

Functional Polymorphisms: Bovine Calpastatin Gene and Meat Tenderness

Mohd Nazmi Bin Abd Manap
(B.Sc)

Thesis submitted to the University of Nottingham for the
Degree of Doctor of Philosophy, May 2012

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Abstract

Calpastatin is widely known as an endogenous specific inhibitor to the ubiquitously expressed calpain an enzyme responsible for proteolysis of myofibrillar proteins during post-mortem degradation of muscle. The presence of the calpastatin polypeptide in muscle indicates that the activity of calpain can be potentially down regulated which could result in meat toughness. Assesment of calpastatin activity in meat could be a predictive marker to meat tenderness and variation in the gene has the potential to become a candidate genetic marker which is associated with meat tenderness. The variability and inconsistency produced in meat tenderness post-mortem could be reduced if animals could be selected based on this potential genetic marker prior to slaughter which in turn will reduce the cost in meat processing and ultimately achieve the main objective of producing consistently tender meat.

Previous studies have successfully sequenced bovine calpastatin cDNA and found that a series of promoters in the 5' region are responsible for transcribing Type I, II and III mRNA for calpastatin. The presence and length of CA tandem repeat sequence 5' to the transcription start site of Type I calpastatin mRNA is believed to play a significant role in regulating the transcriptional activity of this promoter. This thesis investigated the hypothesis that there was a relationship between length polymorphisms of CA repeat located 5' to the promoter region of Type I bovine calpastatin which altered the level of calpastatin transcripts and ultimately influenced meat shear force value due to the variation in calpain inhibition. Apart from this, transcriptional activity of promoter for Type I, II, and Type III calpastatin were also assessed as well as their response towards agents involved in signalling cascade associated with the agents that stimulate hypertrophic growth. In order to investigate the CA tandem repeat polymorphisms, a PCR based cloning strategy was developed in this study which allowed amplification of this region. Cattle (n=6) of different breed and meat tenderness had their CA tandem repeat sequences amplified which were then cloned into a ZsGreen based reporter construct and transcriptional activity of the promoter were measured using fluorescence imager (Typhoon Trio). From the results, there was no direct correlation ($R=0.28$) found between the CA tandem repeat length and the shear force value of the meat. However, transcriptional activity for Type I promoter was significantly affected ($P<0.05$) by changing the length of CA tandem repeat (40-60bp). In general, the calpastatin promoters displayed negative response towards treatment with cAMP($P<0.05$) and there were no significant changes to the promoter activity when it was treated with forskolin. Furthermore, a significant reduction in promoter activity ($P<0.05$) was observed from all calpastatin promoters with calcimycin treatment.

The research shows that the type I calpastatin promoter has transcriptional activity and is regulated by secondary messengers which activate cAMP dependent kinases. Although altering the CA tandem repeat length alters promoter activity, there appears to be no simple relationship between its length and toughness, as determined by shear force. However the differential activity of the three calpastatin promoters indicates that there are potentially multiple mechanisms by which its activity can be regulated.

ABBREVIATIONS

%	percentage
°C	celcius
μ-	calpain micro calpain
μg	microgram
μl	microliter
μM	micromolar
AC	adeneylyl cyclase
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-1	activator protein 1
APS	ammonium persulfate
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
bp	base pair
C/EBPa	CCAAT/enhancer binding protein alpha
C/EBPb	CCAAT/enhancer binding protein beta
C ₆ H ₁₂ O ₆	glucose
Ca ²⁺	calcium ion
cAMP	cyclic adenosine monophosphate
CAST	calpastatin
cDNA	complimentary deoxyribonucleic acid
cM	centimorgan
CMV	cytomegalovirus
CO ₂	carbon dioxide
CP	creatin phosphate
CRE	cAMP response element
CREBP	cAMP response element binding protein
dbCAMP	dibutyryl CAMP
DEAE	diethylaminoethyl
DFD	dark firm dry
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FAOSTAT	Food and agriculture organizations of the United Nations
FCS	fetal calf serum
GAPDH	glyceraldehydes 3-phosphate
GATA-1	GATA binding factor 1
GATA-2	GATA binding factor 2
GATA-3	GATA binding factor 3
gDNA	genomic DNA
GFP	green fluorescence protein
HPLC	high performance liquid chromatography
HPSF	high purity salt free

