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Investigation of variation in the promoter region of the myostatin gene (*GDF-8*) in New Zealand cattle

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A Dissertation

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of the requirements for the Degree of

Bachelor of Science (Honours)

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by

Katianna Louisa Woolley Rasmussen

Lincoln University

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science (Honours).

Investigation of variation in the promoter region of the myostatin gene (*GDF-8*) in New Zealand cattle

by

Katianna Louisa Woolley Rasmussen

Introduction: Myostatin, also called growth and differentiation factor 8 (GDF-8), has the specific function of negatively regulating muscle growth and muscle homeostasis through its interactions with myoblasts during their proliferation to myotubes. As a result, the absence of functional myostatin proteins causes an increase in whole-body muscling. In this study a section of the promoter region of the bovine myostatin gene (*GDF-8*) was analysed to determine whether variation is present. Belgian Blue, South Devon and KiwiCross™ cattle were analysed. The DNA sequence proved uniform across all animals, therefore all carry the same genotype and polymorphisms are not present.

Methods and materials: Blood samples were collected from three cattle breeds, Belgian Blue, South Devon and KiwiCross™, in New Zealand. Electrical side-cutters and FTA cards were used to collect samples. Subsequently, DNA from a section of the myostatin gene (*GDF-8*) promoter was amplified and typed using PCR-SSCP analysis.

Results: The typing results revealed there to be no variation across the samples and breeds that were tested. This suggests that all animals have the same DNA sequence in this section, and therefore it is conserved.

Discussion: Based on these findings, and findings in the literature, this section of the myostatin gene promoter is probably not useful for developing a gene-marker test for myostatin functionality (muscling effects) as variants have not been found. However, further studies into regions upstream from the one studied here may reveal genetic variation and provide useful polymorphisms to establish a gene-marker test for muscling.

Keywords: Myostatin, *MSTN*, bovine, promoter region, *GDF-8*, PCR-SSCP, double-muscling

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Abbreviations

GDF-8	Growth and differentiation factor 8
TGF-β	Transforming and growth factor beta
PCR-SSCP	Polymerase chain reaction-single strand conformation polymorphism
MSTN	Myostatin gene
bp	basepairs
kb	kilobasepairs
SNP	Single nucleotide polymorphism
DNA	Deoxyribose nucleic acid
RNA	Ribose nucleic acid
BMP-1	Bone morphogenetic protein-1
hSGT	Human small glutamine-rich tetratricopeptide repeat containing protein
LAP	Latency-associated protein
T-cap	Titin-cap
Fst	Follistatin
FSTL-3	Follistatin-related gene protein 3
GASP-1	Growth and differentiation factor-associated serum protein-1
LTBP	Latent-TGF-β binding proteins
ActRII/ActRIIB	Activin type two receptors
ActRI	Activin type one receptors
ND	N-terminal domain
p21	Cyclin-dependent kinase inhibitor
Cdk2	Cyclin-dependent kinase 2
Rb	Retinoblastoma
MyoD	Myogenic differentiation
Kd	Dissociation constant
kDa	kilodalton
nM	nanometre
µL	microlitre
µM	micromolar
mM	millimolar
mm	millimetre
cm	centimetre
ng/mL	nanogram per millilitre

Chapter 1

Introduction

Myostatin also called growth and differentiation factor 8 (GDF-8), has the specific function of negatively regulating muscle growth and muscle homeostasis through its interactions with myoblasts during their proliferation to myotubes. As a result, the absence of functional myostatin protein causes hyperplasia (an increase in cell number) and to a lesser extent muscle cell hypertrophy (an increase in cell size). Homozygous non-functional carriers produce a phenotype that has been described as 'double-muscling', where whole-body muscling increases by two to three times compared to average. Heterozygous carriers also show increased muscling from normal, but less than homozygotes.

The overall structure, function, number of exons and introns (and their boundaries) and the number and arrangement of important amino acids in myostatin, is conserved across many animal species. This conservation, along with the whole-body increase in muscling, has led to interest in developing a gene-marker test to enable the determination of an animal's genotype. This would aid in animal selection for increased muscling and thus meat yield, but could also be tailored to other species such as humans, to combat muscle-wasting syndromes.

Carcass weight is of economic importance as a trait for beef farming. Belgium Blue and Piedmontese cattle breeds are examples of breeds that were selected for high muscle producing traits. This inadvertently selected for non-functional myostatin. Traits which increase an animals muscling ability can increase carcass weight and consequently profitability in beef production systems. Establishing a gene-marker test would enable farmers to more accurately select animals carrying non-functional myostatin, and thus animals that will produce more muscle.

In this study a section of the promoter region of the bovine myostatin gene (*GDF-8*) was analysed to determine whether variation was present. These may have proven useful in establishing a myostatin gene-marker test to screen large numbers of cattle, useful for agricultural purposes. Belgian Blue, South Devon and KiwiCross^{TM} bovine breeds were analysed. The DNA sequence proved uniform across all animals, therefore all carry the same genotype and variation is not present. Further research into the DNA sequence further upstream in the promoter may prove useful to finding variation that may be able to be used to establish a gene-marker test.

Chapter 2

Literature Review

2.1 What is myostatin?

Myostatin or GDF-8, is part of the transforming growth factor beta (TGF-β) super-family. This super-family are cytokines, whose function is predominantly to control cell growth, apoptosis, cell differentiation and proliferation from embryonic to mature cells.

Myostatin has the specific function of negatively regulating muscle growth and muscle homeostasis through its interactions with myoblasts during their proliferation to myotubes.

The overall structure, function, number of exons and introns (and their boundaries) and the number and arrangement of important amino acids is conserved in myostatin across many animal species. The gene is located on chromosome 2 in many species, but chromosomal location does vary.

In mammals, the myostatin protein contains three sections, an N-terminal signal peptide, a central propeptide region and the C-terminal myostatin peptide. Whether the polypeptide is a form containing three, two or one section(s); it interacts with other proteins, both intra- and extra-cellularly, and these proteins regulate and assist the proteins function. The inactive protein (prepropeptide) is located in the cytoplasm of skeletal muscle cells, while the active/mature form is in circulation.

Homozygous and heterozygous variation in the myostatin gene (*GDF-8* or *MSTN*) can cause it to become non-functional allowing increased myoblast proliferation to myotubes. This causes hyperplasia (an increase in cell number) and to a lesser extent muscle cell hypertrophy (an increase in cell size), increasing whole-body muscling from the average in heterozygous individuals and 'doubling-muscling' in homozygous individuals. This phenotypic change has been observed across many animal species, but demand for increased lean meat production has led to interest in developing a myostatin gene-marker test for increased carcass muscling.

2.1.1 Primary structure of myostatin

The amino acid sequence of myostatin is conserved across many species (human, cow, rat, mouse, monkey, dog, chicken and turkey) (Jeanplong, Sharma, Somers, Bass, & Kambadur, 2001). Typically, it consists of three exons and two introns, the length and sequence of which can vary slightly. Bovine *MSTN* is located near the centromere on chromosome 2 (2q14-q15) and has a total length of 6673 base pairs (bp), of which 2767 bp make up the coding-region of the myostatin protein. Exon 1 is 506

bp, intron 1 is 1840 bp, exon 2 is 374 bp and intron 2 is 2033 bp. Exon 3 has a variable length of 1701 bp, 1812 bp or 1887 bp, depending on where the polyadenylation site is located (Jeanplong et al., 2001).

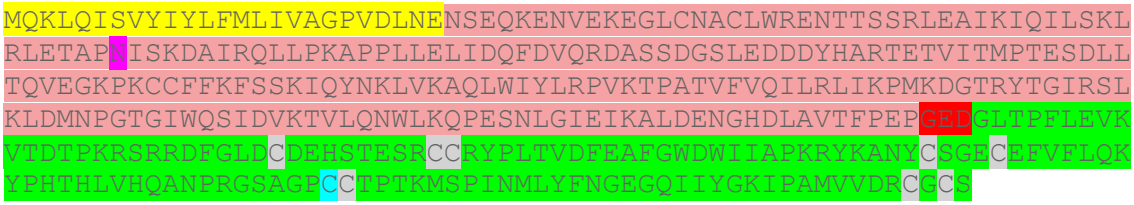


Figure 2.1 The structural and functional domains of bovine myostatin. The yellow sequence is the signal sequence, located at the N-terminal, and that consists of 25 amino acids. The pink region is the propeptide which becomes the N-terminal domain when the signal sequence is proteolytically cleaved. This domain varies in size in different species, ranging from 215-266 amino acids in length and it remains attached to the final portion, making it inactive. The G, E or D (glycine, glutamic acid and aspartic acid) residues highlighted in red, become the C-terminal end depending on the coding sequence. Consequently this amino acid change also alters the initial N-terminal codon of the mature myostatin portion of the protein. The cysteine residues (C) highlighted in grey, are the nine that make up the cysteine knot. These are conserved across all species as they form a major structural component of the three-dimensional structure. The C highlighted in blue, interacts with the same C residue on a second mature myostatin peptide to form an inter-chain disulphide bond, which holds the dimer together. The purple N (asparagine) is highly conserved, as it is involved in an important glycosylation reaction.

2.1.2 Higher level structures of myostatin

The secondary, tertiary and quaternary structures of myostatin are conserved across animal species, and the mature protein consists of two peptides that form a homodimer of approximately 26 kDa. Both bovine and human myostatin are active as homodimers typically consisting of two subunits of 376 amino acids. The propeptide and sometimes the mature myostatin structure can however sometimes differ slightly in length.

