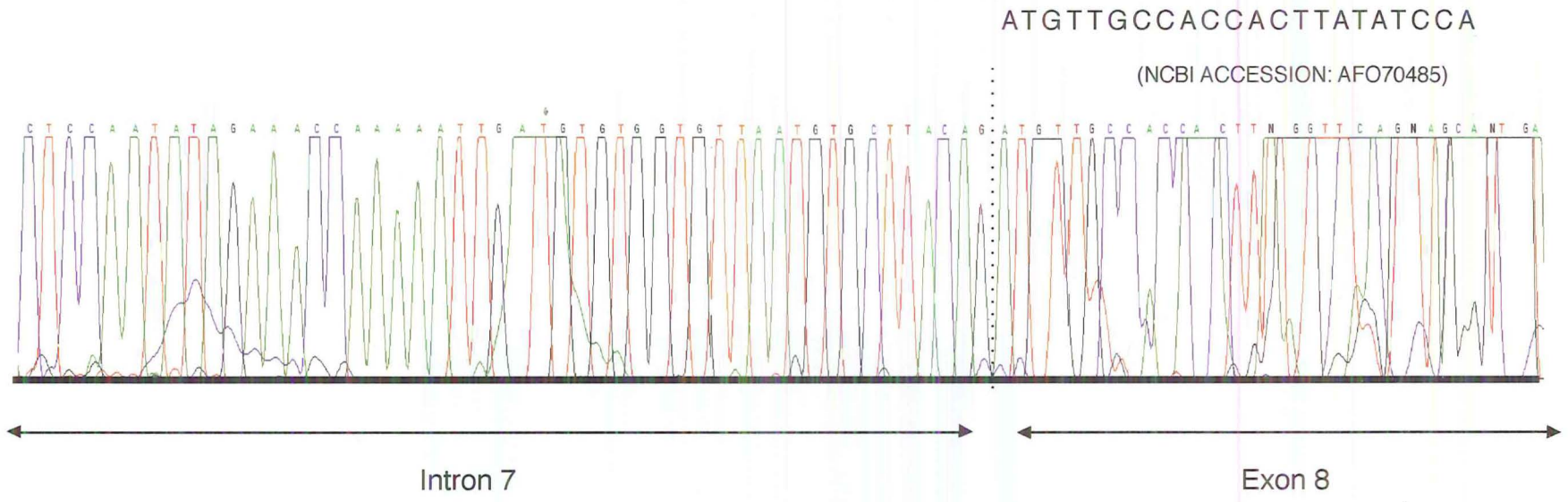


Figure 3.5: Chromatogram of the 3' Splice Site of Intron 7 obtained from the PCR Screening of the GR Phagemid Library

3.4 CONCLUSIONS

The PCR screening of the canine GR genomic DNA phagemid library was successful in amplifying a 350 bp PCR product using the exon 8 reverse and M13 forward primers. Sequencing of this PCR product with the exon 8 reverse primer identified the 3' splice site of intron 7.

CHAPTER 4.0 COSMID LIBRARY CONSTRUCTION

4.1 INTRODUCTION

A genomic library contains the total sequences present in the genome of an organism. The larger the insert of genomic DNA in each recombinant, the lower the number of recombinants needed to completely represent the organisms genome (Strachan & Read, 1996).

Cosmid vectors are synthetic hybrids possessing the desirable features of both plasmid and bacteriophages and permit the cloning and propagation of large fragments of foreign DNA (Strachan & Read, 1996). Cosmid vectors complete with an *in vitro* packaging system can clone genomic DNA in the 32-47 kb range and are therefore preferred for the construction of genomic libraries for screening by conventional hybridisation.

In this experiment, a GR genomic cosmid library was constructed, initially to identify the intronic sequences surrounding exon 6.

4.2 METHODS

4.2.1 The Cosmid Vector

The SuperCos 1 vector supplied by Stratagene was selected for application due to its ability to accommodate genomic DNA fragments ranging in size from 30 to 42 kb.

4.2.1.1 Preparation of Vector DNA

Isolation of SuperCos 1 cosmid vector DNA was based on Sambrook et al (1989) protocol for preparation of vector DNA.

50 ml of LB media supplemented with Kanamycin (5 µg/ml) was innoculated with a single SuperCos clone. The culture incubated overnight at 37°C with shaking at 200 rpm. The culture was centrifuged for 10 minutes at 4°C. The pellet was resuspended in TEG swelling buffer to which 10 ml of alkaline SDS was added. The sample was incubated on ice for 10 minutes after which 7.2 ml of high salt neutralisation buffer (3 M KOAC) was added. The sample was centrifuged for 10 minutes at 4°C and at 13 000 rpm. The supernatant was poured through a sterile gauze into a fresh tube and an equal volume of isopropanol was added. The sample was again centrifuged for 10 minutes at 4°C and at 13 000 rpm. The supernatant was discarded and the pellet resuspended in 2 ml of nuclease free water. 2 ml of LiCl was added to the sample and incubated on ice for 10 minutes. DNA was extracted by the phenol/chloroform method and centrifuged for 13 000 rpm for 5 minutes at room temperature. The phenol/chloroform method of DNA extraction involves extraction with a phenol-chloroform-isoamyl alcohol (25:24:1) mixture saturated with 50mM Tris-HCL (pH 8.0) followed by extraction with chloroform. The aqueous phase was adjusted with 0.3 M NaOAc (pH 5.5) and ethanol-precipitated. The pellet was washed with 70%(w/v) ethanol and the ethanol wash was removed and the DNA pellet air dried for 5 minutes and resuspended in 400 µl of 1X TE. 4 µl of RNAase A (10mg/ml stock) was added to the sample which was incubated for 15 minutes at 37°C. The sample was divided into 4, 50 µl samples. Three of these samples were re-extracted by different methods, the final sample was not treated further.

The first re-extraction method was phenol choloroform extraction as described above. The second re-extraction was by ethanol precipitation as described in Section 3.2.2 (pp. 42). The third re-extraction was by Qiquick purification as described in Section 2.2.2.1 (pp. 31). All samples were resuspended in 50 µl of nuclease free water (except the untreated sample which was already in 50 µl of nuclease free water. The concentration and purity of the isolated superCos vector DNA was assessed by spectrophotometry and agarose gel electrophoresis as described in Section 2.2.1.4 (p. 26) and 2.2.1.5 (p. 27).

4.2.1.2 Digestion and Dephosphorylation of Vector DNA

50 µl, 60 µg of Supercos 1 cosmid vector (Sample B) extracted by ethanol precipitaion was digested with 200 U *Xba*1 at standard buffering conditions (25 µl of 10 x multicore buffer) and dephosphosphorylated with 5 units of SAP to a total volume of 250 µl with nuclease free water. The sample was incubated at 37°C for 1 hour. The reaction was adjusted to 15mM EDTA to inactivate the *Xba*1 followed by incubation at 68°C for 10 minutes to inactivate the SAP. The sample was ethanol precipitated and resuspended in 50 µl of nuclease free water.

A 5 µl aliquot was then electrophoresed on a 0.5% (w/v) agarose gel to check for complete digestion of the cosmid vector which was recognised as a single linear cosmid band of 7.6 kb.

4.2.1.3 Digestion of *Xba*I- and SAP-treated DNA with *Bam*HI

Twently five micrograms (21µl) of the *Xba* I- and SAP-treated vector DNA was digested with 5 U/µg of *Bam*H I enzyme in a total volume of 250 µl at standard

buffering conditions (25 µl of 10 x multicore buffer) for 1 hour at 37°C. The DNA was ethanol-precipitated and resuspended in nuclease free water to a concentration of 1.0µg/µl.

4.2.2 Preparation of Genomic DNA

4.2.2.1 Partial Digestion and Dephosphorylation of Insert DNA

As large DNA inserts were required for cloning into the cosmid vector, the manufacturer recommended that genomic DNA must be ~150 kb before digestion. The genomic DNA extracted from the GR dogs 19C5 and 6B00 (see, Section 2.2.1, p. 24) were considered to be larger than 50 kb and therefore adequate for this application. Utmost care was taken throughout the digestion of the genomic DNA during both mixing and aliquoting steps, gentle inversion and gentle pipettling with wide bore tips to prevent shearing of the DNA.

In order to clone into the *Bam*H I site of the of the SuperCos 1 vector the genomic DNA was partially digested with *Sau*3A I. Using extracted genomic DNA (section 2.2.1, p. 24) two trial digests using 0.5 U and 0.1 U of *Sau*3A 1 were constructed to determine the ideal conditions for obtaining the desired insert size range of 30 to 42 kb. Ten micrograms of genomic DNA, 10 µl of 10 x multicore buffer, 1 µl Bovine serum albumin (BSA), in a final volume of 100 µl made with nuclease free water was pre-equilbrated at 37°C for 5 minutes before the 0.5 U or 0.1 U of *Sau*3A 1 was added. During various time points of 0-, 5-, 10-, 15 minutes, 15 µl aliquots were removed from the reaction. To these samples 6 x GLB loading buffer was added and the samples electrophoresised on a 0.5% (w/v) agarose gel.

After the optimal time interval of the digestion had been determined, a large scale partial digestion of 50 µg of genomic DNA was then performed in a total reaction volume of 500 µl. The reaction was scaled up to best mimic the test conditions of the partial digest including enzyme concentration, temperature and reaction time. Also included in the large scale digestion was 2 U of SAP. After digestion the sample was ethanol precipitated and resuspended in 25 µl of nuclease free water. A 0.1 µl of the large scale digests were electrophoresised on a 0.5% (w/v) to check for digestion compared to undigested λ DNA marker.

4.2.3 Ligation of Insert DNA and the SuperCos 1 Vector

The ligation was constructed by adding the following components to a eppendorf tube: 2.5 µg of the partially digested SAP genomic DNA, 1.0 µl of the SuperCos 1 DNA (Xba-SAP and digested with BamH-1[1 µg/µl]) was added to 2.0 µl 10 x ligase buffer (Promega) in a final volume of 20 µl made with nuclease free water. A 2 µl aliquot was removed and placed at 4°C and stored for gel analysis. To the sample 0.3 U of T4 ligase (supplied by Promega) was added and left to ligate overnight at 14°C. The T4 ligase buffer supplied by Promega did not contain polyethylene glycol (PEG) which can inhibit packaging.

4.2.4 Packaging

4.2.4.1 The Gigapack III XL Packaging Extract and Protocol

Packaging extracts are used to package recombinant phage with high efficiency. The single tube format of Gigapack III packaging extract, simplifies the packaging procedure and increases the efficiency and representation of libraries. The Gigapack III

XL packing extract supplied by Stratagene is designed for use in genomic cosmid library construction and can preferentially package 47- to 51 kb recombinants.

The Giga pack III XL packing extract was removed from -80°C storage and placed on dry ice. The packaging extract was quickly thawed and the experimental DNA (1-4 µl containing 0.1-1.0µg ligated genomic and vector DNA) was combined with the packing extract. The tube was stirred with pipette tip and then incubated at room temperature for 2 hours. Following incubation, 500 µl of SM buffer and 20µl of chloroform were added to the sample and the contents gently mixed. The sample was then centrifuged briefly to sediment the debris. The supernatant was then ready for titering.

4.2.4.2 Gigapack III Packaging Extract Efficiency Test

4.2.4.2.1 Preparation of Host (VCS257) Bacteria

Cells from the VCS257 host strain bacterial frozen glycerol stocks were revived by scraping off splinters with a sterile tip and streaking them on to LB agar plate which was placed at 37°C and incubated at overnight.

Fifty millilitres of LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$ in a sterile flask was inoculated with a single colony of the VCS257 host strain. The flask was then placed at 37°C for 4 to 6 hours until the cells reached an OD_{600} of 0.5. These cells were centrifuged in a falcon tube for 10 minutes at 2000 rpm and the cell pellet resuspended in 15 ml of sterile 10 mM $MgSO_4$. Cells were then diluted to a final OD_{600} of 0.5 with 10 mM $MgSO_4$ and stored until use at 4°C

4.2.4.2.2 Efficiency Test Protocol

The frozen wild type lambda control DNA (λc1857 *sam7*) was thawed on ice and gently mixed. Using 1 µl of the wild type lambda control DNA (~0.2 µg) two consecutive 10^{-2} dilution's of the packaging reaction were made in SM buffer to yield a 10^{-4} dilution of the packaging reaction. Ten microlitres of 10^{-4} dilution was added to 200 µl the VCS257 host strain (producing an effective $4.8X10^{-6}$ dilution of the packaging reaction) and incubated at 37°C for 15 minutes. To the sample 3 ml of of 0.7% top agar (approximately 48°C) was added and the mixture poured onto a dry re-warmed LB agar plate. The plate was then incubated overnight at 37°C. Phage growth was stopped with 1 ml of SM buffer. Approximately 400 plaques per plate were expected from the 210 µl mixture of packaging reaction and VCS257 host strain plated out.