HSF2	heat shock factor 2
IGF	insulin growth factor
IMF	intramuscular fat
kb	kilobase
kDA	kiloDalton
LB	luria broth
lb	pound
LD	longgisimus dorsi
m-calpain	mili calpain
mg	milligram
Mg ²⁺	magnesium ion
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
ml	milliliter
mRNA	messenger ribonucleic acid
MyHC	myosin heavy chain
NaCl	sodium chloride
NFκB	nuclear factor kappa B
Ng	nanogram
nm	nanometer
PBS	phosphate buffer saline
Pbx-1	pre-B-cell leukemia transcription factor 1
PCR	polymerase chain reactions
PKA	protein kinase A
PKC	protein kinase C
PSE	pale soft exudative
qPCR	quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT-PCR	realtime polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	standard error mean
SP-1	stimulating protein 1
SRY	sex-determining region Y gene product
TAE	tris acetate EDTA
TBE	tris boric acid EDTA
TEMED	tetramethylethylenediamine
TF	transcriptional factor
TSS	transcriptional start site
USF	upstream stimulator factor
UV	ultraviolet
v	volt
v/v	volume per volume
w/v	weight per volume
α	alpha
β	beta

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors; Dr Tim Parr and Dr Ronald Bardsley for their continuous support, guidance and encouragement during my PhD study which in turns helps me to progress in my study. Their input and knowledge has been very valuable.

I am also indebted to both of my parents, Mr Abd Manap Bin Din and Mdm Fatimah Bin Abdullah. Their guidance and unconditional faith have kept me motivated throughout the year of my study.

Many thanks to the kind assistance given by colleague and lab technician in the Nutritional Sciences department. Without them, it will not be possible for me to complete my experiment.

Not forgetting close friends, Dr Bee Lynn Chew, Dr Wan Asrina, Dr Hafiza, Dr Nathalie Narraidoo, Nordini Rusli and Nurulhikma Md. Isa as their presence and friendship contributes to the wonderful memories in Sutton Bonington Campus.

Lastly I would like to sincerely thank the Malaysian Higher Education Ministry and University Sains Islam Malaysia for the scholarship given during my PhD study in UK.

Chapter 1.0: Literature Review

1.1 Introduction

Most modern livestock breeding require a stringent quality control to produce meat that has all the quality attributes needed for the market. There are many factors that affect the quality of the meat and this variability is dependent on the conditioning of the meat during post-mortem processing, how the meat is cooked and other environmental factors related to the experiences of the animal prior to slaughter; such as the stress levels of the animal (Terlouw et al., 2008). In addition, meat tenderness is influenced by the means of feeding the animal (French et al., 2001), as altered feeding strategies can improve carcass quality (del Campo et al., 2008). One of the properties of the meat which is greatly desired by the meat industry, in order to forefill the demand of customers, is tenderness. The meat factors which influence consumers' satisfaction ranged from the flavour, colour, texture, juiciness and the tenderness of the meat. The most important aspect of meat quality which relates to consumer satisfaction is tenderness (Robbins et al., 2003).

Genetic diversity can contribute to various differences in meat quality (Williams, 2008) . Research had shown that meat tenderness can be improved by direct selection within breeds using progeny testing. Cattle from different breeds differ in meat quality and this depends on the genetic factors (Oconnor et al., 1997). The diversity in genetic traits linked to meat tenderness is found in *Bos Indicus*, as this subspecies of cattle demonstrates a broader range in beef toughness value compared to *Bos Taurus* subspecies (Crouse et al., 1989) . Thus, the impact of genetic traits that contribute to meat quality is an important factor in the market value of beef therefore there is an interest in research to produce a technique to differentiate between cattle of different meat quality using available genetic markers; for

example those based on microsatellite and diallelic markers (Blott et al., 1999).

Overall, the main work performed in this thesis was to investigate the link between genotypic variation reported in cattle in the form of CA repeat polymorphisms in calpastatin gene and meat tenderness in order to assess its potential as a marker for selection purposes. In order to understand the link between both, the introduction chapter will cover how the main hypothesis was formed based on the evidence reported in previous research. As meat tenderisation which occurs in meat particularly affecting the muscle structure, this aspect will be explained as well as the conversion process from muscle to meat, enzyme system(s) responsible during the post-mortem period and finally the role of calpain-calpastatin and its regulation which affect meat tenderness.