Myostatin in the common house mouse (*Mus musculus*) is a homodimer of polypeptides of 326 amino acids. This difference in length between species does not alter myostatin function, as all the major structural components of the protein are still present.

Myostatin can be inactivated by forming a non-covalent biologically inactive complex with its propeptide. This inactive complex consists of an N-terminal secretory signal, a propeptide domain, and a C-terminal peptide.

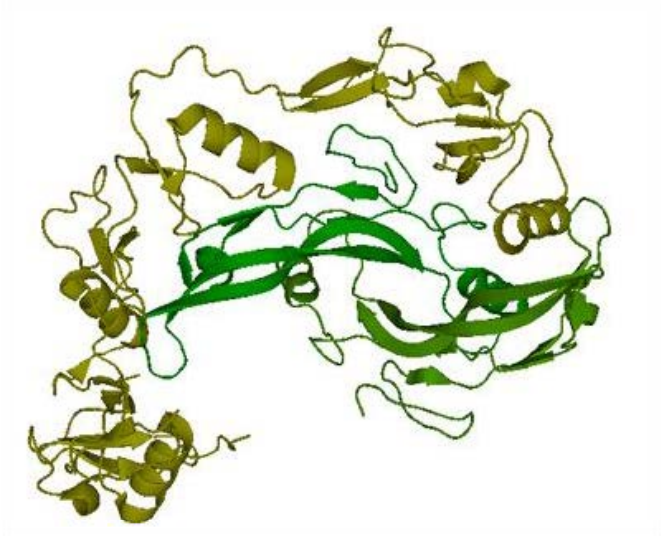


Figure 2.2 The myostatin homodimer (dark green)-follistatin homodimer (brown-green) complex (Song, Harel, Prilusky, & Pinotsis, 2015)

The prepropeptide secretory signal is located in the hydrophobic centre near the N-terminus, while at the C-terminal a proteolytic processing signal, made up of four conserved amino acids RSRR (arginine, serine, arginine, and arginine) is found. A calcium-dependent serine protease, called furin intracellularly cleaves the RSRR site on the signal region, leaving behind the propeptide and the 13 kDa mature peptide. The propeptide is cleaved at a variable site by bone morphogenetic protein (BMP-1), but remains non-covalently bound to C-terminal myostatin. It then forms the C-terminal domain, allowing the homodimer to form by joining to another mature myostatin via a disulphide bond. It also contains nine cysteine residues in the C-terminal region to facilitate the formation of a 'cysteine knot' structure (Jeanplong et al., 2001).

The mature myostatin monomer contains two alpha-helices and seven beta-strands, with the dimers joined by one inter-chain disulphide bond, to form a hand-shaped structure that is characteristic of the TGF-β family. The two monomers, joined by the disulphide bond, are anti-parallel and the surface of which consist of four distinct regions "…two convex type II receptor binding sites on the β sheet or 'finger' region and two concave type I receptor binding sites composed of the 'fingertip' and major helix or 'wrist' region" (Cash et al., 2011).

Attempts to determine the three-dimensional structure of mature myostatin using X-ray crystallography, revealed that it was predominantly found bound to other molecules. Figure 2.2 above depicts myostatin bound to follistatin 288 (resolved at 2.15 Å). The follistatin (brown-green) binds around the outside of myostatin, while the inner dark green structure is the myostatin homodimer.

2.2 Myostatin interactions with other proteins

Myostatin is involved in many inter-protein interactions. The majority of these are associated with its regulation and movement out of, and back into, target cells. Like other TGF-β proteins, the propeptide myostatin requires other proteins to bind to it before it can be secreted across the cell membrane and out of the cell.

2.2.1 The interaction of human small glutamine-rich tetratricopeptide repeat-containing protein with myostatin

Upon synthesis prepropeptide myostatin is translocated into the endoplasmic reticulum, where it forms a homodimer. The human small glutamine-rich tetratricopeptide repeat-containing protein (hSGT) has been shown to interact with the prepropeptide myostatin intracellularly (McFarlane et al., 2005). The C-terminal region of hSGT, containing the third TPR (threonine, proline, and arginine) motif that is vital for the interaction to take place, associates with the N-terminal signal peptide on myostatin. The function of hSGT appears to be to act as a molecular chaperone that is involved in protein folding and processing. During myogenesis in skeletal muscle cells hSGT also mediates myostatin secretion and activation. hSGT is ubiquitous, indicating it has the same functionality across many species (Wang, Zhang, & Zhu, 2003) and the myostatin-hSGT interaction may have additional functions, such as providing a docking site for other myostatin partner proteins: enabling the formation of signalling-complexes that mediate myostatin binding to its cell surface receptor (Wang et al., 2003).

2.2.2 The interaction of furin and myostatin

Upon moving to the Golgi apparatus the signal region of the prepropeptide myostatin structure is proteolytically processed at the C-terminal signal site (RSRR) by a calcium-dependent serine protease, called furin (Huang, Chen, & Chen, 2011). At the same time, it also cleaves the now N-terminal propeptide at the furin site (Anderson, Goldberg, & Whitman, 2008), leaving an N-terminal propeptide (called latency-associated protein (LAP)) and an inactive C-terminal protein, as a consequence of it still being non-covalently bound to LAP (Burks & Cohn, 2011). It has been revealed that the furin protease promoter activity is regulated by mature myostatin and thus myostatin controls the level of furin protease present and hence, how much of itself is produced. This consequently affects the differentiation of myoblasts (McFarlane et al., 2005).

2.2.3 The interaction of the titin-cap proteins and myostatin

Titin-cap (T-cap) proteins interact with the propeptide myostatin and the affinity between the two is very high (K_D of 40 nM). It has been revealed that over-expression of T-cap does not alter the

expression of myostatin, but it does increase its secretion out of the cell in myogenic precursor cells (Nicholas et al., 2002), suggesting its role is to aid in myostatin secretion.

2.2.4 The interaction of follistatin and myostatin

The myostatin propeptide and follistatin-related gene peptides are proteins that are involved in the negative regulation of myostatin activity (they make it inactive), and this results in increased muscle mass (Liu et al., 2012). LAP binds non-covalently to the active myostatin structure making it inactive both intracellularly and extracellularly. Extracellularly, follistatin (Fst), follistatin-related gene protein 3 (FSTL-3), telethonin and growth and differentiation factor-associated serum protein-1 (GASP-1) regulate myostatin, making it inactive *in vivo* and *in vitro* (Burks & Cohn, 2011). Fst, follistatin-related gene protein-3 (FLRG-3) and activin IIB receptor fusion protein (ActRIIB-Fc) bind around myostatin (or activin, which is also a member of the TGF-β super-family) making it unable to bind to the receptors ActRIIA or ActRIIB. These (along with the other extracellular binding-proteins), act on myostatin as it travels throughout the body.

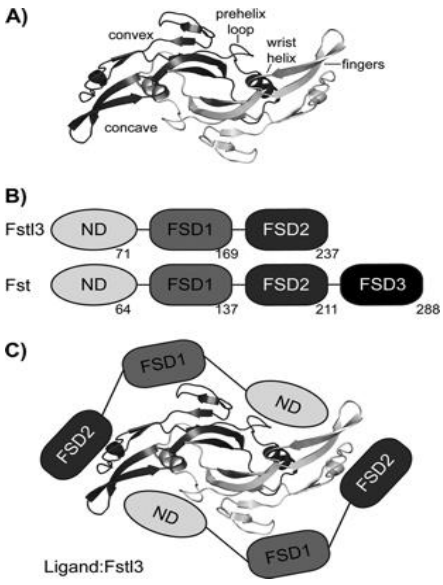


Figure 2.3 Fst-type protein family and TGF-β interaction. A) Predicted structure of mature myostatin. B) Fst and FSTL3 domain layout. C) The interaction of FSTL3 and myostatin, FSD1 and FSD2 block the convex type II receptor-binding site, whereas the N-terminal domain (ND) blocks the concave type I receptor-binding site (Cash et al., 2011)

2.2.5 The interaction of activin and myostatin

Once the activin type II receptors are activated (by myostatin and/or activin), they then activate type I receptors (shown in figure 2.4), which initiates phosphorylation of the intracellular mediators called small mothers against decapentaplegic (SMAD) 2 and 3 (Lee & Glass, 2011). Signalling can only be

initiated when two type I and two type II receptors have been activated (Cash et al., 2011). The alpha-helix on myostatin plays a significant role in this process ensuring specificity when binding to type I transmembrane receptors, while the β-sheet 'fingers' are important in type II receptor binding (Cash et al., 2011). FLRG-3 interacts with myostatin-LAP structure via its N-terminal domain. Point mutations in *FLRG-3* stop the proteins ligand-binding activity, whereas corresponding point mutations in the Fst gene (*Fst*) have little effect (Cash et al., 2011). This is in part due to the crossing-over of N-terminal domain (ND) and FSD3 (the third domain of FSTL-3) when bound to TGF-β molecules.

Many studies have looked into the differential binding of Fst-type molecules to TGF-β ligands, but few have looked specifically at myostatin (Cash et al., 2011).