4.2.5 Titering the Unamplified Cosmid Library

A fresh overnight culture of XLI-Blue MR host cells was prepared as in Section (4.2.4.2.1, p. 60). Two dilutions, 1:10 and 1:50 of the unamplified library was made in sterile SM buffer. Twenty-five microlitres of the two dilutions were mixed with 25 µl of the XLI-Blue MR host cells and incubated for 30 minutes at room temperature. 200 µl of the LB media was added to each sample and placed at 37°C for 1 hour with gentle shaking at each 15 minute intervals. The microfuge tube was centrifuged for 1 minute and the pellet resuspended in 50 µl of fresh LB media. Using a sterile spreader, the cells were plated on LB plates supplemented with 50 µg/ml amplicilin. The plates were then placed at 37°C and incubated overnight.

4.2.6 Amplifying Cosmid Libraries

In a series of 6X15 ml culture tubes, the following was added: 80 µl of the SuperCos canine genomic library, 70 µl of SM buffer and 100 µl of XLI-Blue MR host cells. The samples were incubated at room temperature for 30 minutes. Four volumes of LB broth was added to each sample and the tubes placed at 37°C for 1 hour with shaking at 200 rpm. Each sample was plated out onto 150 mm-LB amplicillin plates and incubated overnight at 37°C. 3 ml of LB broth was poured onto each plate and the colonies scraped off and pipetted into a 50 ml falcon tube. To this sample sterile glycerol was added to a final concentration of 20% and ampicillin was added to 50 µg/ml. The sample was mixed throughly and 1 ml aliquoted into individual sterile 1.5 ml screw capped tubes and stored at -80.°C.

4.3.2 Titering Amplified Cosmid Libraries

One of the 1 ml aliquots of the canine genomic DNA cosmid library of GR dogs 19C5 and 6B00 were removed from -80.°C and placed on ice. A 1:10 and 1:25 dilution were created in LB supplemented with ampicillin (50 µl/ml) and plated out on to LB plates using a sterile spreader. The plates were incubated overnight at 37°C. The colony forming units per millilitre (cfu/ml) of each library was calculated.

4.3.8 Integrity of the Cosmid Library

The integrity of the canine genomic DNA cosmid library was assessed by amplifying exons 6, 10, 52 and 53 from the dystrophin gene using conventional PCR (Section 3.2.5.1, p. 44). Primers are shown in (Table 4.1). A positive control was constructed for each of the exons using pre-existing genomic DNA from a normal male GR (Chip number 711). The PCR reaction products were then electrophoresised on a 2% (w/v) agarose gel, stained with ethidium bromide and photographed for analysis.

Table 4.1: *Primers used to Amplify Canine Dystrophin Exons 6, 10 52 and 53*

Exon	Primer	Sequence 5' to 3'	Expected size of amplification products
6	Can6F	GTCAAAAATGTAATGAAAAATATCAT	173 bp
	Can6R	CTATGACTATGGATGAGAGCATTCA	
10	Can10F	CCT GGA CAG TTA TCA AAC AGC	90 bp
	Can10OR	AATCTCTCCTTGGGCTTGGAG	
52	DMD Ex52F	AATGCAGGATTTGGAACAGAGCCGTCC	110 bp
	DMD Ex52R	TTCGATCCGTAATGATTGTTCTAGCCTC	
53	DMD Ex53-F	TTG AAA GAA TTC AGA ATC AGT GGG ATG	210 bp
	DMD Ex53-R	CTT GGT TTC TGT GAT TTT CTT TTG GAT TG	

4.3 RESULTS

4.3.1 Preparation of SuperCos Vector DNA

Spectrophotometry of the SuperCos 1 vector DNA isolated from the different extraction methods is shown in Table 4.2. The phenol/chloroform extraction method failed to isolate the SuperCos vector DNA. This failure was probably the result of the loss of the DNA pellet during one of the ethanol washes. For the other extraction methods the purity was consistent while concentration ranged from approximately 291 to 610 µg/ml. $OD_{260/280}$ was 1.9 for all four samples.

Table 4.2: ***Concentration and Purity of Extracted Vector DNA.***

Extraction method	$OD_{260/280}$	Concentration (μg/ml)
Phenol/Chloroform	0.0	0.0
Ethanol Precipitation	1.9	291.2
Qiaquick Purification	1.9	553.5
Untreated	1.9	613.0

Agarose gel electrophoresis of these samples revealed the presence of RNA contamination in both the Qiaquicked and untreated SuperCos vector DNA (Figure 4.1). Complete Digestion of the SuperCos vector DNA was therefore performed on only the ethanol precipitated sample, and successfully produced a complete digested Supercos which was observed at ~ 7.6 kb.

Lanes 1 2 3 4 5 6

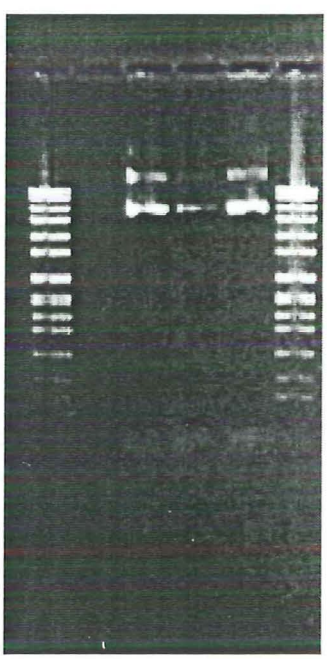


Figure 4.1: Extraction of SuperCos 1 Vector DNA. Lane 1: SPP-1 molecular weight marker. Lane 2: Phenol/chlorofom Extraction. Lane 3: Ethanol precipiation. Lane 4: Qiaquick purification. Lane 5: Untreated. Lane 6: : SPP-1 molecular weight marker.

4.3.2 Digestion of Genomic DNA

Based on the optimal condition indicated by trial partial digests, large scale digests were performed. Optimal conditions indicated by the trial digests for dog 19C5 was 0.1 U of *Sau*3A 1 at 37°C for 15 minutes while for GR dog 6B00 optimal conditions was obtained at 0.5 U of *Sau*3A 1 at 37°C for 10 minutes .

4.3.3 Ligations

Figure 4.2 shows the digested SuperCos 1 vector DNA and the digested SuperCos 1 Vector DNA with GR Genomic DNA (completely digested) before and after ligation with T4 ligase. Lanes 2 and 4 show that ligation worked. The 'bands' in these lanes were shifted upwards relative to the control lane of 1. Of note was the fact that ligation still worked in the presence of GR genomic DNA (see, lane 4).

Lane 1 2 3 4 5

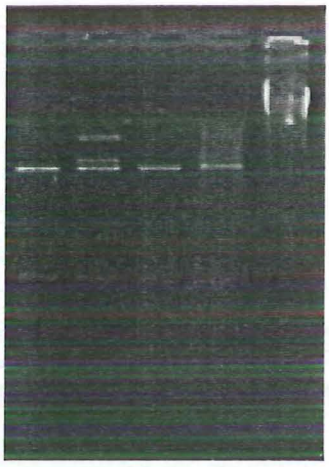


Figure 4.2: Ligations of SuperCos Vector DNA and the SuperCos Vector DNA with GR Genomic DNA. Lane 1: Complete Digest of SuperCos Vector DNA before Ligation. Lane 2: Complete Digest of SuperCos Vector DNA after Ligation. Lane 3: Complete Digest of SuperCos Vector DNA with GR genomic DNA before Ligation. Lane 4: Complete Digest of SuperCos Vector DNA with GR genomic DNA after Ligation. Lane 5: Undigested λ marker.

4.3.4 Packaging

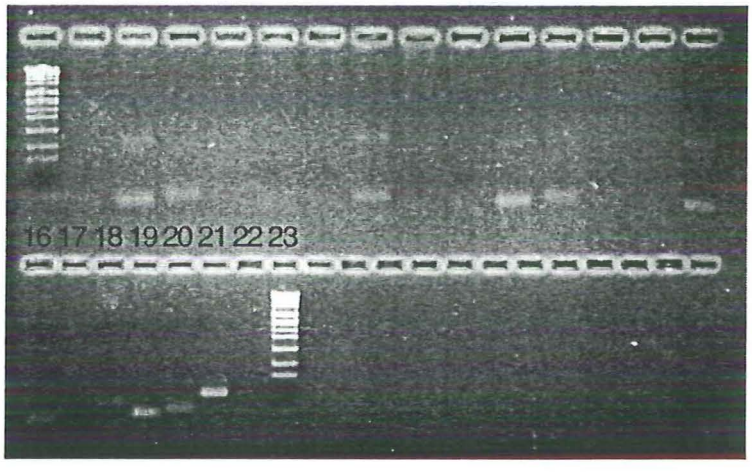
The packaging control showed 100% eficiency with approximately 400 plaques counted on the LB plate. Titering of the amplified GR genomic cosmid library showed that the efficiency of the packaging of the recombinants was not as effective

with only 21 and 22 clones observed at the 1:10 and 9 and 11 clones observed at the 1:25 dilutions for GR dogs 19C5 and 6B00 respectively.

4.3.5 Integrity

The intergrity of the library was assessed by PCR for exons 6, 10 , 52 and 53. All exons were present in the positive GR genomic DNA control. Only exons 10 and 52 amplified in the GR cosmid libraries constructed in this project. Further optimisation to amplify exons 6 and 53 by decreasing and increasing the annealing temperature from 55°C to 50°C and from 55°C to 58°C, respectively and varying the amount of target through dilution failed to produce any significant products. The dystrophin gene in the two GR genomic DNA cosmid libraries constructed in this experiment was, therefore, most probably under represented. Screening for the intronic boundaries surrounding exon 6 by conventional hybridisation was therefore abandoned.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



- As the samples had run below the SPP-1 molecular weight marker an aliquot of the Amplification products were electrophoresied on a 3% agarose gel (w/v) for 80V for 40 minutes. All exons corresponded to expected sizes.

Figure 4.3: Amplification of Canine Dystrophin Exons 6, 10, 52 and 53 from the two GR Cosmid Libraries. Lane 1: SPP-1 Molecular weight marker. Lane 2: 10 ng of GR 19C5 Cosmid Library, Exon 6. Lane 3: 10 ng of GR 19C5 Cosmid Library, Exon 10. Lane 4: 10 ng of GR 19C5 Cosmid Library, Exon 52. Lane 5: 10 ng of GR 19C5 Cosmid Library, Exon 53. Lane 6: 20 ng of GR 19C5 Cosmid Library, Exon 6. Lane 7: 20 ng of GR 19C5 Cosmid Library, Exon 10. Lane 8: 20 ng of GR 19C5 Cosmid Library, Exon 52. Lane 9: 20 ng of GR 19C5 Cosmid Library, Exon 53. Lane 11: 10 ng of GR 6B00 Cosmid Library, Exon 6. Lane 12: 10 ng of GR 6B00 Cosmid Library, Exon 10. Lane 13: 10 ng of GR 6B00 Cosmid Library, Exon 52. Lane 14: 10 ng of GR 6B00 Cosmid Library, Exon 53. Lane 15: 20 ng of GR 6B00 Cosmid Library, Exon 6. Lane 16: 20 ng of GR 6B00 Cosmid Library, Exon 10. Lane 17: 20 ng of GR 6B00 Cosmid Library, Exon 52. Lane 18: 20 ng of GR 6B00 Cosmid Library, Exon 53. Lane 19: Control GR 711 Genomic DNA, Exon 6. Lane 20: Control GR 711 Genomic DNA, Exon 10. Lane 21: Control GR 711 Genomic DNA, Exon 52. Lane 22: Control GR 711 Genomic DNA, Exon 53. Lane 23: PCR negative control. Lane 24: SPP-1 Molecular weight marker.