1.2 Meat: consumer perception and commercial value

An analysis by Food and Agriculture Organisations of The United Nations (FAOSTAT Statistical database) have shown that meat consumption in the world has been steadily increasing every year between the period from 1961 to 2002. This was shown with meat consumption per capita, which is described as total meat retained for use in country per person per year showing 4 to 14 fold increase (dependent on the country) from its initial consumption value recorded in 1961. This is especially noticeable in developing countries such as Malaysia and China. However, this increase in meat consumption has not been shown in US and UK, as in these countries the consumption level remained relatively stable throughout this period although the demands is still considered high in UK with recorded value of 79.6kg consumed per person in 2002.

In order to meet consumer's demands, production of meat at a global level has to increase. This is clearly demonstrated by world meat production increasing from 44 million tons in 1950 to 242 million tons in 2002 (FAOSTAT Statistical database). It has been projected that by the year 2050, total meat production in the world will have to increase from 265 million tons in 2006 to 624 million tons (Elam, 2006). The global increase in meat demand and production means that the commercial value of meat will increase (Bansback, 1995). Therefore, it is important to increase the meat production to meet increases in demand but at the same time improve the drive for efficiency and reduce the produced waste in order to meet the demands of food security; this being the ability to ensure that there is a balance between availability of food and the ability to supply this at a consistent rate(Sansoucy and Jabbar, 1995).

In general, the perception of consumers on meat quality is described as their view towards the attributes and the properties of the meat, rather than the biological safety of the product. The attributes that describe the perception of consumers towards meat quality are tenderness, juiciness, flavour and

colours. These attributes have been shown to influence consumers in their choice when purchasing meat, at least in European countries (Glitsch, 2000). These attributes, which define meat quality, are influenced by several factors the main ones being described below.

The juiciness attributes in meat are associated with the higher level of intramuscular fat content (IMF) in the meat, thus high IMF improves eating quality (Warriss, 2000). It is defined as the feeling in the mouth of moisture from cooked meat while chewing. In addition, the juiciness attribute is strongly related to the attribute of flavour, as this latter attribute is also affected by the level of IMF in meat. The reason for this is believed to be as a result of the volatile fatty acids composition in meat (Kerry et al., 2002).

The major determinant of the appearance of meat is the colour attribute, which is affected by the changes in pH post-mortem. At the extremes of colour development, the produced meat from pig can have either a pale soft exudative characteristic, if the pH drops rapidly, or be dark, firm and dry, if the pH remains high throughout the post-mortem period (Warriss, 2000). These effects were influenced by changes in the myoglobins which are present in the meat. The bright red colour produced in meat are the results of reaction between myoglobin pigments and oxygen forming oxymyoglobin which are associated with meat freshness by consumers while the purple and grey-brown colour are less desirable (Lawrie, 1998).

Of all the mentioned attributes, the study by Glitsch (2000) identified tenderness and flavour as the most important aspects, when it comes to the “eating quality” for Europeans. Therefore, it is crucial to investigate the mechanisms and factors affecting the attribute of meat tenderness which is one of the main attributes sought by the consumers.

A study showed a willingness of consumers to pay for guaranteed tender steak through a series of tests. In the first test, when given meat which was not labelled to identify meat tenderness, 72% of consumers surveyed were able to correctly differentiate between meat which was ‘guaranteed tender’

and that as being 'probably tough' based on their own taste. Moreover, 36% of these consumers from the blind test were willing to pay an average of \$2.73/kg meat for a tender meat (Lusk and Koohmaraie, 1999). In the second test where information regarding tenderness was revealed, 90% of the surveyed consumers showed a preference for tender meat as they selected meat labelled 'guaranteed tender' compared to the meat which is labelled 'probably tough', with 51% of them willing to pay the additional cost at an average of \$4.09/kg for a guaranteed tender meat (Lusk and Koohmaraie, 1999).

This research showed that consumers are willing to pay premium for guaranteed tender meat and the majority of them are aware of meat quality in terms of tenderness. There was also preference toward meat packaging which informed customers regarding the tenderness of the meat. To conclude a complete understanding on the factors contributing to meat tenderness is important as consumers perceived tenderness as the major aspect and determinant of meat quality, therefore it is commercially important to develop a technique which can precisely predict tenderness in meat.