2.2.6 The interaction of Smads and myostatin

Smads (a family of proteins that are involved in the translocation of signals from TGF-β receptors, bone morphogenetic protein receptors and other surface receptors to the cell nucleus (Medicine, 2015)) are the intracellular signals, initiated by myostatin binding. They translocate into the nucleus where they activate downstream gene transcription by acting as transcription factors.

2.3 The mechanisms of myostatin action

Furin (calcium-dependent serine) proteases, proteolytically process the three fragments of myostatin intracellularly (primarily in the Golgi apparatus) cleaving off the N-terminal signal peptide and cleaving at the furin site on the C-terminal propeptide (Jeanplong et al., 2001). This leaves the propeptide (now called LAP) non-covalently bound to the mature, but inactive, myostatin (Wintgens, Dschietig, Stoveva, Paulsson, & Armbruster, 2012). Further proteins, such as titin-cap and hSGT, interact with the myostatin structure intracellularly, aiding in its secretion into circulation. While the structure is in circulation, follistatin and other latent TGF-β binding proteins (LTBP) can bind to it; forming a larger complex. Bone morphogenetic protein-1 (BMP-1) of the tolloid family of metalloproteases, locally activates mature myostatin from the circulatory complex formed, and this enables it to bind to its transmembrane serine/threonine kinase receptors, the activin type II receptors, ActRIIA or ActRIIB (McFarlane et al., 2005).

BMP-1 cleaves between Arg-75 and Asp-76 (Anderson et al., 2008) and is located in the Golgi apparatus, trans-Golgi network, extracellular space and extracellular matrix (Anderson et al., 2008). Myostatin is activated locally and extracellularly (near muscle cells), thus BMP-1 located in the extracellular matrix or space activates it (the specific location is unknown).

Although LAP inhibits myostatin from becoming active, it appears to be required for the correct fold, dimerization, and secretion of the mature peptide (McFarlane et al., 2005). The propeptide state also allows for the regulation of active myostatin and the regulation of other binding molecules.

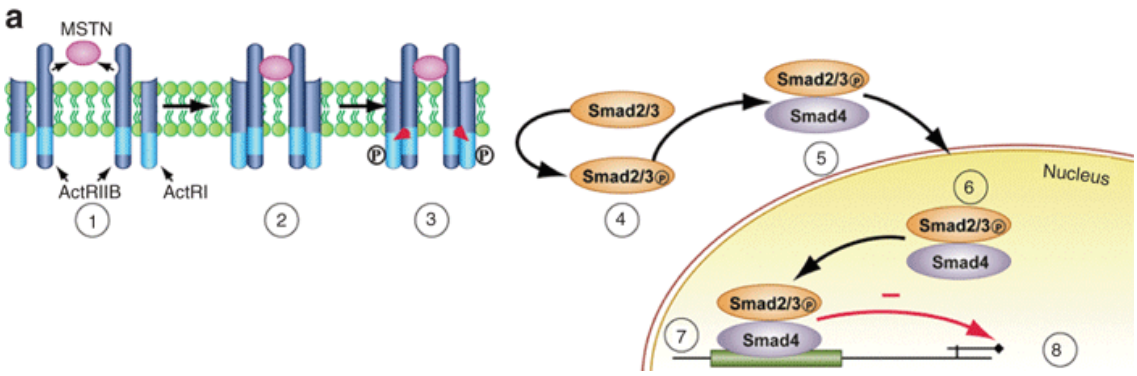


Figure 2.4 Myostatin signalling. (1) Mature myostatin binds to activin receptor type IIB (ActRIIB) dimer. (2) This recruits two activin receptors type I (ActR1). (3) Trans-phosphorylation occurs, activating the kinase activity of ActRI. (4) Phosphorylation activates Smad2 and Smad3 intracellularly (5) and they form a heterodimer with Smad4. (6) This is translocated into the nucleus (7, 8) which initiates transcription of other genes (Fakhfakh, Michaud, & Tremblay, 2011) *Note that this image was cropped from the original.*

The activin type II receptors, ActRIIA and ActRIIB, are embedded in the cell membrane, which then activates signals inside the cell (as shown in figure 2.4). This occurs along with the binding of TGF-β1 to activin type I receptors, ALK5 or ALK4. This then initiates a downstream phosphorylation of R-Smads 2 and 3, which then bind to Smad 4.

The intracellular proteins that myostatin and TGF-β1 signal to, can interfere with the normal cascade of interactions that occur inside the cell, which can then impact the effectiveness of the processes. The Smad complex is translocated inside the nucleus where it acts as a transcription factor for gene expression (Burks & Cohn, 2011).

Functionally, mature myostatin regulates embryonic myogenesis (progression and differentiation of myotubes from myoblasts) through interaction with the myoblasts. During the proliferation of non-differentiated myoblasts to committed myoblasts, p21 (a cyclin-dependent kinase inhibitor) is up-regulated by myostatin signalling (Spiller et al., 2002; Thomas et al., 2000). The role of p21 is to regulate cyclin-dependent kinase-2 (Cdk2) by inhibiting its action. Its' up-regulation therefore inhibits Cdk2 protein activity. As a result one of the Cdk2 targets, retinoblastoma (Rb), is hyper-phosphorylated (inactivated), which affects E2F transcription factors essential for myoblasts to progress from the G_1 to S-phase in the cell cycle (Thomas et al., 2000).

Myostatin also works as a negative regulator through Rb-independent pathways and can inactivate MyoD which inhibits myoblast terminal differentiation (as shown in figure 2.5) (McFarlane et al., 2005). This offers one explanation into the syndrome known as 'double-muscling', whereby defective myostatin genes result in greater hyperplasia and hypertrophy, as myoblast proliferation is deregulated.

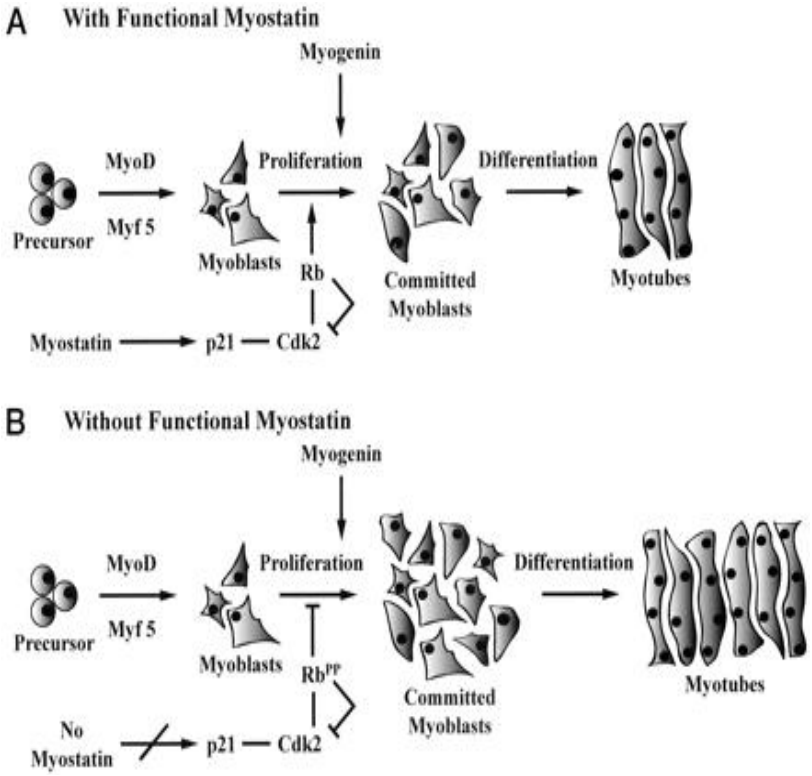


Figure 2.5 A) The interaction of functional myostatin on myoblast differentiation. B) The effects of non-functional myostatin on myoblast differentiation, with increased myotube formation. (Thomas et al., 2000)

2.3.1 Myostatin redundancy

Despite TFG-β1 and activin (both members of the TFG-β super-family) functioning in a similar way to myostatin and binding to similar transmembrane receptors, they do not set off the same cascade of intracellular Smad events as myostatin. The current literature has found no other protein which sets off the same intracellular Smad events or initiates the same genes to be expressed as myostatin. This suggests that redundancy does not exist between myostatin and other proteins.

2.4 Determining the location and characterising the myostatin gene (*MSTN*)

Researchers in the Faculty of Veterinary Medicine at the University of Liege, and scientists in the Department of Molecular Biology and Genetics at the John Hopkins University School of Medicine in Baltimore, worked simultaneously to discover the location and function of myostatin.

2.4.1 The University of Liege's findings about *MSTN*

In 1929 it was proposed the double-muscling "syndrome" was inherited and due to a single gene defect, although further research was not carried out until 1995 when the Faculty of Veterinary Medicine at the University of Liege, Belgian mapped the defect.

Through their marker-assisted genetic mapping, the defect was located on the centromeric end of bovine Chromosome 2 (Chr 2), and it was confirmed that the phenotype was the result of a single, autosomal, gene defect (Flynn & Flynn, 2015). The approach used a panel of 213 bovine microsatellite markers, and an experimental pedigree obtained by back-crossing Belgian Blue x Friesian dams to double-muscle sires (Charlier et al., 1995). Microsatellites (simple sequence repeats) are DNA sequences which contain 2-4 nucleotide tandem repeats. They are a class of genetic polymorphism that are known to have high mutation rates, and which are used as reference points along a chromosome in mapping, in tracing inheritance patterns and in linkage analysis (BioScience, 2016). Charlier et als (1995) resulting genetic map indicated seven microsatellites specific to Chr 2, from which the muscling locus (referred to as mh (muscular hypertrophy)) was positioned two centiMorgans from the closest marker.