4.4 CONCLUSION

The cosmid library constructed in this experiment contained insufficient dystrophin clones. There are a large number of factors that could account for this under representation: non-random distribution of restriction sites, restriction activity in the packaging extracts, deletion of sequences by recombination, and selection against representation of the clone of interest during amplification of the library (Sambrook et al., 1989, p. 3.55).

CHAPTER 5.0 PCR SCREENING OF THE CANINE λ LIBRARY

5.1 INTRODUCTION

In a final attempt to obtain intronic sequence for the canine dystrophin gene, a pre-made canine genomic DNA λ (FIX II) library was purchased from Stratagene. This was constructed from beagle genomic DNA. Since it was the only such library available, it was purchased on the assumption that the dystrophin exonic and intronic sequence of the beagle and the GR dogs were identical. This assumption could be tested, at least for exonic sequences.

The basic strategy of this experiment was to biologically amplify this library in a multiwell plate, using a total of 96 wells, such that 3 canine genomes become available for PCR screening. In particular, this amplified genome was to be screened for the presence of the intron boundaries surrounding exon 6.

5.2 METHODS

5.2.1 The λ FIX II Library

The λ FIX II vector is a replacement vector used for cloning large fragments of genomic DNA. λ phages that contain active *red* and *gam* genes are unable to grow on host strains that contain P2 phage lysogens. λ phages without these genes are able to grow on host strains lysogenic for P2. The *red* and *gam* genes in the λ FIX II DNA are located on the stuffer fragment, therefore the wild-type λ FIX II phage cannot grow a P2 phage lysogen such as the bacterial strain XL-Blue MRA (P2). When the stuffer fragment is replaced by an insert, the recombinant λ FIX II vector becomes *red/gam*,

and the phage is able to grow on the P2 lyosgenic strain. Therefore, by plating the library on the XL-Blue MRA (P2) strain, only recombinant phages are allowed to grow. The polylinker for the λ FIX II vector has T3 and T7 bacteriophage promoters which can be used for PCR amplification of inserts.

5.2.5 Integrity of λ FIX II Library: Exon 6 Assay

The integrity of the λ FIX II unamplified library was tested for exon 6 prior to PCR screening of the multiwell plate. Two approaches were adopted: conventional PCR using: *Tth* DNA polymerase (Biotech International); and Platinum *Taq* DNA polymerase (GibcoBRL, 1999b). The primer set shown in Table 5.2 was used to amplify an exon 6 product of approximately 90 bp from the unamplified λ FIX II Library. The components of the conventional PCR using *Tth* DNA polymerase is outlined in Table 5.1.

Table 5.1: ***Components of Conventional PCR***

Component	Volume (µl)	Final concentration
Sterile water	16.9	-
5.2^{100}	5	1x PCR buffer, 2mM $MgCl_2$ + 100µM dNTP's
Primers (50ng/µl)	2	4 ng/µl
T. th DNA polymerase (5.5U/µl)	0.1	0.22 U/µl
Target DNA (various dilu[n])	1	-
TOTAL	25	-

Table 5.2: ***Exon 6 Primers***

Primer	Primer Orientation	Sequence 5' to 3'
Can 6F	Forward	GTCAAAAATGTAATGAAAAATATCAT
Exon 6IR	Reverse	CG(A/G)ACCCAGCTCAG(A/G)AGAATC

Thermal cycling was performed under the conditions described in Section 3.2.5.1 (p. 44). The PCR reaction products were then electrophoresed on a 3% agarose gel, stained with ethidium bromide and photographed for analysis. Optimization was attempted by: varying annealing temperature over the range 50°C-60°C; and by varying the volume of the library target DNA added to the PCR from 1 µl -> 0.01 µl (by dilution).

Platinum *Taq* DNA polymerase (GibcoBRL, 1999b) was then used as the enzyme to attempt this amplification. Its hot start, which acts to increase sensitivity, specificity and yield were considered possibly advantageous for the optimization of this PCR. To improve the liklehood that exon 6 would be amplified with Platinum *Taq* DNA polymerase, 5 ng, 25 ng, 50 ng and 250 ng masses of genomic DNA were used as targets. In addition 1 and 5 µl aliquots from the unamplified library were also used as target in this PCR series. The components of the Platinum *Taq* reaction are outlined in Table 5.3.

Table 5.3: ***Components of the Platinum Taq PCR reactions***

Component	Volume (µl)	Final concentration
Sterile water	17.4	-
10x PCR Buffer	2.5	1x
50mM $MgCl_2$	1	2mM
5mM dNTPs	1	200µM
Primers (50ng/µl)	2	
Platinum Taq polymerase (5.5 U/µl)	0.1	0.22 U/µl
Target DNA	1	-
TOTAL	**25**	-

Thermal cycling was performed under the same conditons as conventional PCR (see, p. 44). The PCR reaction products were then electrophoresed on a 3% (w/v) agarose gel, stained with ethidium bromide and photographed for analysis.

5.2.3 Amplifying the Phage Library

Screening of this library was based on a modified version of the Hughes (1998) protocol for arraying bacteriophage λ libraries for screening by PCR. Hughes protocol involved the transfer of phage particles on to a gridded nylon membrane. This membrane was then divided up into individual pieces and placed in multiwell plates. Phage particles were eluted into SM buffer for pooling and screening by PCR. This novel method involved the amplification of the λ library in liquid suspension in a 96 well PCR plate for pooling and screening by PCR.

In the modified technique reported here, the gridded nylon membrane was eliminated in the interests of economy and because there seemed to be a risk of cross contamination as the nylon membrane was cut into cm^2 pieces. Phage particles were simply amplified in the multiwell plates.

The complexity of the canine library was provided at 6 x 10^9 plaque-forming units per millilitre (pfu/ml). To reduce the labour of screening for single positive clones, library complexity was reduced to 3 canine genomes, spread over 96 wells in a single multiwell plate. A $3x10^3$/µl (3000 phage per well) dilution of the unamplified library was made in sterile SM buffer. To each well of the 96 well plate, 1 µl of $3x10^{-3}$/µl dilution was added to 100 µl of host XLI-Blue MRA (P2) cells (OD_{600} = 0.1) as prepared using same procedure as section 4.2.4.2.1 (p. 60). The plate was then incubated at room temperature for 15 minutes. 200 µl of sterile LB medium was then added to each well and the plate was covered with a plastic cover to prevent inter-well contamination and left at 37°C with gentle shaking overnight.

5.2.4 Titering the Library Array for Amplification

To check the library array for amplification, 1 µl of the diluted phage ($3x10^{-3}$/µl) and 1 µl from 5 randomly picked wells across the plate were combined with 9 µl SM buffer and 200 µl of host bacteria and incubated at 37°C for 15 minutes. Following incubation, 3 ml of 0.7% top agar, at approximately 48°C and supplemented with 10 mM $MgSO_4$, was mixed with each sample and poured on to pre-warmed LB plates supplemented with 10 mM $MgSO_4$. The plates were then incubated at 37°C overnight. Plaques were counted to confirm amplification of the library array.

5.2.5 Screening with Platinum *Taq* PCR

Five microlitres of chloroform supplemented with sodium hydrogen carbonate was added to each well and left at room temperature for 15 minutes with gentle shaking. The cell debris was allowed to settle for 30 minutes.

A strategy of pooling well contents was adopted to reduce, from 96 to 20, the number of exon 6 screening PCRs that would be required. This was achieved by dividing the 96-well plate into twelve columns labelled 1 to 12, and eight rows labelled A to H (see, Figure 5.1, p. 74). Five microlitres from each well in a row was sampled and pooled into a single well. This gave 8 pooled samples (labelled A-H), each containing 12 phage amplifications. Columns were pooled in a similar fashion to give 12 pooled amplifications (labelled 1-12), all containing 8 phage amplifications (see also, Figure 5.1). Since the screening of the genomic FIX II library showed Platinum *Taq* DNA polymerase amplification to be most successful with this library, it was the amplification method employed to screen all pooled samples. The reaction components and cycling conditions employed are described in section 5.2.2 (p. 70). A positive control was set up for exon 6 using 5 µl of the unamplified library.

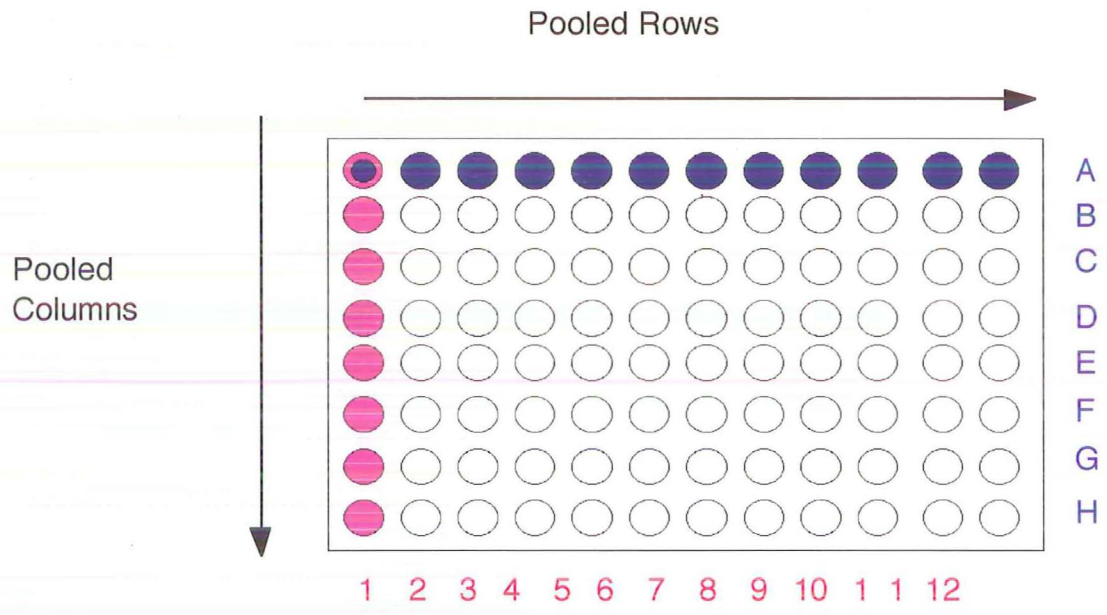


Figure 5.1: Pooling Strategy of the Amplified Multiwell Plate.

Screening of positive well(s) was performed using combinations of an exon specific primer with either the T3 or the T7 vector primer. An inspection of the PCR product size indicates the orientation of the cloned fragment. The T3 and the T7 vector primers are shown in Table 5.4.

Table 5.4: *T7 and T3 primers*

Primer	Primer Orientation	Sequence 5' to 3'
T3 promotor	Forward	TAATACGACTCACTATAGGG
T7 promotor	Reverse	AATTAACCCTCACTAAAGGG

5.2.6 Screening with Elongase PCR

Exon 6 may be located in cloned fragments too far from T7 and/or T3 primers for amplification by Platinum *Taq* polymerase. Its maximum range is approximately 3 kb. To cover this possibility, positive exon 6 clones which Platinum *Taq* polymerase failed to amplify with either T3 or T7 primers, were tested using ELONGASE. The conditions that were employed were described in Section 2.2.2 (p. 28).

5.2.7 Sequencing of Exon 6 Boundaries

PCR products to be sequenced were first purified by the Qiaquick purification system as previous described in Section 2.2.2.1 (p. 31). Following purification, a 5ul aliquot was run on a 2% agarose gel and the intensitiy compared to the DNA markers. The sequencing reaction and analysis of results were performed as outlined in Section 2.2.2.2 (p. 32).

5.3 RESULTS

5.3.1. Integrity of the λ FIX II library: Exon 6 Assay

Despite 17 attempts at optimising conventional PCR using *Tth* DNA polymerase, this approach failed. Fortunately, attempts to amplify exon 6 from the canine (GR) genomic DNA and the unamplified λ FIX II library using Platinum *Taq* DNA polymerase, all succeeded. These results are shown in Figure 5.2 (p. 76).