1.3 Skeletal muscle structure and composition

The skeletal muscle is the predominant system which after death goes on to become meat. Therefore it is important to understand the properties of skeletal muscle to give a basis for the process involved in the development of meat and thereby how various factors during the post-mortem period influence tenderness of meat.

1.3.1 Muscle structure

The structure of skeletal muscle is characterized by the complex organization of proteins which interacts to enable simple voluntary movements in a mammalian system. An average of 30-40% of the live weight of cattle is composed of skeletal muscle whilst 35-68% of the carcass weight is skeletal muscle which will ultimately become meat (Lawrie, 1998). An individual anatomically defined skeletal muscle is surrounded by epimysium (Figure 1.1), a layer of connective tissue, while a bundle of myofibres inside the skeletal muscle are enclosed by another type of connective tissues known as perimysium and the individual unit, the myofibre is enclosed by another connective tissue named endomysium (Tornberg, 1996).

The muscle is based on a hierarchy of components. Muscle fibres or myofibres are cells which are multinucleated containing a range of quantities of components such as mitochondria, ribosomes, soluble protein, glycogen and lipids (Pearson and Young, 1989). The diameter for the muscle fibre is around 10-100µm (Lawrie, 1998). Inside the multinucleated muscles fibres, there are multiple long and thin cylindrical structures of protein filaments which lie parallel to each other, known as myofibrils. The rod shape myofibril is estimated to be 1 to 3µm in diameter and its structure defines the contractile properties of the muscle (Lawrence and Fowler, 2002). Each individual myofibril is composed of repeating units of sarcomeres which give the appearance of striated muscle under the microscope (Lonergan et al., 2010) (Figure 1.1). The sarcoplasmic reticulum, which surrounds the myofibrils, is responsible for sequestering and storing Ca^{2+} which is utilised,

on its release into the sarcoplasm, to initiate contraction, thereby regulating the intracellular level of Ca^{2+} in the system.

1.3.2 Structure of the sarcomere

The length of mammalian sarcomere is $\sim 2\mu\text{m}$ and can be shortened to $\sim 70\%$ of its initial length during contraction (Au, 2004). The main features defining the structural properties of the sarcomere are the longitudinal Z or M line (or known as disk or bands) (Figure 1.2). A sarcomere unit is defined as being between successive Z-disks, this structure being built from α -actin but being specifically associated with cytoskeletal proteins such as α -actinin (Taylor et al., 1995). Within the sarcomere are two distinct areas, the A-band and the I-band areas. The A-band areas are occupied with two alternating filaments, the thick (myosin) filament and the thin (actin) filament, whilst the I-band area contains the thin actin filaments anchored to the Z-disk, which divides one sarcomere from the adjacent sarcomere unit. In the middle of the A band area, there is an H zone area where there are no thin filaments overlapping on the thick filaments, the centre of this region being the M-line. For contraction to occur, the thick myosin and the thin actin filaments slide passed each other which involve the formation of cross bridges formation between the myosin and actin. The complex formed during the interaction of myosin and actin is referred to as actomyosin (Huff Lonergan et al., 2010). The length of sarcomere is reduced during muscle contraction with the length of the A-band not changing but the I-band and the H-zone will shorten. While muscle is in the relax state, myosin is blocked from binding with actin by a regulator protein known as tropomyosin, a coiled-coil protein which is wrapped around thin actin filaments (Au, 2004). The tropomyosin protein prevents the crossbridges from occurring by blocking the myosin binding site on actin. In addition, another protein complex called the troponin complex controls the positioning of the tropomyosin on the actin filament. During nerve mediated stimulation of the muscle, Ca^{2+} is released from the sarcoplasmic reticulum which then binds onto troponin causing a change in the position of tropomyosin on the actin filament therefore exposing the

myosin binding site (Reece and Campbell, 2011). The interaction between actin and myosin filaments requires the use of energy from ATP which bind to the myosin head and is subsequently hydrolyse by ATPase to provide the energy for contraction (Holmes and Geeves, 2000).