2.4.2 The Johns Hopkins University findings about GDF-8 (subsequently called myostatin)

At the same time as the research at Liege, investigation of the TGF-β superfamily was being undertaken by scientists at the Department of Molecular Biology and Genetics at the Johns Hopkins University School of Medicine in Baltimore (Flynn & Flynn, 2015). During their research they discovered an unidentified protein they called growth differentiation factor-8 (GDF-8) which contained 376 amino acids.

Degenerative polymerase chain reaction (PCR) was used to find the gene producing GDF-8 (called *GDF-8*), followed by gene-targeting in mice to confirm their discovery. Finding new proteins in the TGF-β superfamily involved utilising conserved regions of known family members to scan genomic mouse DNA with specially designed degenerate oligonucleotides that act as primers for PCR amplification. By using one of the 110 complementary cloned sequences obtained from PCR (subsequently determined to be a portion of *GDF-8*) as a probe for screening a murine skeletal muscle cDNA library, a complete *GDF-8* cDNA clone (that encoded a protein of 376 amino acids) was

isolated. The putative polypeptide contained a signal sequence, proteolytic processing site and nine conserved cysteine residues in the carboxy-terminal region, all of which are hallmark characteristics of TGF-β family members (McPherron & Lee, 1997).

Once the GDF-8 gene was found, its expression pattern in mice was determined using *in situ* hybridisation on sectioned mouse embryos isolated at different stages of development. This indicated that mRNA expression is restricted to developing skeletal muscle during foetal development and continues, almost exclusively, in skeletal muscle tissues in adult life (Kambedur, Sharma, Smith, & Bass, 1997)(McPherron, Lawler, & Lee, 1997).

The biological activity of GDF-8 was further investigated using gene knock-out mice (both in foetal and adult stages of development). This involved injecting target clones that deleted the entire C-terminal region resulting in it becoming non-function or null into blastocysts (McPherron et al., 1997). Compared to wild-type and heterozygous animals, homozygous mutant individuals were around 30% larger, irrespective of age or gender. The increase was due to the mice having larger muscles throughout their bodies, these being 2-3 times larger than the wild-type animals. Heterozygous mutant animals also showed an increase in muscle size compared to wildtype individuals, but they were not as muscled as their homozygous counterparts.

From these findings it was concluded that GDF-8 functioned specifically as a negative regulator of skeletal muscle growth (McPherron et al., 1997) and because of this, they renamed GDF-8, myostatin. Myostatin (abbreviated to *MSTN* for the GDF-8 gene) has been used from this point on in this dissertation.

2.4.3 Additional functions of myostatin

More recently it has been revealed that myostatin is found in foetal and adult hearts (Sharma et al., 1999) and mammary glands (Ji et al., 1998), specifically being identified in cardiomyocytes and Purkinje fibres. Upregulation of myostatin has also been recorded in cardiomyocytes following myocardial infarction. This, as well as its presence in foetal and adult hearts, indicates that myostatin may play a significant role in cardiac development and physiology (Sharma et al., 1999). Myostatin may also play a role in mammary gland development and/or lactation due to its detection in porcine mammary glands (Ji et al., 1998).

A systemic wasting syndrome (called cachexia) occurs as a result of ectopic expression of myostatin. This has been found to occur naturally in mice and results in premature death. This suggests the balance between muscle growth and muscle wasting through myostatin signalling and production, is vital for maintaining homeostasis (Anderson et al., 2008). A study into myostatin involvement in human AIDS-related wasting syndrome, found that higher concentrations of myostatin were

recorded in adult men with the syndrome than men without (Gonzalez-Cadavid et al., 1998). This correlated with weight loss in these infected men. These findings support the hypothesis that myostatin acts as a negative regulator of muscle growth and contributes to muscle wasting in HIV-infected men (Gonzalez-Cadavid et al., 1998).

2.4.4 Links between myostatin activity and the Belgian Blue cattle double-muscled phenotype

In many species, naturally occurring mutations or variation in myostatin result in abnormally high levels of muscling or the so-called 'double-muscled' phenotype. For example variation within Belgian Blue cattle is typically under-pinned by six nucleotide variations (Flynn & Flynn, 2015), as shown in figure 2.6 and table 1.

2.4.5 Disadvantages in having variant forms of myostatin

It was determined that either one, or two copies of the variant form of the gene, resulted in an increase in muscle development, or hyperplasia (an increase in cell number); and to a lesser extent muscle cell hypertrophy (an increase in cell size). Further research into the effect revealed that homozygous cattle increased muscling two-fold, but birthing difficulties also arose when calving; while heterozygous animals increased muscling markedly, but did not appear to have birthing difficulties. This led to the conclusion that heterozygosity was the preferred genotype for farmed cattle (Flynn & Flynn, 2015), unless the occurrence of birthing difficulties could be reduced.

2.5 Documented variation in *MSTN*

2.5.1 Variation in the bovine *MSTN* coding region

Although myostatin is conserved across species, variation does occur. A number of myostatin variants have been identified in other species, most of which are silent or neutral. However, some of these are mutations can result in the protein becoming either non-functional, or having altered functionality.

This is well documented in bovine species. Six sequence variants have been described in exons 2 and 3 of bovine *MSTN*, which are depicted in figures 2.6 and table 1 below. One of these mutations is a deletion of eleven base pairs in exon 3, and this is found in Belgian Blue cattle. This causes a frameshift that eliminates almost all the mature myostatin protein and leads to its inability to function. Another sequence variant that is found in Piedmontese cattle is the substitution of cysteine 313 with tyrosine. The result is a distorted cysteine-knot, making the secondary structure unattainable and therefore the protein becomes non-functional.

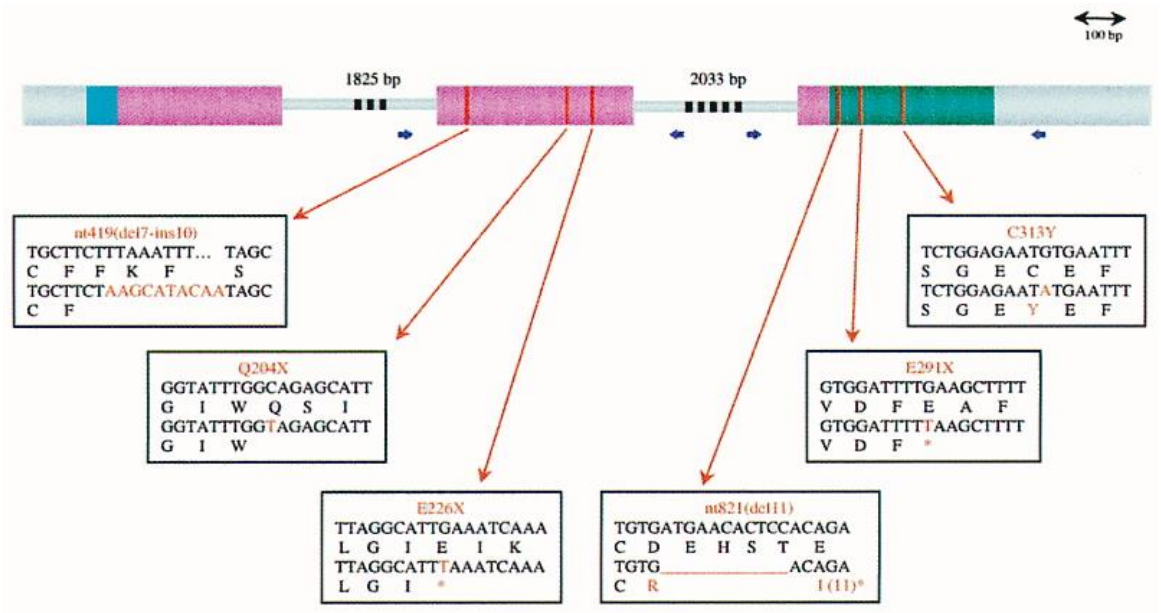


Figure 2.6 Six common bovine myostatin sequence variations. Highlighted in blue is the conserved signal sequence, in pink the central propeptide region containing three or six variations in red, and highlighted in green is the active myostatin C-terminal region which is the site of a further three sequence variations. The portions highlighted in grey are non-coding regions. Table 1 further describes the variation. (Flynn & Flynn, 2015)

Table 1 Six myostatin sequence variants found in cattle including; what the variation is, the resulting effect on the protein and in which breed the variation has been found (Flynn & Flynn, 2015).