Lane 1 2 3 4 5 6 7 8 9

Figure 5.2: Optimization of PCR using Platinum *Taq* to Amplify exon 6 from both Genomic DNA and the Undiluted Phage Library. Lane 1: 200 ng DMW-100M molecular weight marker. Lane 2: pUC molecular weight marker. Lane 3: 5 ng of genomic DNA. Lane 4: 25 ng of genomic DNA. Lane 5: 50 ng of genomic DNA. Lane 6: 250 ng of genomic DNA. Lane 7: 1 µl of the undiluted lambda library. Lane 8: 5 µl of the undiluted lambda library. Lane 9: Negative control

5.3.2 Titering the Library Array for Amplification

All 5 wells randomly picked from the multiwell plate, gave total lysis of phage following overnight incubation, indictating that amplification of the multiwell plate had taken place and that PCR amplification was possible.

5.3.3 Identification of Positive Wells

Platinum *Taq* amplification (Section 5.2.2) for exon 6 from the pooled wells A to H indicated that a well in row B was positive for exon 6 (see Figure 5.3, p. 77), while amplifcation of the pooled wells 1 to 12 indicated that a well in column 10 was positive for exon 6 (see, Figure 5.4, p. 78). Using these positive co-ordinates, only one well, 10B, contained exon 6 (see, Figure 5.5, p. 78). Screening of well 10B was performed with the exon 6 forward primer and with either the T3 or T7 vector primers (see, Figure 5.6). Amplification of the exon 6 forward and T7 vector primer set produced a PCR product of 1.3 kb (Figure 5.6).

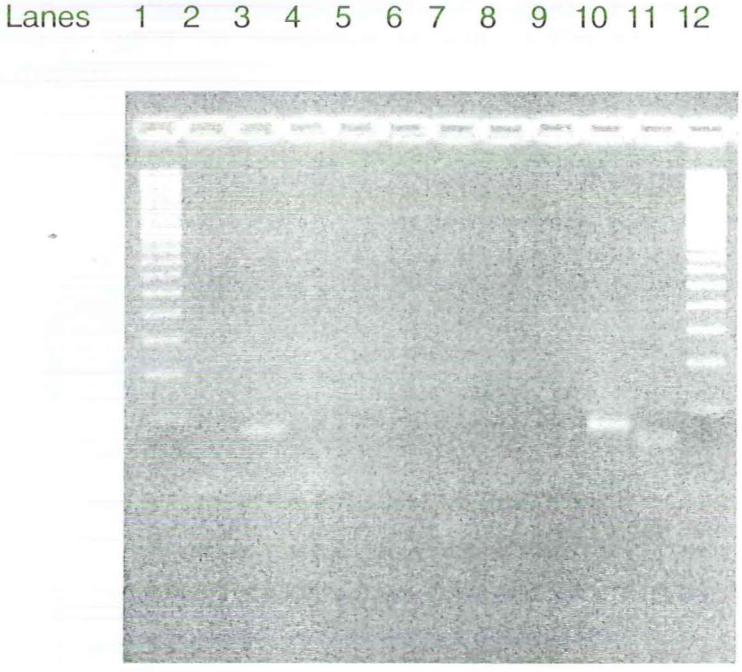


Figure 5.3: PCR Screening of the Pooled Wells A to H from the Library Array. Lane 1: 200ng DMW-100M molecular weight marker. Lanes 2 to 9: pooled wells A to H. Lane 10: Positive control exon 6 from undiluted library. Lane 11: Negative control. Lane 12: 200ng DMW-100M molecular weight marker.

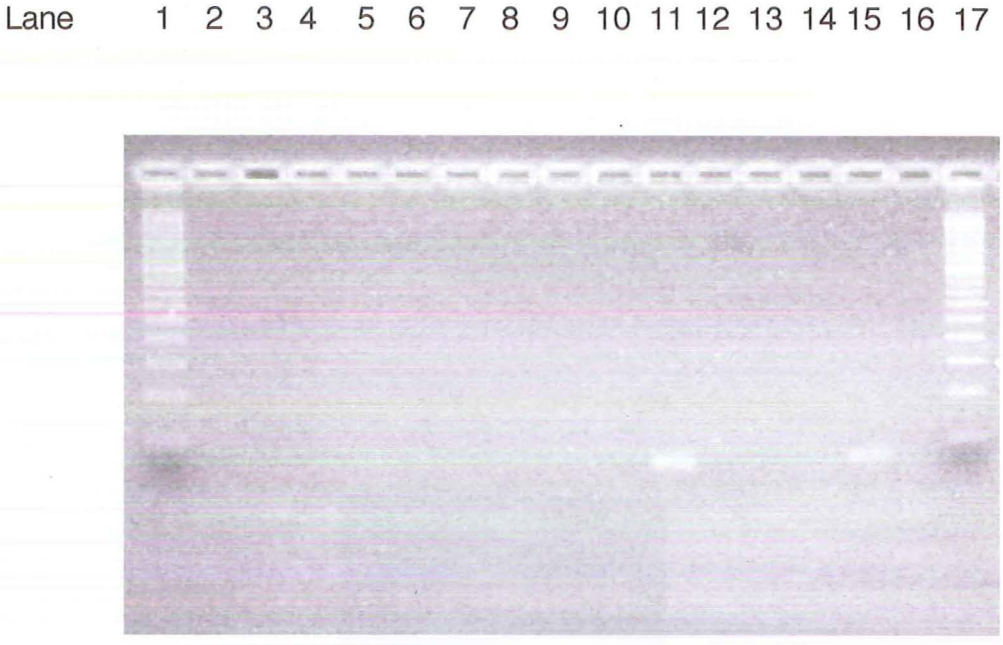


Figure 5.4: PCR Screening of the Pooled Wells 1 to 12 from the Library Array. Lane 1: 200ng DMW-100M molecular weight marker. Lane 2 to 13: Pooled wells 1 to 12. Lane 14: Positive control exon 52. Lane 15: Positive control exon 6 from undiluted library. Lane 16: Negative control. Lane 17: 200ng DMW-100M molecular weight marker.

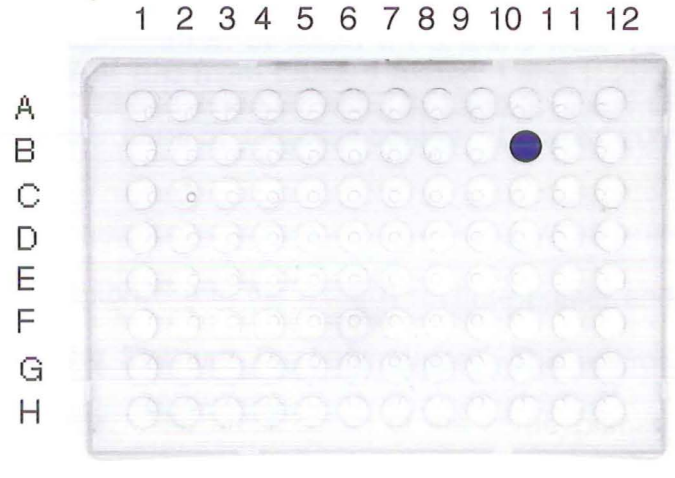


Figure 5.5: Multiwell plate Showing the Positive Well (10B) Containing Exon 6.

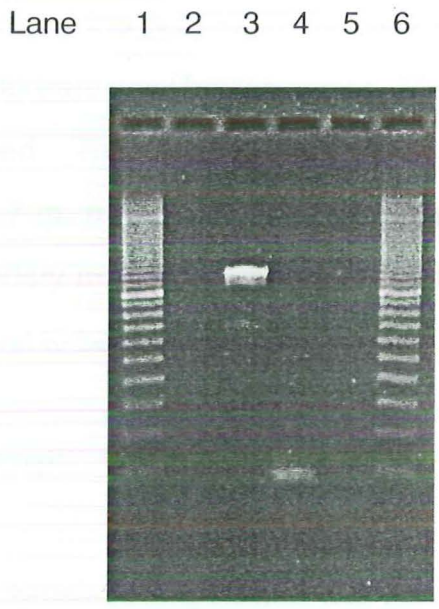


Figure 5.6: Amplification of Exon 6 Forward with T7 and T3 Vector Primers Focusing on Well 10B from Library Array. Lane 1: 200 ng DMW-100M molecular weight marker. Lane 2: Exon 6F with T3. Lane 3: Exon 6F with T7. Lane 4: Exon 6 positive control. Lane 5: negative control. Lane 6: 200 ng DMW-100M.

Amplification of exon 6 with the exon 6 reverse primer and the T3 vector primer set was attempted by Platinum *Taq* polymerase. This approach failed to produce any significant products. The amplification of the same primer set was performed with ELONGASE. This approach also failed to produce any significant PCR products.

5.3.4 Sequencing

The PCR product obtained by the exon 6 forward and T7 primer set was purified by the Qiaquick protocol and an aliquot was electrophoresed to determine the integrity and intensity for sequencing. A discreet band was observed at 1.3 kb and was approximately the same intensity as the band corresponding to 1 kb on the 100 bp ladder. Two microlitres of this purified product was used as template for sequencing with only the exon 6 forward amplification primer. The sequence of the 5' splice site of intron 6 was obtained. The chromatogram of this exon 6 / intron 6 boundary is illustrated in Figure 5.7 (p. 81). A 2'-O-methyloligonucleotide was designed to block this exon/intron boundary to induce skipping of exon 6 is also illustrated in Figure 5.7 (p. 81). This AO is yet to be ordered.

5.4 CONCLUSIONS

Platinum *Taq* DNA polymerase amplification was successful in amplifying exon 6 from both genomic DNA (GR) and from the unamplified canine λ FIX II library.

Growth of the λ FIX II library in liquid suspension on the multiwell plate was successful. The modified method pioneered here offers unique advantages over the Hughes method. It saved money and time and avoided the possibility of cross contamination. The PCR screening of the λ FIX II library using the pooled well strategy, successfully identified a positive well containing exon 6 positive clones.

The amplification of the positive well using Platinum *Taq* DNA polymerase with the exon 6 forward and T7 vector primer successfully amplified a 1.3 kb PCR product that gave a possible intronic stretch for sequencing. Sequencing of this product identified the canine 5' splice site of intron 6.

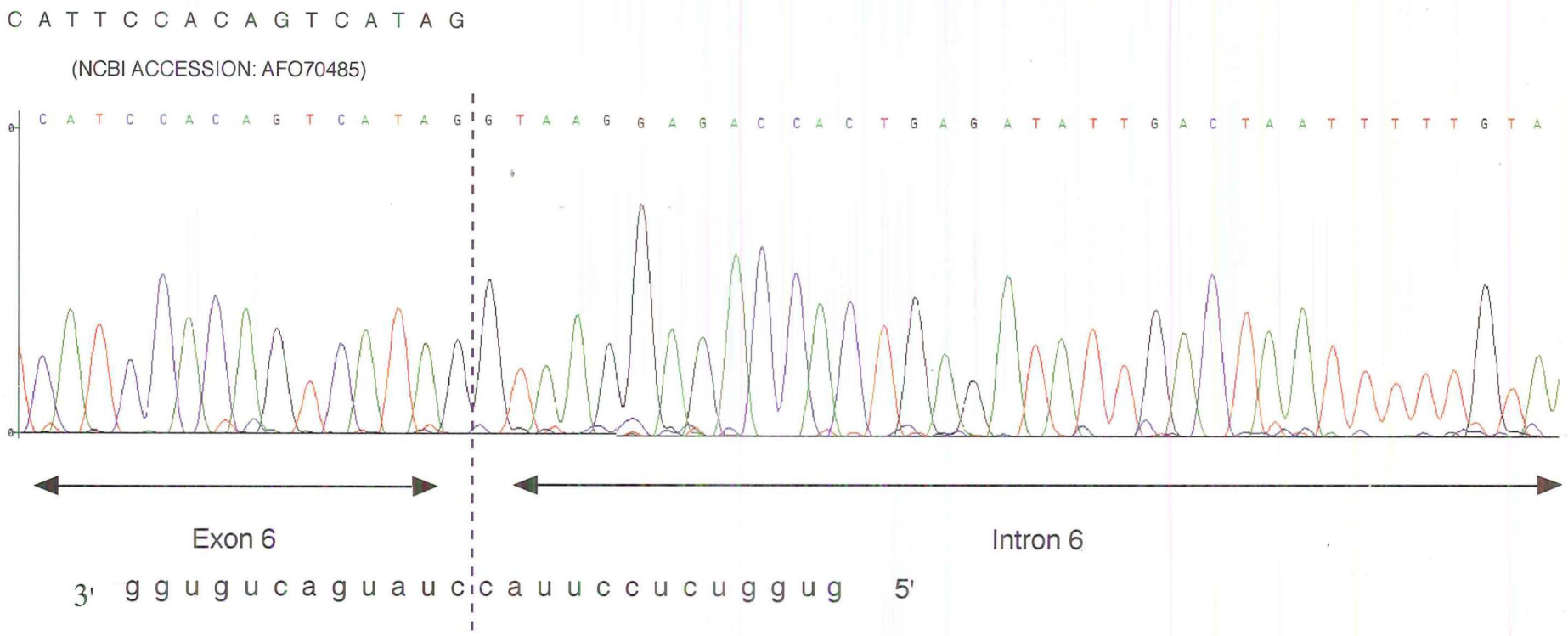


Figure 5.7: Chromatogram of the 5' Splice Site of Intron 6 obtained from the PCR Screening of the Canine λ FIX I I Library and below the AO Designed to Block this Site

CHAPTER 6.0: AO TRANSFECTION STUDY

6.1 INTRODUCTION

As the project reached its conclusion the availability of the 2'-O-methylribooligonucelotide complementary to the 5' splice site of intron 8 permitted a "one shot" transfection study. This transfection study was designed to deliver the AO to cultured primary muscle cells derived from the normal GR dog. This AO should hybridise with its complementary mRNA sequence and induce skipping of exon 8 during splicing of the canine dystrophin gene.