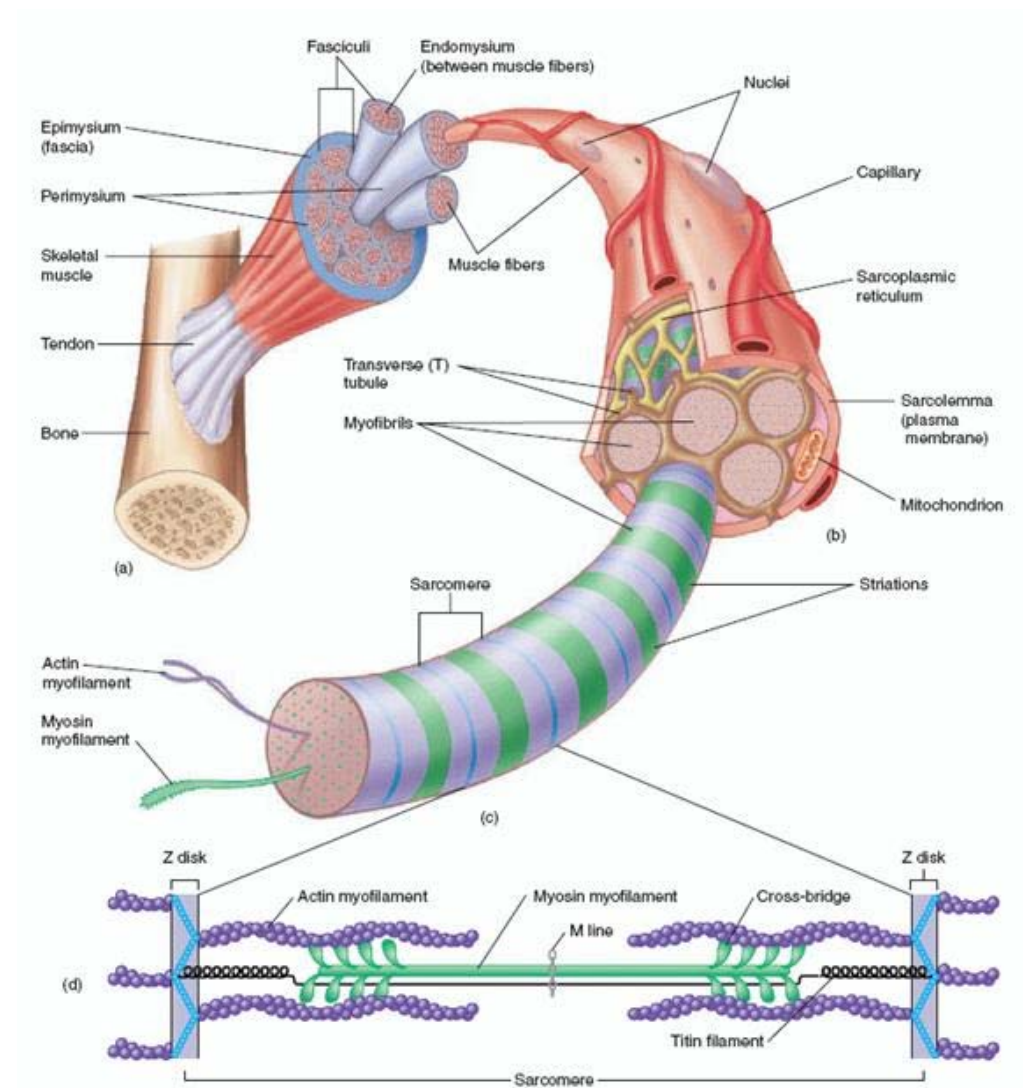


Figure 1.1 The complex structure of skeletal muscle composed of muscle fibres and myofibrils. The whole complex of muscle structure composed of bundled fascicles that are built from collection of muscle fibres (myofibres). The muscle fibres contain bundles of myofibril which characterize the contractile properties of the muscle. (a) bone, (b) myofibre, (c) myofibril (d) sarcomere unit. Adapted from Marieb (2009).