Type of gene variation (nucleotide position after the initiation codon)	Change in myostatin protein	Cattle breeds
nt419(del7-ins10) **Deletion of seven base pairs and replacement with ten others**	**Truncation of the protein due formation of a premature STOP codon**	**Maine-Anjou**
Q204X **C --> T substitution**	**Truncation of the protein due to formation of a premature STOP codon**	**Charolais Limousin**
E226X **G --> T substitution**	**Truncation of the protein due to formation of a premature STOP codon**	**Maine-Anjou**
nt821(del11) **Deletion of eleven base pairs**	**Truncation of the protein due to formation of a premature STOP codon**	**Belgian Blue Blonde d'Aquitaine Limousin South Devon**
E219X **G --> T substitution**	**Truncation of the protein due to formation of a premature STOP codon**	**Marchigiana**
C313Y **G --> A substitution**	**Substitution of cysteine with tyrosine**	**Piedmontese**

2.5.2 Variation in the promoter region of bovine *MSTN*

Variation has also been described in regions upstream of the *MSTN* coding sequence. SNPs have been described in some breeds (Crisa, Marchetelli, Savarese, & Valentini, 2003), for example a T>A substitution -371 and G>C substitution at -805 have been described in some European cattle breeds (Marchigiana, Chianina, Romognola, Piedmontese, Holstein-Friesian, Italian Red Pied, Brown Swiss, Belgian Blue and Limousine). Promoter region SNPs have also been found in Qinchuan and Red Angus cattle (He, Wu, Quan, Liu, & Zhang, 2013).

E-boxes (CAnnTG) are sequence motifs recognized as critical regulatory components in muscle gene expression (Apone & Hauschka, 1995) (Catala et al., 1995). Multiple E-boxes are located in enhancers or promoters of muscle-specific genes, such as the creatine kinase gene, the myogenin gene and the myosin light chain gene. These motifs regulate transcription by working cooperatively (Rao, Donoghue, Merlie, & Sanes, 1996). They have also been revealed to control variable axial skeletal muscle gene expression and accessibility in mice, by acting as a sensor responsible for regional differences (Ceccarelli et al., 1999).

Myogenic regulatory factors (MRFs) (including MyoD, Myf5 and myogenin) are a group of basic helix-loop-helix transcription factors which bind to E-boxes (Spiller et al., 2002). E-boxes are vital in providing a binding site for MyoD and Myf5, are expressed in myoblasts and myotubes, and are required for myogenesis (Spiller et al., 2002) (as shown in figure 5). Myogenin has been shown to be vital for myotube formation. Studies have found significant increases in myogenin mRNA during myoblast differentiation (Apone & Hauschka, 1995).

MSTNs promoter region contains ten E-boxes (Spiller et al., 2002) and it is clear that myostatin expression is partly dependent on the presence of the MRFs. How critical each separate E-box is to bovine myostatin expression has been studied, with findings showing that E6 (the sixth E-box) interacts with MyoD and is critical for myostatin promoter activity (Spiller et al., 2002).

The ten E-boxes are arranged in three clusters. The cluster containing E1 and E2 is located near the TATA box and ATG site, while the cluster containing of E3-E6 spans -175 to -667 and the distal cluster containing E7-E10 is further removed(Spiller et al., 2002). The presence of only the proximal cluster (E1 and E2) was sufficient to drive gene activity, while deletion of the E5 and E6 E-boxes reduced (15- to 9-fold reduction) myostatin promoter activity. On further investigation, deletion of both the distal and E5 and E6 E-boxes further reduced (six-fold reduction) promoter activity. A similar result was found when only E6 was deleted, highlighting the importance of this E-box. It was concluded that the distal cluster is dispensable for the maximum promoter activity (Spiller et al., 2002).

This study also analysed E1 and E2 within the promoter section genotyped and found no variation (Spiller et al., 2002). The position and sequence of these E-boxes is well conserved across different species (mice, cattle and pigs) and contributes to the functional conservation of the myostatin promoter (Spiller et al., 2002).

2.5.3 Ovine and caprine *MSTN* variation

Variation in ovine (sheep) and caprine (goat) *MSTN* has also been reported, which in some cases results in non-functional myostatin being produced. Eight haplotypes have been described in ovine myostatin, with 28 nucleotide substitutions described, of which only one is located in a coding region. This results in an amino acid change of glutamic acid (Glu) to glycine (Gly) (Hickford et al., 2010). Variation in intron 2 of goat myostatin has also been recorded, with seven polymorphic sites being identified (Li et al., 2006).

2.6 Overall conclusion

Myostatin is part of the TGF-β superfamily of cytokines and has function in the control of cell growth, apoptosis, cell differentiation and cell proliferation. Myostatin negatively regulates muscle growth from early in embryonic development into adult life. It is translated as a three part prepropeptide consisting of an N-terminal signal peptide, a middle propeptide portion and a C-terminal myostatin portion. Mature myostatin is ubiquitous and differs very little in sequence and size between species. Whether myostatin is located intra or extracellularly, it interacts with other proteins that regulate and assist its function. Lack of one or two functional myostatin alleles results in increased muscling across all species.

Chapter 3

Methods

3.1 Blood sample collection

In this study 116 dairy cow (predominantly KiwiCross™) blood samples were collected from two farms and 378 beef cow samples were collected, 371 of which were Belgian Blue samples from one Northland property and 7 of which were South Devon samples from one farm. Samples were collected between 2014 and 2015 with farmer consent and according to normal farm management practices.

Where possible, samples were taken from all cattle on the farm with zero prejudice towards animal condition, gender or age.

Blood samples were collected by cutting the lower part of the ear, near the tip where the vein ends. To reduce contamination, electrical side-cutters were used to clip the ear, as blood does not come into contact with these.

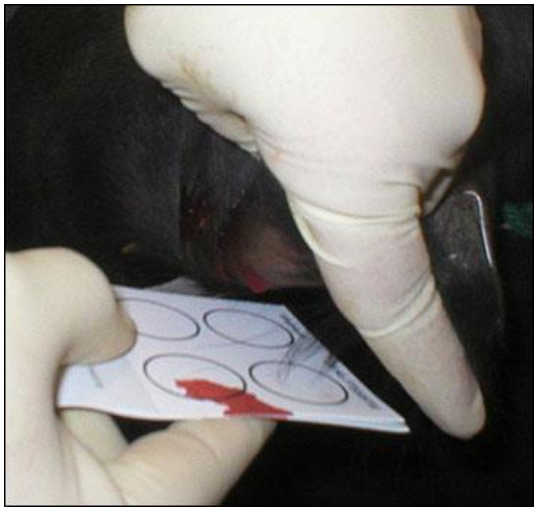


Figure 3.1 Bloody collection on FTA cards (McClure, Weaber, & Olson, 2005)

The extracted blood drops were collected on FTA cards that were labelled according to the farm, farmer and breed. Blood needed to soak through the card to ensure a good quality sample was collected.

The KiwiCross™ samples were collected prior to the start of this study for a separate study being undertaken by the Lincoln University Gene Marker Laboratory. From the hundreds of samples collected, 116 were randomly selected to be genotyped. The South Devon and Belgian Blue samples were collected during the study by the method described, and all they were all genotyped in this

study. The FTA cards were stored at room temperature and in the dark at the Lincoln University Gene Marker Laboratory.

3.2 DNA extraction

DNA was extracted from 1.2 mm discs punched from the blood on the FTA cards. The discs were then placed in 200 µL tubes and incubated at 60^{o}C in 200 µL of 20 mM NaOH for 15-20 minutes, or until the discs became white. The NaOH solution was aspirated and the discs were washed in 200 µL of 1x TBE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) for 5 minutes before that was aspirated. The discs were left in their tubes to dry overnight.

3.3 PCR-SSCP analysis

Dr Huitong Zhou (Lincoln University) designed two primers for this investigation. The forward PCR primer sequence was 5′-GAAGTAGTCAAATGAATCAGC-3′ and the reverse primer sequence was 5′-CAAGGGAAAAGATTGTATTGAT-3′. These were designed based on the published *MSTN* promoter sequence in (Crisa, Marchitelli, Savarese, & Valentini, 2003) accession number AJ438578.1. These primers specifically amplified a portion of the promoter region of the myostatin gene that was upstream from the ATG start site. Integrated DNA Technologies (Coralville, IA, USA) synthesised these primers.

```
cGDF8        GAAGTAGTCAAATGAATCAGCTCACCCTTGACTGTAACAAAATACTGTTT    50
cGDF8        GGTGACTTGTGACAGACAGGGTTTTAACCTCTGACAGCGAGATTCATTGT   100
cGDF8        GGAGCAAGAGCCAATCACAGATCCCGACGACACTTGTCTCATCAAAGTTG   150
cGDF8        GAATATAAAAAGCCACTTGGAATACAGTATAAAAGATTCACTGGTGTGGC   200
cGDF8        AAGTTGTCTCTCAGACTGGGCAGGCATTAACGTTTGGCTTGGCGTTACTC   250
cGDF8        AAAAGCAAAAGAAAAGTAAAAGGAAGAAGTAAGAACAAGGGAAAAGATTG   300
cGDF8        TATTGATTTTAAAACCATG                                  319

Primer binding regions are shown in red. The start codon and the putative TATA
box are shown in green.
```

Figure 3.2 MSTN promoter sequence depicting the primer binding regions in red and ATG and TATA site in green

PCR amplification followed. Amplifications were performed in a 15 µL reaction containing the genomic DNA on a 1.2 mm punch of FTA card, 0.25 mM of each primer, 150 mM dNTPs (Eppendorf, Hamburg, Germany), 2.5 mM of $MgCl^2$, 0.5 U of Taq DNA polymerase, and 1 x the reaction buffer supplied with the enzyme (containing 1.5 mM $MgCl_2$) (Qiagen, Hilden, Germany).

The reagents listed above were added in aliquots of 15 µL to the tubes containing the discs punched from the FTA card. The PCR thermal profile consisted of an initial 2 minute extension phase at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. A final extension phase of 5 minutes at 72°C finished the profile. PCR amplification was carried out in an S1000 thermal cycler (Bio-Rad, Hercules, CA, USA).