As this transfection experiment was the first of its kind on cultured GR myoblasts, the transfection conditions were based on optimized conditions developed for the transfection of *mdx* myoblasts.

6.2 METHODS

6.2.1 AO Delivery Systems

Two transfection reagents were used, Lipofectin and LipofectAMINE (LF2000) both supplied by GibcoBRL.

LIPOFECTIN reagent is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium choride (DOTMA), and dioleoyl

phosphotidylethanolamine (DOPE) in membrane filtered water. It is suitable for the transfection of DNA into tissue culture cells.

LF2000 is a new proprietary formulation that has been found to have the highest trasfection efficiency in many cell types.

6.2.2 Pimary GR Myoblast Culture Preparation

The normal primary myoblast culture was approximately 90% confluent after two days of incubation at 37°C with 5% CO_2 in proliferation media: 20% foetal calf serum (FCS), 0.5% Chick embryo extract (CEE) and Hams F10 media. This proliferation media was removed 3 hours prior to transfection and replaced OPTI-MEM and incubated at 37°C with 5% CO_2. Once the transfection mixtures were ready to add to the normal primary myoblast culture, the differention media was removed.

6.2.3 AO Transfection

As two different transfection systems were used in this project the 24 well culture plate containing the primary myoblast culture was divided so that each reaction condition was made in duplicate, except for the controls.

6.3.4.1 Lipofectin Transfection

The lipofectin transfection experiment was constructed using two transfection ratios, 1:2 and 1:5, of AO to transfection reagent. A lipofection reagent control was also constructed (Table 6.1).

The lipofectin transfection protocol consisted of two solutions, A and B (Table 6.1). Solution A was constituted by adding the lipofectin to the Opti-MEM. The solution was mixed thoroughly and incubated at room temperature for 45 minutes. Following the incubation period Solution B was constituted and combined with the solution A.

This mixture was mixed gently and incubated at room temperature for 10 to 15 minutes. 800 µl of HAMS F10 supplemented with 5% FCS was then added. 500 µl of this mixture was subsequently added to each of the two wells containing the primary GR myoblasts.

Table 6.1: ***Components of the lipofectin Solutions A and B***

	Ratio of AO to lipofectin reagent		
	1:2 (made two)	**1:5** (made two)	**Lipofectin Control** (made one)
Solution A	8.8 µl Lipofectin 92 µl Opti-MEM	22 µl Lipofectin 78 µl Opti-MEM	22 µl Lipofectin 78 µl Opti-MEM
Solution B	4.4 µg AO 96 µl Opti-MEM	4.4 µg AO 96 µl Opti-MEM	100 µl Opti-MEM

6.2.3.2 LF2000 Transfection

The LF2000 transfection experiment was constructed using two transfection ratios, 2: 3 and 1:2, of AO to transfection reagent. A LF2000 reagent control was also constructed (Table 6.2).

The LF2000 protocol consisted of two solutions, A and B (Table 6.2). Solution A was constituted by adding the LF2000 reagent to the Opti-MEM. The solution was mixed

thoroughly and incubated at room temperature for 5 minutes. Following the incubation period Solution B was constructed and added to Solution A. The mixture was then incubated for 20 minutes. 800 µl of HAMS F10 supplemented with 5% FCS was added to the minture and 500 µl of this mixture was subsequently added to each of the two wells containing the primary GR myoblasts.

Table 6.2: ***Components of the LF2000 Solutions A and B***

	Ratio of AO to LF2000 Reagent		
	2:3 (made two)	**1:2** (made two)	**LF2000 Control** (made one)
Solution A	6.6 µl Lipofectin 94 µl Opti-MEM	8.8 µl Lipofectin 92 µl Opti-MEM	8.8 µl Lipofectin 92 µl Opti-MEM
Solution B	4.4 µg AO 96 µl Opti-MEM	4.4 µg AO 96 µl Opti-MEM	100 µl Opti-MEM

6.2.3.3 Controls

6.2.3.3.1 AO Control

The 2'-O-methyloligoribonucleotide designed to target the 5' splice site of intron 8 was syntheized by Geneworks. This AO was resuspended in sterile water to a final concentration of 1 µg/µl.

1 ml of HAMS F10 supplemented with 5% FCS was added to 4.4 µl (or 2.2 µl per well) of the AO. 500 µl of the mixture was then addedto each well.

6.2.3.3.2 Untreated

For the untreated wells the media was removed and 500 µl of Hams F10 supplemented with 5% FCS was added to each well.

When all samples had been added to the wells, the culture dish was then incubated for 20 hours at 37°C with 5% CO_2.

6.2.4 Total RNA Extraction RNAzol B

Total RNA was extracted from primary cultured myotubes using RNAzol B (Tel Test) as described by the manufacturer.

The media from each reaction condition (two wells) was removed. These wells were pooled to obtain a suitable quantity of RNA. 300 µl of RNAzol B was added to the first well and mixed thoroughly. This mixture was transferred to the second well and mixed thoroughly again to ensure a thorough lysing of the cells. The 300 µl of RNAzol B containing the cell extracts was transferred to a sterile eppendorf tube and 30 µl of chloroform was added. The sample was vortexed thoroughly and incubated for 15 minutes at 4°C followed by centrifugation at 13 000 rpm for 15 minutes at 4°C. After centrifugation, the aqueous phase (containing the RNA) was removed and placed into a fresh 1.5 ml eppendorf tube to which an equal volume of isopropanol was added. This sample was vortexed thoroughly and incubated for 15 minutes at 4°C followed by centrifugation at 13 000 rpm for 15 minutes at 4°C. The supernatant was removed from the RNA pellet and 150 µl of 100% ethanol and 50 µl of 0.3 M sodium acetate (pH 5.2) was added. The tubes were placed at -80°C for 5 to 10 minutes and centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was carefully removed and the

RNA pellet washed in 100 µl of 75% ethanol followed by immediate centrifugation at 13 000 rpm for 10 minutes. After removing the 75% ethanol wash, the pellet was air dried under a tissue (to avoid contamination from the air) for 15-20 minutes until all residual moisture had evaporated. The RNA sample was then resuspended in 40 µl of RNase-free water.

6.3.4.1 Spectophotometry

Spectophotometry was performed on 5 µl of RNA in 55 µl of water (1/12 dilution). An OD_{260} value of 1.0 represents a concentration of 40ng/µl of RNA. The concentration of the RNA sample was determined as follows:

Concentration of RNA (µg/ml) = OD_{260} x dilution factor X 40

The ratio of $OD_{260/280}$ of 2.0 was assumed to represent pure samples of DNA.

6.2.5. Reverse Transcriptase PCR (RT-PCR)

The Titan expand kit supplied by GibcoBRL is a one tube system allowing PCR amplification to occur in the same tube following cDNA synthesis by the reverse transcriptase. Primary amplification of the RNA extracted from the transfected GR primary myoblast was performed form exons 1 to 10. Components of the Titan reaction are described in Table 6.3. Primers for the amplification of exons 1 to 10 are shown in Table 6.4.

Table 6.3: ***Components of the Titan Expand RT-PCR***

Component	Volume (µl)	Final Concentration
Sterile Water	3.125	-
DTT (100mM)	0.625	5mM
RT-PCR 5X Buffer with Mg^{2+}	2.5	1x (1.5 mM $MgCl_2$)
dNTP's (5 mM)	0.5	0.2 mM
Primer mix (25 ng/ul)	1.5	1.5 ng/ul
Enzyme mix	0.25	Not specified
Target RNA	4	~4 ng/ul
Total	**12.5**	

Table 6.4: ***Primers used to Amplify Canine Dystrophin Exons 1 to 10***

Primer	Primer Orientation	Sequence 5' to 3'
Can1F	Foward	GTGGGAAGATAGAGGACTG
ConsDys10R	Reverse	ACATCATT(A/T)GAAATCTCCTTG

Thermocyling was performed under the following conditions: 25°C for 2 minutes, 48°C for 30 minutes, 94°C for 2 minutes followed by 45 cycles of 94°C for 15 seconds, 55°C for 1 minute and 72°C for 2 minutes.

Amplified primary samples were fractionalised by agarose gel electrophoresis and visualised by UV transillumination.

6.2.6 Secondary PCR Amplification

Secondary amplification was performed using a 1µl aliquot of the primary PCR products and re-amplified in a 50 ul reaction using the nested primer sets to amplify dystrophin exons 5 to 10 and 5 to 9. These primers sets are shown in Table 6.6. The secondary

amplifications using conventional PCR as outlined in Table 6.5. Thermocycling was performed as outlined in Section 3.3.5.2 (p. 44).

Table 6.5: ***Components of Secondary Amplification of PCR Products***

Component	Volume (µl)	Final Concentration
Sterile Water	34.8	-
5.2^{200}	10	200 mM dNTP's, 2 mM $MgCl_2$
Primers (50 ng/ µl)	4	4 ng/ µl
Taq (5.5 U/µl)	0.2	0.022 U/ µl
Target	1	
Total	**50**	

Table 6.6: ***Primers used to Amplify Canine Dystrophin Exons 5 to 10 and 5 to 9***

Exons	Primer	Primer Orientation	Sequence 5' to 3'
5 to 10	CanDys5F*	Forward	GGAAATCA(C/T)AAACTGACTCTTGG
	Can10OR	Nested Reverse	AATCTCTCCTTGGGCTTGGAG
5 to 9	CanDys5F*	Forward	GGAAATCA(C/T)AAACTGACTCTTGG
	ConDys9R	Reverse	CATAAGCAGCCTGTGTG(A/T)AGGCAT

Amplified samples were fractionalised on a 2% agarose gel and visualised by UV transillumination.

6.3 RESULTS

6.3.1 The Primary GR Myoblast Culture and Transfection

Examination of the myoblasts prior to transfection showed signs of cell death. As a result the transfection was done in the presence of serum but the AO-lipofectin/LF2000 reagent complexes was done in serum free OPTI-MEM.

Observation of the plate following transfection revealed that the untreated and AO controls showed ittle cell death while the wells containing the lipofectin/LF2000 reagents showed signs cell death, indicating a toxic effect of these reagents.

6.3.2 Total RNA extractions

Total RNA extractions from the transfected GR primary myoblasts using RNAzol B ranged in purity and concentration. Spectrophotometry of the RNA extractions are shown in Table 6.7. For consistency all samples with concentrations over 100 µg/ml were diluted 100 µg/ml in RNAase free water prior to Titan RT-PCR amplification.