Gel electrophoresis was used to visualise amplicons. One to 2 µL of PCR product was electrophoresed on 1% agarose (Quantum Scientific, Queensland, Australia) gels after each refinement to the PCR method (these refinements are described in the discussion). This gel confirmed the presence of a DNA band and therefore that amplification had occurred before undertaking SSCP analysis. The agarose gel consisted of 30 mL 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA), 0.3 g agarose and 200 ng/mL of ethidium bromide. Gels were left to set at room temperature for 2 hours before use. The gels were loaded with a mix of 1 µL loading dye and 2 µL of PCR product and run for 10 minutes at 100 volts in approximately 300 mL of 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide. Ultraviolet trans-illumination was used to visualise the band. When a band was present then samples were subjected to SSCP analysis.

The SSCP protocol started with each amplicon being mixed with 10 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol) and denatured at 105°C for 5 minutes, before being place on wet ice to cool.

For the KiwiCross™ cattle, 15 µL of the mix was added to each well, while only 10 µL was added for South Devon and Belgian Blue samples (the reasoning for this is outlined in the discussion). In addition to any new samples that were loaded, reference variants were also run through the SSCP analysis. The SSCP gels were 20 cm x 18.5 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and the reagents used in the gels are listed in table 3. Protean II xi cells (Bio-Rad) machines were used to run electrophoresis at 200 volts for 18 hours in 10x TBE buffer at 20°C.

Table 2 Reagents used in each 14% SSCP 20 cm x 18.5 cm gel

Reagent	1x Gel
10x TBE (mL)	1.7
40% Acrylamide/Bis Solution 37.5:1 (mL)	11.8
Water (mL)	20
10% APS (µL)	203.3
TEMED (µL)	20.3

Following electrophoresis, the gels were silver-stained according to the method of (Byun, Fang, Zhou, & Hickford, 2009).

3.4 Statistical analysis

Microsoft Office Excel 2013 was used to analysis the data collected. As all samples that typed appeared to be of the same genotype, the statistical approaches that could be used to analyse the data were limited.

Chapter 4

Results

4.1 SSCP gel results

Of the 494 samples extracted, 395 (80% overall) were able to be genotyped with bands being visible on the SSCP gels. The possible reasons underlying the failure to amplify all of the samples are explained in the discussion.

4.2 Genotyping results

The PCR-SSCP analysis aimed to determine whether variation was present in this gene region. This would typically be revealed as differences in banding patterns observed on the SSCP gels. However, as described above, not all of the cattle samples analysed produced bands upon PCR amplification and SSCP analysis. The DNA typing results are summarised in table 3 and figure 4.1.

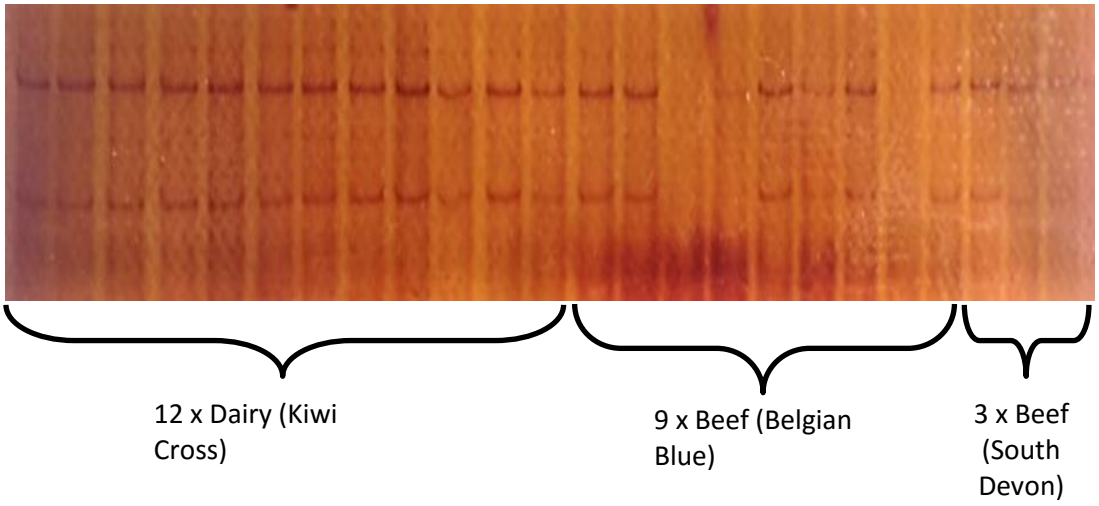


Figure 4.1 A representative SSCP gel demonstrating the apparent lack of genetic variation in the fragment of the *MSTN* promoter region that was analysed. In three of the lanes, no bands can be seen, suggesting that no PCR amplicon was produced.

Table 3 Genotyping Summary. Cattle included 116 KiwiCrossTM individuals of which 86 produced bands, seven South Devon all of which were typed and 371 Belgian Blue individuals, 302 of which were typed. All cattle that were typed had the AA genotype.

Breed	n (tested)	n (typed)	Genotype
KiwiCross™	116	86	AA
Belgian Blue	371	302	AA
South Devon	7	7	AA

There was no discernible difference between each breed, so very little statistical analysis could be undertaken.

It needs to be noted that some of the samples did not amplify, when others from the same herd did. All of the South Devon samples produced bands on SSCP analysis, whereas 86/116 = 74% KiwiCross™ cattle produced bands. This was less than might be expected given the 100% success in typing the South Devon cattle. If the proportion that produced bands with the KiwiCross™ cattle has been observed for the South Devon cattle, then only five of the South Devon cattle might have been expected to have produced bands. Equally 302/371 = 81% of the Belgian Blue cattle produced bands upon SSCP analysis, so once again it might have been expected that fewer than seven of the South Devon cattle may have produced bands.

To test if there was a difference in the success rate of typing, the results for the various breeds were subjected to a Chi-square analysis. The results are shown in figure 4.2.

Results						
	Typed	Tested				*Row Totals*
South Devon	7 (6.22) [0.10]	7 (7.78) [0.08]				14
KiwiCross	86 (89.75) [0.16]	116 (112.25) [0.13]				202
Belgian Blue	302 (299.03) [0.03]	371 (373.97) [0.02]				673
Column Totals	395	494				889 (Grand Total)

The chi-square statistic is 0.5113. The *p*-value is .7744. The result is *not* significant at $p < .05$.

Figure 4.2 Chi-square analysis of the success rate of genotyping

This suggests that despite some of the Belgian Blue and South Devon cattle not being typeable, there does not appear to be a difference in the success rate of typing. However, it needs to be remembered that the populations are independent, thus this statistical approach may be flawed.

Chapter 5

Discussion

The aim of this study was to determine whether polymorphic variation was present in a section of the promoter region of bovine *MSTN*; the presence of which could have been used in a gene-marker test to indicate cattle myostatin functionality and the resulting phenotype.

KiwiCross^{TM} and South Devon cattle blood samples were available to type, while Belgian Blue samples were collected from a North Island property. Variation was expected to be seen, especially in Belgian Blue cattle, as variation is present within the coding region in this breed. However, all the samples that could be typed appeared to produce the same genotype (AA) for the promoter fragment that was amplified.

Previous studies have reported SNPs in the promoter region of different cattle breeds including the Marchigiana, Chianina, Romognola, Piedmontese, Holstein-Friesian, Italian Red Pied, Brown Swiss, Belgian Blue, Limousine, Qinchuan and Red Angus cattle (Crisa, Marchitelli, et al., 2003; He et al., 2013). This variation was upstream of the promoter section under investigation in this study, which was taken to suggest that sequence variation could occur in this part of the promoter and hence that upon PCR-SSCP analysis different banding patterns would be seen.

This suggests that a number of things might have occurred. First, the region amplified may not actually be variable in cattle because its structural integrity needs to be maintained to maintain the functionality of *MSTN*. Any mutations that arose in this region would accordingly be strongly selected against and thus they might only occur at a very low frequency. If this were the case, then in a heterozygous state, it might be expected that the selection pressure against individuals carrying such mutations might be somewhat less and any 'mutant' variant of the promoter might still be found at a low frequency in the population.

Next, the PCR-SSCP typing approach might not have sufficient resolving power to identify subtle sequence difference in the amplified region and thirdly, the primers may have bound to regions in the promoter that were variable, but this variation would not have been detected if PCR amplified the region. Variation in the primer binding region may also have inhibited PCR amplification in some cattle, which is consistent with some samples not amplifying and finally the cattle chosen for typing may have not been variable in this region in the first place, although variation may occur in other cattle from the same breeds. Each of these possibilities is discussed below:

5.1 Bovine *MSTN* variation and the possibility for conservation of key promoter regions

The first studies of *MSTN* variation that caused the 'double-muscling' syndrome were simultaneously completed by (Crisa, Marchitelli, et al., 2003) and (McPherron & Lee, 1997). Each study discovered the location and function of *MSTN* and linked its function to the 'double-muscling' phenotype observed in Belgian Blue cattle. Before these studies it was clear that the 'double-muscling' phenotype was not sex-linked, as both female and male cattle and mice presented with it. It therefore came as no surprise that both studies found *MSTN* to be located on chromosome two.

Nucleotide variations in the myostatin gene that cause functional protein changes are likely maintained in the population because they are non-life threatening or cause insufficient phenotypic change, as to be selected against.

A non-functional myostatin protein cannot negatively regulate muscle proliferation and growth resulting in hyperplasia and hypertrophy. Homozygosity for myostatin alleles producing myostatin of affected functionality results in the phenotype often referred to as 'double-muscling', where two to three times the average muscle is produced. This is often selected for in beef production systems to increase profit and is one of the defining characteristics of both the Belgian Blue and Piedmontese cattle breeds. The six most common sequence variants are described in the literature review and these occur in exons 2 and 3, mostly resulting in formation of a premature stop codon. Mutation C313Y eliminates one of the nine critical cysteine residues needed to form the cysteine knot in the proteins secondary structure causing incorrect protein folding and making the protein non-functional. These all produce reasonably major changes in myostatin and yet they occur naturally in the cattle population.

Variation occurring in the promoter region in close proximity to the ATG start codon is not well documented or researched. This study aimed to identify any that were present, particularly in cattle where this variation may be missed as phenotypic change was wrongly attributed to exon variation and thus overlooked. SNPs have been located further upstream from the region targeted in this study, but they have not been linked to functional changes within the myostatin protein.

Disruptive mutations within the myostatin gene coding sequence are well documented, but they are not always the reason behind its inability to function. When this is the case, studies into upstream or non-coding sequences of the gene are carried out to account for phenotypic change. The pig, human and cattle promoter regions have been sequenced for this very reason. Promoter variation in cattle has been reported (Crisa, Marchitelli, et al., 2003; Spiller et al., 2002), but it was found that there were no obvious associations with variation in phenotype. This may be because the major

transcription binding sites such as TATA box, CAAT site and E-boxes are not altered allowing transcription of the gene to still occur.

However in some, but not all of the ten E-boxes (CAnnTG), sequence variation has been located(Spiller et al., 2002). The distal E-boxes have been found to contain polymorphisms that had little effect on promoter activity. However, when E5 and E6 are not present or altered and the remaining eight are intact, promoter activation is reduced (Spiller et al., 2002). The presence of only the proximal cluster (E1 and E2) is sufficient to drive gene activity while the deletion of only E5 and E6 E-boxes reduced (15- to nine-fold reduction) myostatin promoter activity. This suggests these upstream sections of the promoter play an important role in the gene's expression while the presence of the distal E-box cluster is not vital for maximum promoter activation (Spiller et al., 2002).

In this study the PCR amplicon encompassed E1 and E2 and no variation in this region appears to occur. This is consistent with previous studies (Spiller et al., 2002) and supports the conclusion that despite there being variation elsewhere in the gene, the structural integrity of this region needs to be maintained to maintain the functionality of *MSTN*.

5.2 Were the cattle studied representative of the individual breeds and cattle overall

South Devon and KiwiCross™ blood samples were already available for use in the Lincoln University Gene Marker Laboratory as farmers had sent them in for alternative gene-marker testing. While the location or origin of these herds are known, they may not be representative of the New Zealand population of each breed, and as a result this should be classed as an observational study.

Also, as a consequence of having limited time only a selected portion of the KiwiCross™ samples were analysed.

Only seven South Devon samples could be analysed in this study. The Belgian Blue samples were collected during the study from one of the few Belgian Blue farms in New Zealand, but blood was collected from all animals on the property (dams, calves and bulls), except one herd (around 30 animals) due to buyer interest. All 371 Belgian Blue samples were studied, but not all yielded typing results. As a consequence of the limited number of farms breeding Belgian Blue cattle in New Zealand, samples could only be collected from one location. This weakens any analysis that could be carried out on the effect of the environment on the phenotype of the cattle. The number of South Devon samples available, at seven, was insufficient to draw sound conclusions about the whole breed. The results should therefore only be taken as an indication of the allele frequencies for any of the three breeds, rather than as conclusive evidence of there being no variation in the region amplified in all cattle.

5.3 Limitations imposed by the use of PCR- SSCP typing

PCR-SSCP has been proven to be an effective method to describe DNA sequence variation, even when a single nucleotide change has occurred (Hayashi, 1991; Orita, Iwahana, Kanazawa, Hayashi, & Sekiya, 1989). Samples are run in lots of 25 on acrylamide gels making this method cost effective and analytically efficient, for studying large numbers of samples. These benefits outweigh the three days it takes to extract, amplify and type the samples.

The standard PCR and DNA extraction methods used in the Lincoln University Gene Marker Laboratory were used in this study. Both were optimised for the amplicon that was studied. To ensure the optimal protocol was used for this section of DNA to produce clear DNA bands, various components of the PCR-SSCP (Zhou & Hickford, 2008) were altered. The initial temperature at which extension was set during PCR was 60^{o}C. This was increased to 62^{o}C and 61^{o}C for trial runs after some banding became unclear, but was dropped back to 60^{o}C when non-specific bands appeared. The standard amount of SSCP loading dye used in the Lincoln University Gene Marker Laboratory was added to the amplified DNA however, the amount of the SSCP loading dye-DNA mix loaded into each well of the gels was reduced to 10 µL for Belgian Blue and South Devon samples and increased to 15 µL for KiwiCross^{TM} samples. This was done to ensure clear bands were produced for the varied quality of each breed's DNA. Blood samples from Belgian Blue and South Devon breeds were fresh (analysed within weeks of collection) while the KiwiCross^{TM} samples were older (collected in 2014) and the DNA therefore potentially of lower quality. More DNA appeared to be needed to produce clear bands.

These alterations improved band quality, yet not all DNA samples produced visible bands on the acrylamide gels. The DNA extraction method, designed by Zhou & Hickford (2008), is suited for samples up to 5 years old and is used in the Lincoln University Gene-Marker Laboratory on ovine and bovine dried blood samples. Ovine DNA from these samples seems to decay more slowly than bovine as many bovine samples less than five years old did not produce clear bands in the initial stages of this study when ovine samples have in other studies. This may be the reason that KiwiCross^{TM} samples collected in 2014 needed greater concentrations of DNA in the wells to produce the same clarity bands as newly collected (2016) Belgian Blue and South Devon samples.

The quality of blood extracted may also play a role in why some of the samples produced unclear bands. The DNA is extracted from dried blood on FTA cards, the amount of DNA present on the card is influenced by the amount and quality of the blood collected from each animal. Some animals bleed more quickly and/or produce more blood from the tips of their ears than others, affecting the DNA collected. Although one punch of a FTA card is usually sufficient to obtain enough DNA, cards are

sometimes punched numerous times for other studies and the quality of blood left around the edges of the bloody stain may not contain enough DNA for adequate analysis.

Taq polymerase, in conjunction with 5x Q solution, were used to ensure DNA templates with high levels of GC-rich areas separate, thus improving the accuracy of amplification (Hayashi, 1991). This is vital in this method to optimise the PCR solution for amplification.

During the initial stages of DNA analysis agarose gels were used to ensure DNA was present and in sufficient quantities, for further analysis. This check is vital to ensure the PCR protocol is adequate for this DNA section. After which the use of agarose gels to check amplification is unnecessary as the specificity of SSCP gels far surpass agarose gels.

5.4 Limitations to primer specificity

One reason some samples did not amplify may be the result of non-specific binding of the primers. Although the primers were designed specifically for one promoter region in bovine myostatin, identical DNA sequences may also be present in other areas of the bovine genome. This results in multiple DNA sections being amplified, which could interfere with the amplification of the target DNA section. The preference for non-specific DNA sections to amplify, over the target sequence would result in the absence of bands, or presence of bands depicting non-specific DNA sections rather than the target sequence.

The literature alludes to possible non-specific primer binding in genes associated with myostatin, but does not specify non-specific binding in the myostatin gene (Girven et al., 2016; Welle, Cardillo, Zanche, & Tawil, 2009).

In this study no variation was found between the breeds studied. Although the results appear uniform, they may be the result of primers binding to polymorphic regions throughout the genome which differs between breeds. The resulting bands, or lack thereof, would not disclose the poor primer binding in some or all cattle breeds.

Equally, the region in which the primers bind (i.e. directly base-pairing with the primers) may be polymorphic. This could result in two outcomes. First the difference between the primer and its target may not compromise primer binding and thus amplification would occur, but the amplicons would have the sequence defined by the primer and not the promoter template region. Variation in the promoter may therefore be hidden. Secondly, sequence variation if present in the primer binding site of some cattle might lead to no amplicon being produced. This would be consistent with the results, and a valid conclusion might then be that at very least the Belgian Blue and KiwiCross^{TM} cows typed where in some cases homozygous for primer-binding site variation. Heterozygous individuals

would not however be detectable as the wild-type form of the promoter that matched the primers would still amplify.

Variation on this theme might also occur, with the possibility of one or both primers having their binding affected, and in different combinations in different genotype. This possibility is further addressed in section 5.6 below.

5.5 Future research

This study is classified as an observational study only due to many samples already being used by the Lincoln University Gene-Marker Laboratory for genetic testing at the request of farmers. These samples were selected through an unbiased selection process. However if this research was to be extended, more herds of the same and different breeds should be analysed to expand on the limited genetic variation expected in one herd. Multiple herds from multiple environments should be typed to reduce biased results from breeding programs and gain a representative group of New Zealand cattle. In an ideal study, a random sample of farms (with farmer permission) located throughout New Zealand should be selected with herds exposed to a variety of selection pressures. Environmental effects on myostatin variation could then also be analysed and the allele frequencies, if variation was present, could be compared. A number of randomly selected animals from each farm would be analysed, rather than the whole herd as a representative population of the whole, including animals of both sexes and all ages. This detailed information on the samples analysed would offer not only a much wider range of genotypes but information about allele frequency variants under differing selection pressures.