Table 6.7: ***Concentration and Purity of Extracted RNA***

Sample	$OD_{260/280}$	Concentration µg/ml
Lipofectin control	1.8	110.4
LF2000 control	2.2	142.3
AO only	1.9	117.2
Untreated	1.8	121.6
Lipofectin 1:2	1.8	57.1
Lipofectin 1:2	1.7	161.7
Lipofectin 1:5	1.7	183.6
Lipofectin 1:5	1.7	104.1
LF2000 1:1.5	1.7	66.4
LF2000 1:1.5	1.7	71.9
LF2000 1:2	1.7	108.8
LF2000 1:2	1.6	70.8

6.3.3 Titan RT-PCR

Figure 6.1 shows the primary amplification of cDNA ,exons 1 to 10 derived by reverse transcription of the extracted RNA from transfected GR primary myoblasts using the Titan expand RT-PCR kit. Strong amplification products were observed at 1.1kb corresponding to exons 1 to 10 of the dystrophin gene. A smaller 1.0 kb was also observed. This product corresponds to exons 1 to 10 missing exon 9 (124 bp). A fragment of ~ 915 bp skipping exon 8 was not observed. The 300 bp fragment observed in all amplifications except the negative
has yet to be identified by sequencing.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

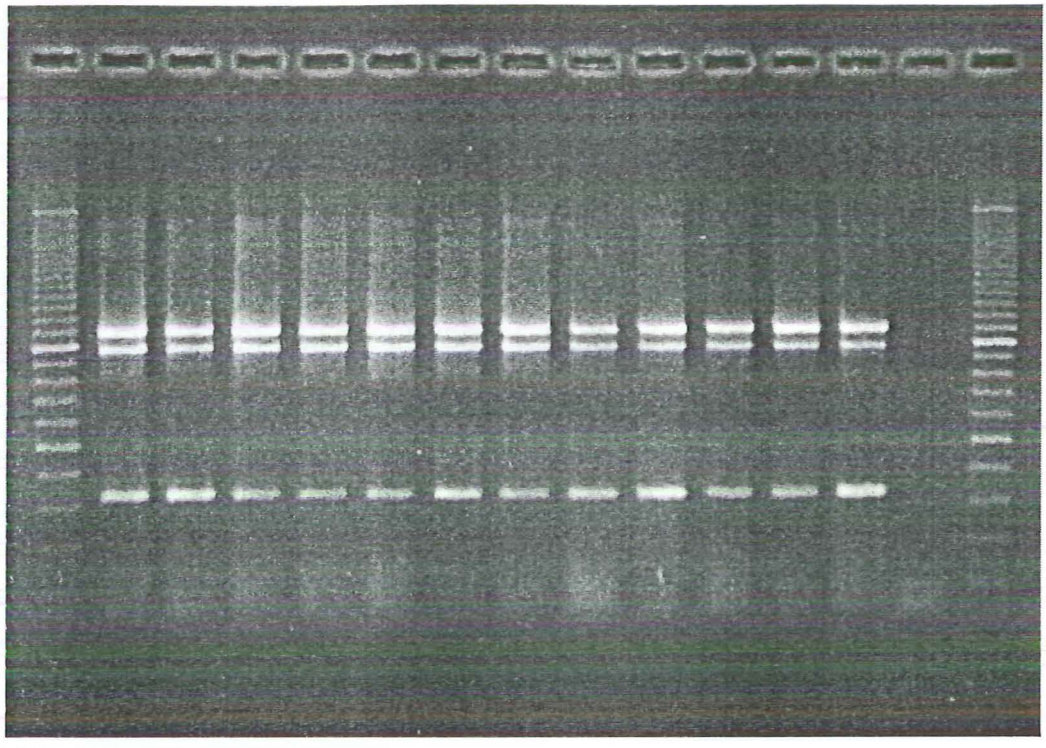


Figure 6.1: Primary Amplification of Exons 1 to 10 from the Dystrophin Gene of Transfected Primary GR Myoblast Cultures using Titan Expand RT-PCR. Lane 1: DMW-100M. Lane 2: Lipofection control. Lane 3: LF 2000 control. Lane 4: AO control. Lane 5: Untreated control. Lane 6: Lipofectin/AO 1:2 ratio. Lane 7: Lipofectin/AO 1:2 ratio. Lane 8: Lipofectin/AO 1:5 ratio. Lane 9: Lipofectin/AO 1:5 ratio. Lane 10: LF2000/AO 2:3 ratio. Lane 11: LF2000/AO 2:3 ratio. Lane 12: LF2000/AO 1:5 ratio. Lane 13: LF2000/AO 2:3 ratio. Lane 14: Negative PCR control. Lane 15: DMW-100M.

6.3.4 Secondary Amplification

Nested amplification with the exon 5 forward and 10 reverse primers generated strong bands observed at 800 bp, representing exons 5 to 10, and a 700 bp fragment corresponding to the exons 5 to 10 with skipping of exon 9. Only the lipofection 1:2 ratio sample showed a alternative transcript that coresponded to the predicted size (~ 483 bp) of a fragment skipping both exon 8 and 9 (Lane 7 of Figure 6.3). Other alternative transcripts were also observed in the LF2000 2:3 ratio of approximately 400 bp (Lane 11, Figure 6.2) and the LF2000 1:2 ratio of 200 bp (Lane 13, Figure 6.2). These products are yet to be identified by sequencing.

To increase the specificity and sensitivity nested ampliifcation exons 5 to 9 was performed (Figure 6.3). A strong 600 bp product was observed in all samples except the negative control and represented exons 5 to 9. The smaller 500 bp product skipping exon 9 was also observed in all samples. No alternative transcripts were observed.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

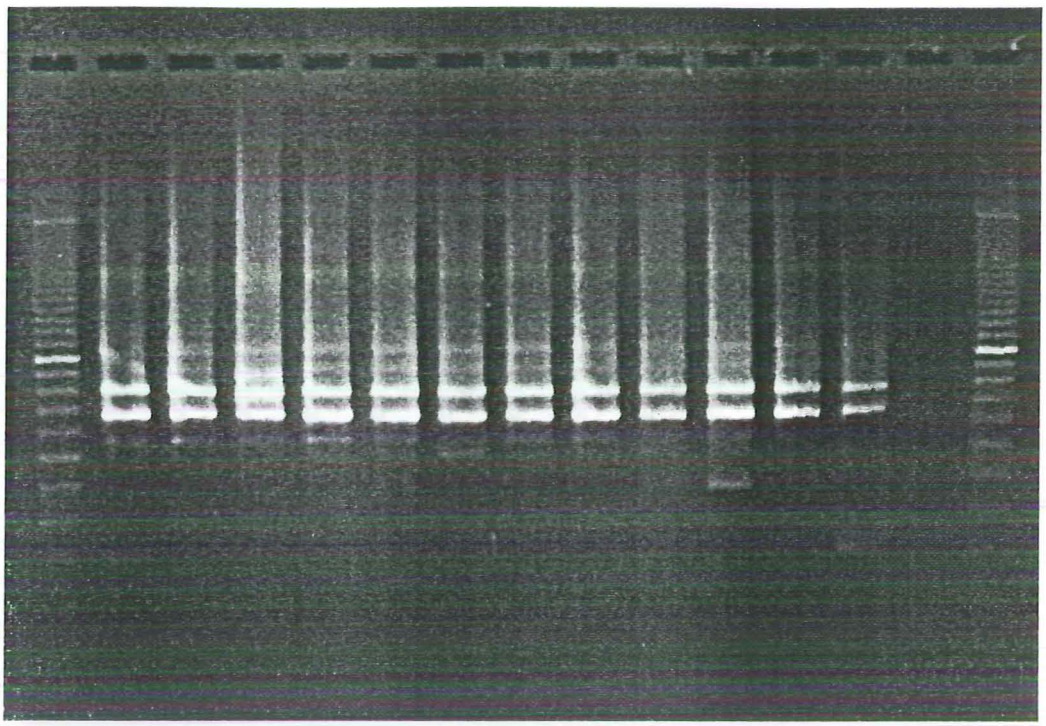


Figure 6.2: Secondary Amplification of Exons 5 to 10 from the Dystrophin Gene of Transfected Primary GR Myoblast Cultures using *Tth* DNA Polymerase PCR. Lane 1: DMW-100M. Lane 2: Lipofection control. Lane 3: LF 2000 control. Lane 4: AO control. Lane 5: Untreated control. Lane 6: Lipofectin/AO 1:2 ratio. Lane 7: Lipofectin/AO 1:2 ratio. Lane 8: Lipofectin/AO 1:5 ratio. Lane 9: Lipofectin/AO 1:5 ratio. Lane 10: LF2000/AO 2:3 ratio. Lane 11: LF2000/AO 2:3 ratio. Lane 12: LF2000/AO 1:5 ratio. Lane 13: LF2000/AO 2:3 ratio. Lane 14: Negative PCR control. Lane 15: DMW-100M.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

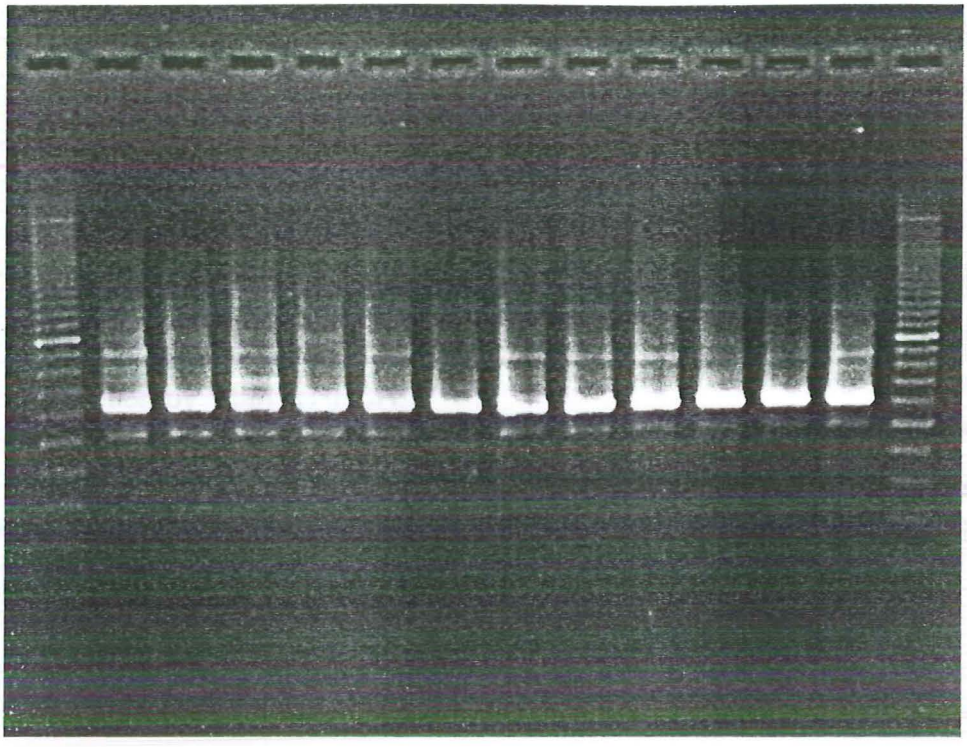


Figure 6.4: Secondary Amplification of Exons 5 to 10 from the Dystrophin Gene of Transfected Primary GR Myoblast Cultures using *Tth* DNA Polymerase PCR. Lane 1: DMW-100M. Lane 2: Lipofection control. Lane 3: LF 2000 control. Lane 4: AO control. Lane 5: Untreated control. Lane 6: Lipofectin/AO 1:2 ratio. Lane 7: Lipofectin/AO 1:2 ratio. Lane 8: Lipofectin/AO 1:5 ratio. Lane 9: Lipofectin/AO 1:5 ratio. Lane 10: LF2000/AO 2:3 ratio. Lane 11: LF2000/AO 2:3 ratio. Lane 12: LF2000/AO 1:5 ratio. Lane 13: LF2000/AO 2:3 ratio. Lane 14: DMW-100M.

6.4 CONCLUSION

This was the first study to transfect GR primary myoblasts culture. Problems with cell death and the consequent requirement of serum in the transfection would have affected the effiecny of the transfection reactions especially for the lipofectin reagent.

Spectrophotometric anaylsis of the total RNA extracted from the transfected primary myoblasts using the RNAzol B protocol was of sufficient quantity and quality for primary amlification by Titan RT-PCR.

Amplification of both primary and nested amplifications failed to produce consistent alternative transcripts skipping of exon 8.

Further study is required for the transfection of the AO designed at the 5' splice site of intron 8 and to optimize the transfection conditions for primary GR and GRMD myoblasts.

CHAPTER 7.0 DISCUSSION

At the start of any new scientific project, a considerable amount of time and effort are devoted to optimising protocols. It is a period of trials, modification and more trials to determine the best way forward. Such was the nature of the laboratory work reported in this thesis. it was intended to be a project which did the basic research, subsequently enabling others to progress to *in vitro* AO trials with GRMD dogs. For the most part, this project was just that. Five different strategies were employed to identify the intronic sequences of interest, each strategy consisting of trial followed by trial followed by even more trials as the elusive optimisation of experimental conditions were pursued. In essence it was a shotgun approach to find out what would work, and since four intronic sequences were discovered it has to be considered to be a success. In fact there was enough time at the end of this project to attempt *in vitro* AO-induced exon skipping in the GR dystrophin gene (see, Chapter 6). Although this attempt showed little signs of success, this was not surprising. It was, after all, a 'one-off', first ever, *in vitro* attempt to induce exon skipping in the GR dystrophin gene This attempt was well beyond project expectations as indicated in the the aims of this project (see, Aims, p. 21). The following sections review the strategies adopted.

7.1 Identification of GR Dystrophin Intronic Sequences

Five different strategies were tested in the search for GR dystrophin intronic sequences. These were: long-range amplification, inverse PCR, PCR screening of a phagemid library, construction of a GR cosmid library for hybridization screening and PCR screening of a phage canine genomic library.

7.1.1 Long-range Amplification

The long range amplification of canine genomic DNA was successful in yielding intron 8 from GR genomic DNA which, without exonic sequences, was approximately 1.2 kb. McNaughton et al. (1997) characterised the 1.1 kb human dystrophin intron 8, so the fact that long-range PCR managed to amplify GR intron 8 indicates complementary size between humans and GR for this intron. Following this line of reasoning, it was not surprising that long-range PCR failed to amplify the GR dystrophin intron 7 given that the human dystrophin intron is 110 kb (Nicholoson et al., 1997). The sizes of the human dystrophin introns 5, 6 and 9 are not known. At this stage, therefore, it is not known if the failure of long-range PCR to amplify these introns was due to their excessive size.

7.1.2 Inverse PCR

Inverse PCR was a strategy tested, but not reported elsewhere in this thesis on account of its failure to amplify any intronic sequences of interest. It was a valid approach, however, and most probably only required some further optimisation to get it working. Inverse PCR is an extension of conventional PCR to allow amplification of unknown nucleotide sequences flanking previously characterised regions, without resorting to conventional cloning (Ochman et al., 1988). The first step involves the partial digestion of the genomic DNA with a suitable restriction enzyme (normally a restriction enzyme with a four-based cleavage site) that almost certainly cleaves within intron sequences, but not within the exon of interest, in order to liberate small DNA fragments containing characterised exon sequence and its two flanking intronic regions (Ochman et al., 1988; Hartl & Ochman, 1994; Strachan & Read, 1996). In this project *Sau*3A was used to digest GR genomic DNA.

After cleavage, the ends of the two flanking regions bound to the known sequence are brought together by forcing the fragment to circularise. This is achieved by conducting the ligations at very low DNA concentrations. Under these conditions intramolecular reactions are favoured rather than intermolecular ones, hence the possibility of circularisation, followed by ligation (Ochman et al., 1988; Hartl & Ochman, 1994; Strachan & Read, 1996).

Amplification of the uncharacterised flanking regions is then carried out utilising two primers that hybridise to the ends of the known sequence. Inverse PCR refers to the fact that primer orientation is reversed, primers are orientated away from the other primer instead of towards it, as is customary with respect to their normal orientation in PCR. Because of the circular configuration of the molecule, the product of each polymerisation can serve as a template for annealing of the opposite primer. Hence repeated rounds of denaturation, annealing and polymerisation can result in a exponential increase in the number of copies of the target sequence if the intronic distance between primers is not too great (Ochman et al., 1988; Hartl & Ochman, 1994; Strachan & Read, 1996). In this project GR dystrophin exon primers will be used.

The inverse PCR attempted in this project resolutely failed to produce any amplification products. Alteration of PCR reaction conditions, varying Mg^{2+} concentration and decreasing primer annealing temperature, all failed to yield amplification products. This suggested that the problem lay in the ligation step and specifically with the concentration of DNA employed. Titrating ligation DNA concentrations would be a worthwhile trial.

7.1.3 PCR Screening of a Phagemid Library

PCR screening of the phagemid library successfully identified the 3' splice site of intron 7. Attempts to identify the intronic boundaries of surrounding exon 6 and the 5' splice site of intron 9 were unsuccessful. Secondary amplification using semi-nested exonic primers should have increased specificity but as reported in Section 3.3.3, this failed despite may attempts at optimising PCR conditions. This failure remains unexplained.

7.1.4 Construction of a GR Cosmid Library for Hybridization Screening

As reported in Chapter 4 a GR genomic DNA cosmid library was successfully constructed as demonstrated by the successful amplification of exons 10 and 52. However it was an under represented library because other exons failed to amplify. The difficulties that can be encountered in the construction of cosmid libraries, and leading to under representation, were reviewed in the Conclusion to Chapter 4.

7.1.5 PCR screening of a Phage Beagle Genomic Library

In this strategy a phage beagle genomic library was screened for intron boundaries surrounding exon 6 on the premise that it would be homologous to the GR exon 6. This turned out to be a correct assumption, as far as this assumption could be tested. The beagle exon 6 sequence was found to be identical to the published GR exon 6 sequence. The assumption generated by this discovery was that the beagle intron 6 5' sequence, determined for the first time in this project, was homologous to the GR intron 6 5' sequence. This assumption would be proven to be correct if the AO designed around the exon 6/intron 6 boundary could be shown to induce exon skipping in GRMD dogs.

7.1.6 GRMD AO-Induced Exon Skipping: Future Directions

There are only two previous report of successful *in vitro* AO-induced exon skipping in the dystrophin gene, and both of these employed the *mdx* mouse dystrophin gene (Dunckley et al. 1995; Wilton et al. 1999). But the success of these authors at using AOs to induce in *vitro* exon slipping raises confidence that, with sufficient trials, experimental conditions for *in vitro* AO-induced GR dystrophin exon skipping will be optimised. The AOs designed from the work reported here will eventually be employed to induce the precise splicing of exons 6 and 8, to remove the mutation causing GRMD and, hopefully, produce sufficient quantities of functional dystrophin to encourage *in vivo* GRMD trials.

New difficulties will then confront *in vivo* genetic therapy mediated by AO technology.

7.2 Human Gene Expression, AOs and Genetic Therapy

In its application to genetic therapy, AO intervention attempts to alter, in a precise way, an enormously complex system. That it can be done for *mdx* dystrophin *in vitro* has been demonstrated (Dunckley et al. 1998; Wilton et al. 1999). That it will be done *in vitro* GRMD dystrophin is very likely. To achieve such success *in vivo* requires consideration of two further factors.

7.2.1 Redefining Genes

Traditionally, genes were considered as entities that controlled specific activities in cells or organisms. More recently, genes have been redefined as sequences of nucleotides, each coding for one specific polypeptide product. This definition is now inadequate and is under review. The vast majority of genes in humans are transcribed individually and the term 'gene' and transcription unit are interchangeable. But a variety

of alternative processing events are now known to occur commonly in all complex eukaryotes where gene expression has been studied (Strachan & Read, 1996, pp. 160-163). The net result of alternative processing events, is that individual transcription units encode several products. Alternative processing can occur at transcriptional or post-transcriptional levels of human gene regulation. It is at this point that the term 'gene' and its meaning becomes blurred. They can no longer be described as sequences of nucleotides, each coding for one specific polypeptide product. Two alternative processing events seem to be relevant to *in vivo* AO technology and the expression of the dystrophin gene. Both operate at the transcriptional level of gene regulation.

First, in the application of AO technology to genetic therapy described here, AOs were designed to directly interfere with the function of the spliceosome. Improved understanding of the operation of this complex molecule can only improve the optimisation of conditions for AO-induced exon skipping. How does this molecule splice and alternately splice? What and how does the spliceosome assist the production of several products from individual transcription units?

The second alternative processing event that appears to be of significance to genetic therapy AO, is the use of alternative promoters in some human genes. These promoters appear to be largely cell-specific promoters.

7.2.1 The Spliceosome

Recent investigation of the spliceosome, the central eukaryotic enzyme complex that removes intronic sequences from mRNA precursors, has shown the behaviour and interactions of its individual proteins to be extremely complex. Twenty-five spliceosome proteins were identified by amino acid sequencing and a further 19 from

expressed sequence tag (EST) databases (Neubauer et al., 1998). These authors also identified 4 novel spliceosome proteins. In addition, the spliceosome contains several RNA components, many of its proteins exist in sub-stoichiometric amounts and complex patterns of post-translational modification occur (Neubauer et al., 1998). How spliceosome components interact with each other and with other cell components, will require the evaluation of an enormous number of interactions.

Direct fluorescent microscope evidence for the location of proteins can be obtained from *in vivo* expression of green fluorescent protein (GFP) fusions. This technique involves the fusion of full-length cDNA to the *Aequorea victoria* jellyfish gene encoding GFP. These constructs were used to transfect host cells where the gene under investigation is expressed. Naeuber et al. (1998) used this technique to locate their newly discovered spliceosome protein to the nucleus of HeLa cells, strengthening the case for their status as spliceosome proteins.

Decoding the functional genomics of the spliceosome presents a formidable problem. The major components have been identified, now the huge number of possible interactions of functional significance must also be identified for the systematic optimisation of exon skipping AO genetic therapy. For example, decoding the way in which upstream and down stream intronic pyrimidine tracts interact with components of the spliceosome during splicing (Kennedy et al. 1998) may enable highly specific AOs to be developed to improve the efficiency of exon-skipping.

7.2.3 Alternative Promoters in Dystrophin

At the transcriptional level, human genes have complex *cis*-acting sequence elements, which include, response elements, enhancers, silencers and promoters (Strachan & Read, 1996, pp. 160-163). The presence of alternative promoters in one gene are

of interest here. We now know that human genes can have several promoters, a fact that could complicate the application of *in vivo* AO technology as a genetic therapy. Promoters are usually upstream from a gene, frequently within 200 bp of the transcription start site. They comprise short sequence elements like the TATA box, the CCAAT box and the GC box that are recognised by *trans*-acting transcription factors. Alternative promoters in a gene are frequently recognised only by cell-specific transcription factors.

In 1989 Feener et al. reported that the first exon of the human dystrophin transcript was different in brain and muscle. They concluded that dystrophin expression could be differentially regulated in these tissues by usage of distinct promoters.

By 1993 knowledge of the complexity of *DMD* alternative promoters led Ahn and Kunkel (p. 283) to state:

> At least five independent promoters specify the transcription of their respective alternative first exons in a cell-specific and developmentally controlled manner. Three promoters express full-length dystrophin, while two promoters near the C terminus express the last domains in a mutually exclusive manner.

Strachan and Read (1996, p. 166-167) reported that the dystrophin gene has at least 7 different promoters and, as a consequence of these alternative promoters, produces 7 dystrophin isoforms. Three of these lead to the transcription of the full 78 exons in the dystrophin gene, resulting in a 427 kDa (Dp427) full-length dystrophin protein. These are the cortical, muscle and Purkinje dystrophins. These three promoters use different first exons and therefore these dystrophin isoforms differ in their N-terminal amino acid sequence. The other promoters are located immediately before exon 30 (Dp260, retinal dystrophin), exon 45 (Dp140, central nervous system dystrophin), exon 56 (Dp116, Schwann cell dystrophin), and exon 63 (Dp71, glial cell dystrophin).