5.6 Suggestions for future study of *MSTN* promoter region variation

Future research should focus on a larger section of, or entire *MSTN* promoter region. Although no variation was found in this study, it does not conclusively rule-out the presence of sequence variation occurring in this section or other sections of the *MSTN* promoter region.

For further study, the use of recorded cattle would enable pedigrees and lineages to be traced. This could help pin-point the source of genetic variation and the best animals within a family. Similarly, increasing the number of animals in the trial would provide a gauge as to whether variation is present in other animals. The more animals typed, the more recombinant events have occurred, the higher the chance of mutation and the more variation will be present in the sample size. Also, increasing the number of breeds in the trial will help ascertain whether variation is limited to one breed, a small number of breeds, breeds other than these trialled or is not present in cattle.

Where possible, multiple herds of the same breed across the country should be analysed to analyse the effect, if any, of environmental pressures on phenotype and genotype. The locality of the herds studied may have caused genetic drift from those in other areas. Extending the study to take this into account may provide insight into promoter polymorphic variation. The environmental conditions of each specific area should also be recorded as a reference for potential favoured genotypes in an environment.

The selection pressures applied to each herd should be recorded in future studies. This may help when determining when mutations have occurred in a herd, the homozygosity/heterozygosity of a herd or individuals in the herd and the preferred phenotypes for certain environments.

The entire *MSTN* promoter region has already been analysed for variation and many sequence variants have been described, however this has not been completed in New Zealand cattle. Typing the entire promoter region may reveal genetic variation already found in offshore studies or *MSTN* variation specific to New Zealand cattle breeds may be found.

Extending the study to look at those proteins, particularly furin, associated with myostatin inter or intracellularly may also highlight downstream effects of polymorphisms found within the open reading and non-coding regions of myostatin.

New primers should be designed and tested alongside new amplicons selected in close regions. Nonspecific primer binding is one of the major unknowns in this study which may be why no variation was visualised and why some bands were not produced. Trialling new primers in multiple locations may reveal sequence variation that was not discovered using these primers.

It is clear there is still sufficient research to be completed before we fully understand the intricacies of myostatin function and its various polymorphisms or lack thereof.

Chapter 6

Conclusion

It is well documented that the functional conservation of the myostatin promoter attributes to the tissue-specific and developmental expression of the myostatin gene in many animal species. This study found no evidence for sequence variation in one section of the myostatin gene promoter in three cattle breeds and this appears to be consistent with the literature. However, the lack of variation found in this study does not rule-out the presence of sequence variation occurring in this section of the promoter region, or in other sections. The advantages of establishing a gene-marker test to assist in bovine selection highlights the need to continue to search for genetic variation.

Appendix A

Published myostatin promoter region sequence

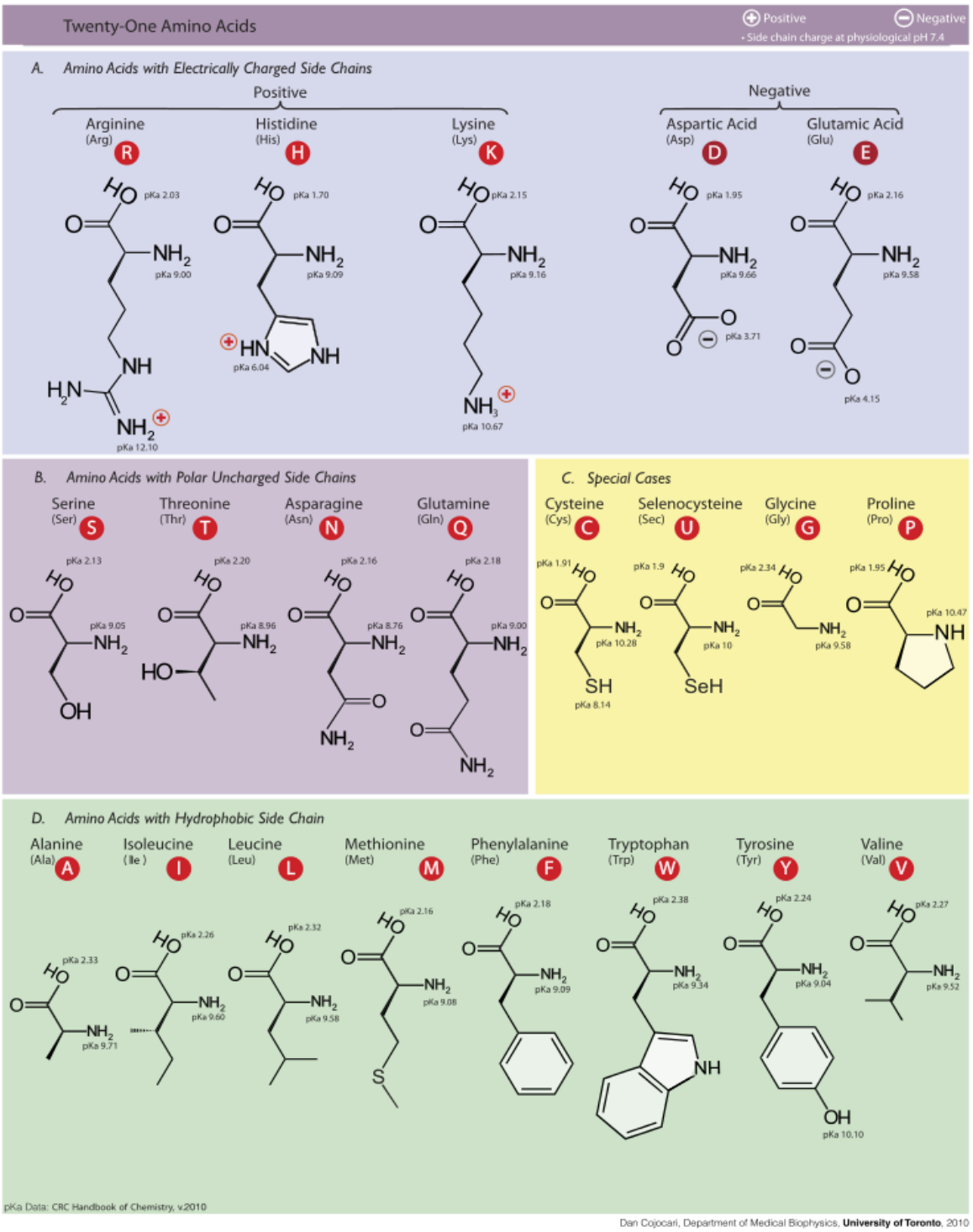
A.1 Coding sequence of *Bos taurus* promoter region (AJ438578.1)

```
1     gctcccagac cttaccccaa atccctgcca ggtgtctgcc tccggtcaaa tgagaaactg
61    gcaaaggaag tactaggagg tcgcacagta ctaggaagta gaaaaatgga ctagcacact
121   actgagaagc agaaaaatgg gcacccttca tgatggtgtt cctttccttt tctgtgttca
181   caatgctccg atataattta cagagggtag ataactacat ttttttcttt taccactgga
241   aagctgagga aaactttgtt acccatcata aaattcacta tcttctaagt cattctatgt
301   tattctaaga tcaaatagct gacaatatcc tctttgtaat aaacaatgaa aaaacacatc
361   ctctsagcaa tattaatctg caactttagg ataggaagta acttaatagt agtcaattga
421   aactgaaata caatttttcat atgaataaaa gatattattt aaaagtaatt ccatgagcaa
481   tttaatatta aagtaggatt ttcattatgt gttaagaatt tattcaggga aacaagtttc
541   tcaaattata gcagaaaatc ttttactagt atcacagtct tttcatttaa gtcttcctga
601   ataaatctgt attttctaat tatacaagac taaaaataat ttaatataac aaataaaatt
661   atttttactt caaatgctta cttaaatagt ataaaatcat tttattttct gagggaaaag
721   catatcaact ttttaagtat gaagtgtaaa ttaagattta ttcacttaaa ttataatttt
781   taaagtttca catataaaga tgaataagat ctaagtgtat atgttattgt taataaagtt
841   tttaattttt cgaatgtcac atacagcctt tattattcat agatttattc cttttaagaa
901   gtagtcaaat gaatcagctc acccttgact gtaacaaaat actgtttggt gacttgtgac
961   agacagggtt ttaacctctg acagcgagat tcattgtgga gcaagagcca atcacagatc
1021  ccgacgacac ttgtctcatc aaagttggaa tataaaaagc cacttggaat acagtataaa
1081  agattcactg gtgtggcaag ttgtctctca gactgggcag gcattaacgt ttggcttggc
1141  gttactcaaa agcaaaagaa aagtaaaagg aagaagtaag aacaagggaa aagattgtat
1201  tgattttaaa accatgcaaa aactgcaaat ctctgtttat atttacctat ttatgctgat
1261  tgttgctgg
```

Appendix B

Amino acids

B.1 Amino acid list including abbreviations



(Betts & Russell, 2003)

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