These dystrophin isoforms have at their N-terminal the first exon translated which will be homologous to the equivalent region in larger dystrophin isoforms. So, in the regions that they overlap, all dystrophin isoforms are homologous, unless alternative splicing has changed the reading frame. This means however that each dystrophin isoform must be in register, each starting at a dystrophin codon, and not within a codon.

When induced exon skipping involves the first exon(s) of a cell type-specific expression of a gene, genetic therapy AO could run into problems. Consider the possibility of skipping, *in vivo*, exons that included exon 30 of the dystrophin gene to produce a reduced length, but in-frame, 'functional' dystrophin molecule. Consider that this exon skipping effectively reduced DMD symptoms to those of BMD. Unless the exon-skipping AOs could be prevented from entering retinal cells, the production of the retinal dystrophin might be abolished by the very same exon skipping AOs delivering genetic therapy to muscle cells. This would happen if exon skipping were induced in these cells because such skipping would almost certainly remove the all-important translation initiation codon, most probably located in exon 30. The initiation codon is almost always AUG (rarely AUC, CUG or GUG) and it is usually the first AUG encountered by the 40S ribosomal subunit (Strachan & Read, 1996, pp. 22-23). AUG is most efficiently recognised as the start codon when it is embedded in the optimal surrounding sequence GCC**A/G**CC*AUG***GG** where bold indicates the most crucial determinants of this surrounding sequence. The removal of AUG in its initiation sequence would reduce translation to a very low level, determined by the efficiency with which the remaining AUG codons were recognised as start codons. The inevitable low-level production of variously sized in-frame and out-of-frame products would effectively abolish translation. Whether this would have clinical consequence, would, of course, depend upon the precise timing and function of the retinal dystrophin isoform. Developing systems that deliver AOs to specific cell-types would seem to be a priority for genetic therapy AO intervention.

Alternative promoters have not been identified for the GR gene. This does not mean that they do not exist. The developing picture of cell-type alternative promoters in the human dystrophin gene suggest a cautionary approach be adopted for *in vivo* AO-induced exon skipping GR trials. At the present state of knowledge, AO-induced exon skipping of exons 6 and 8 in the human dystrophin gene would not be compromised by alternative promoters because the seven known alternative promoters are well upstream or downstream from these exons.

7.3 Concluding Comments

This thesis aimed to discover intronic sequences flanking exons 6, 8 and 9 in the GR dystrophin gene. This aim was achieved. It was intended that these sequences be used by fellow workers to design AOs that might induce exon skipping in GR myoblast tissue culture. As it eventuated, this thesis also reported the first attempt at AO-induced GR dystrophin exon skipping in GR primary myoblast tissue culture. In effect, this thesis helped to launch *in vitro* AO genetic therapy for the muscular dystrophies, and paved the way for *in vivo* AO genetic therapy trials in GRMD dogs.

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APPENDIX A

APPENDIX A

DYSTROPHIN EXONIC PRIMERS

All primers designed prior to this project were ordered through Bresatec (now known as Geneworks, Australia Inc). Primers ordered through Geneworks during this project are indicated by *.

Primer	Primary/Nested	Sequence 5' to 3'
Can1F	P	GTGGGAAGATAGAGGACTG
CanDys5F*	P	GGAAATCA(C/T)AAACTGACTCTTGG
Can5R	P	TATGTCAGTACTTCCAATATTATTCAC
Can6F	P	GTCAAAAATGTAATGAAAAATATCAT
Can6R	P	CTATGACTATGGATGAGAGCATTCA
Exon6IR*	N	CG(A/G)ACCCAGCTCAG(A/G)AGAATC
Can7F	P	GAACATGCATTCAACATTGCC
CanDys7R*	P	TTGTGTGGCTGACTGCTGGC
ConDys8F	P	CAACAAGTGAGCATTGAAGCGAT
Can8OR	N	GATGGCCTTGGCAACATTTCC
Can8R	N	CATTAAGATGGACTTCTTATCTGG
DysEX9F	P	CCTAAGCCTGGATTCAAGAG
PIGDYS9F	N	CACAGTCAGTCTAGCACAGG
ConDys9R	P	CATAAGCAGCCTGTGTG(A/T)AGGCAT
Can10F		CCT GGA CAG TTA TCA AAC AGC
ConsDys10R	P	ACATCATT(A/T)GAAATCTCCTTG
Can10OR	N	AATCTCTCCTTGGGCTTGGAG
DMD Ex52F	P	AATGCAGGATTTGGAACAGAGCCGTCC
DMD Ex52R	P	TTCGATCCGTAATGATTGTTCTAGCCTC
DMD Ex53-F	P	TTG AAA GAA TTC AGA ATC AGT GGG ATG
DMD Ex53-R	P	CTT GGT TTC TGT GAT TTT CTT TTG GAT TG

Can: primer specific for Canine cDNA sequence; **Con**: Consensus of cDNA sequences bwtween species ie human, dog and mouse. **DMD**: primer specific for the human cDNA sequence

VECTOR PRIMERS

Primer	Sequence 5' to 3'
M13 universal - F	CGCCAGGGTTTTCCCAGTCACGAC
M13 universal - R	AGCGGATTTCAATTTCACACAGGA
T7 promotor	AATTAACCCTCACTAAAGGG
T3 promotor	TAATACGACTCACTATAGGG

APPENDIX B

MATERIALS

List of reagents and chemicals

Reagent/chemical	Supplier
Ampicillin	Amresco®
ABI Prism BigDye Terminator cycle sequencing Ready reaction kit	Applied Biosystems
Bacto®agar	Difco laboratories, USA
Bacto®tryptome	Difco laboratories, USA
BactoCyeast extract	Difco laboratories, USA
BamH I	Promega, USA
Bovine serum albumin (BSA)	Promega, USA
Bromo phenol blue – xylene cynol	Sigma®, USA
Chloroform	Sigma®, USA
DNA Isolation Kit for Cells and Tissues	Roche Diagnostics, Australia
DMW- 100M molecular weight marker	Geneworks, Australia
EDTA	Sigma®, USA
Elongase enzyme mix	GibcoBRL, USA
Ethanol	AnalaR®, Australia
Ethidium Bromide	Sigma®, USA
Glacial acetic acid	AnalaR®, Australia
Gelatin	Sigma®, USA
Glycerol	Sigma®, USA

Isopropanol	AnalaR®, Australia
Kanamycin	Amresco®
LipofectAMINE® reagent	GibcoBRL, USA
Lipofectin® reagent	GibcoBRL, USA
Low melt analytical grade agarose	Promega, USA
Magnesium sulfate	Aldrich, USA
Maltose	Sigma®, USA
Molecular biology grade agarose	Scientifix Scientific supplies
Platinum Taq DNA Polymerase	GibcoBRL
Paraffin oil	Sigma®, USA
Platinum *Taq* DNA polymerase	GibcoBRL, USA
PUC DNA marker	Geneworks, Australia
OPTI-MEM® reagent	GibcoBRL, USA
Qiaquick Purification Kit	QIAGEN
RNAase A	Tel Test, USA
RNAzol B	Tel Test, USA
Sau 3A	Promega, USA
Shrimp alkaline phosphatase (SAP)	USB, USA
Sodium acetate	AnalaR®, Australia
Sodium chloride	AJAX chemicals
Sodium Hydrogen Carbonate	AJAX chemicals
SPP-1/*Eco*RI molecular weight marker	Geneworks, Australia
T. th DNA polymerase	Biotech International, Australia
T4 ligase	Life technologies
	Promega, USA
Titan Expand RT-PCR	GibcoBRL, USA

Tri-Hydrocloric acid	Sigma®, USA
Tris base	Sigma®, USA
*Xba*1	Promega, USA

APPENDIX C

APPENDIX C: Stock Reagents

All stock solutions were made according to Sambrook *et al.* (1989) unless denoted by *.

POLYMERASE CHAIN REACTION

Deoxynucleotide Buffer (5.2^{100} and 5.1^{50})

A stock of deoxynucleotide buffer, containing deoxy- adenine, thymine, guanine and cytosine was prepared and stored in 100 µl aliquots at -20^{0}C.

5.2^{100} PCR Buffer	Amount (µl)
10x Reaction buffer	500
1M $MgCl_2$	10
5mM dNTPs	100
water	390

5.1^{50} PCR Buffer	Amount (µl)
10x Reaction buffer	500
25Mm $MgCl_2$	200
5mM dNTPs	50
water	250

AGAROSE GEL ELECTROPHORESIS

Glycerol loading buffer

A stock of 6x glycerol loading buffer was prepared and stored in 1 ml aliquots at 4^0C.

0.25%	Bromophenol blue
0.25%	Xylene cyanol
30%	Glycerol in water

TAE electrophoresis buffer (50X)

A 1 litre stock 50x TAE was prepared by combining 232 g of Tris base, 57.1 ml Glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) and marking up to 1 L with millipore H_20. The pH was then adjusted to 8.2 with glacial acetic acid. The working concentration of 1x TAE was obtained by diluting 80 ml of 50x TAE to a final volume of 4 L in millipore H_20. Both the stock and 1x TAE solutions were stored at room temperature.

Ethidium Bromide

A 0.5 µg/ml working solution of ethidium bromide was prepared by adding 100 µl of 10mg/ml stock to a final volume of 2 L in millipore H_20. The working solution was stored in a light resistant bottle at room temperature. The stock solution was stored at 4^0C.

BACTERIAL MEDIA AND REQUIRED SOLUTIONS

LB Media

1 litre stock solutions of LB Medium (*Luria-Bertani Medium*) were prepared. The pH was adjusted to 7.5 with 5N NaOH and the media was autoclaved on liquid cycle at 15 lbs/sq. inch for 20 minutes.

Component	Amount	Concentration
Bacto-trytone	10g	1%
Bacto-yeast	5g	0.5%
NaCl	10g	1%
Agar (for agar plates only)	15g	1.5%

0.7% Top Agar

To 1 litre of LB medium 7 g of agarose was added. The solution was then sterilized by autoclaving on liquid cycle at 15 lbs/sq. inch for 20 minutes. Once autoclaved, the solution was swirled gently to distribute the melted agarose evenly throughout the solution.

Antibiotics

All antibiotics were dissolved in H_20 and sterilized by filtration through a 0.22 micron filter and stored as 1 ml aliquots at -20^{0}C.

Antibiotic	Stock concentration	Working concentration
Ampilicillin	50mg/ml	20mg/m
Kanamyclin	10mg/ml	10mg/ml

0.5M Ethylene diamine tetra-acetic acid (EDTA) (pH 8.0)

18.6 g of EDTA was added to 80 ml of millipore H_20. The solution was then mixed vigorously by a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets. The solution was then autoclaved on liquid cycle at 15lbs/sq. inch for 20 minutes.

1M Magnesium Sulfate ($MgSO_4$)

74 g $MgSO_4$ was dissolved in 300 ml of H_20. The solution was then autoclaved on liquid cycle at 15lbs/sq. inch for 20 minutes.

20% Maltose

20 g of maltose was dissolved in 80 ml of millipore H_20. The solution sterilised by filtration through a 0.22-micron filter (Nalgene). The solution was stored at room temperature.

SM buffer

1 litre of SM buffer was prepared by combining the following 5.8 g of sodium chloride, 2.0 g of magnesium sulfate, 50 ml of 1M Tri-Hydrocloric acid (pH 7.5), 5 ml of 2% (w/v) gelatin and adding millipore H_20 to a final volume of 1 litre. The solution was then autoclaved on liquid cycle and store at room temperature.

VECTOR DNA EXTRACTIONS

TEG swelling Buffer

25 mM Tris pH 8.0

50 mM EDTA

50 mM Glucose

made to 1 litre with ddH_2O

LiCL

106 g LiCl

5.24 g MOPS

pH to 8.0 made to 1 litre with ddH_2O

3 M KOAc High salt Neutralisation Buffer

124.9 g KOAc

25 ml formic acid

made to 1 litre with ddH_2O

DNA SEQUENCING

3M Sodium acetate

A 3M sodium acetate working solution was prepared by dissolving 40.8g of sodium acetate per 100ml of sterile millipore H_20. The pH was adjusted to 5.2 by adding glacial acetic acid. The solution was stored at room temperature.

75% Ethanol

Absolute ethanol was diluted in sterile H_20 to make a 75% (v/v) working solution of ethanol. The solution was stored at room temperature.