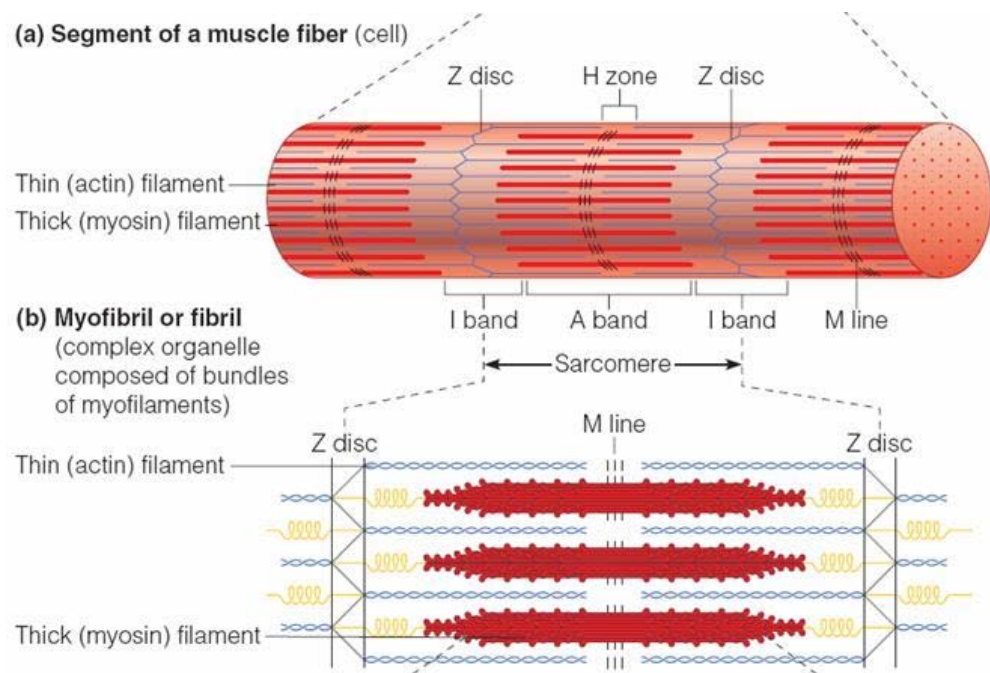


Figure 1.2 The schematic representation of a sarcomere unit from the muscle fibre. (a) myofibril segment (b) the ultra-structure of the sarcomere of the a myofibril. Adapted from Seeley et al. (2002) .

1.3.3 Types of muscle fibre

A unique characteristic of mammalian skeletal muscle is that it is heterogenous and is also capable of altering its phenotypic properties based on metabolic demands. Originally, muscle fibres were differentiated by colour of the meat, being either red or white. Subsequently, muscles were classified based on the muscle fibre composition. Muscle fibres are classified into groups based on their metabolic characteristics that are related to contractile activity of the myosin heavy chains (MyHC) profiles it contains. At first, muscle fibres were classified into three types based on histochemistry stains that detected the activity of the Myosin ATPase (adenosine triphosphate). The three types are slow twitch oxidative (Type I), the fast twitch oxidative glycolytic (Type IIa) and the fast twitch glycolytic (Type IIb)(Maltin et al., 2003)(Figure 1.3). Later this classification was expanded with another type of fibre the (Type IIX) (Schiaffino et al., 1989). This fibre type, also known as Type IIC, is thought to be a transition fibre from Type I to Type II, being between Type IIa and IIb fibres (Pette and Staron, 1990).

Technological advancement in immunohistochemistry has enabled differentiation of muscle fibres based on their oxidative capacity by staining using a combination of myosin ATPase and succinate dehydrogenase staining (Karlsson et al., 1999). In general, the size proportions (cross sectional diameter) for the glycolytic fibres are greater than oxidative fibres because of the higher requirement of the latter for oxygen diffusion (Maltin et al., 2003). The contractile properties of the fast twitch fibre types are faster contraction than the slow twitch and they also enter the relaxation state faster. Summary of the properties of these fibre types is shown in Figure 1.3.




MyHC type	Twitch duration	Shortening velocity	Cross-sectional area	Metabolism	Endurance	Energy efficiency
I		Slow	Small	Oxidative	High	High
Ila						
Ilx						
Ilb						
	Fast	Fast	Large	Glycolytic	Low	Low

Figure 1.3 The properties of the major fibre types observed in mammalian muscle adapted from Gundersen (2011)

1.4 Conversion of muscle to meat

The process which involves conversion of muscle to meat starts after slaughter of the animal during post-mortem development and involves three important processes which are the pre-rigor phase, the rigor phase and the post-rigor tenderization phase (Sentandreu et al., 2002).

1.4.1 Pre-rigor phase

Energy in the form of ATP is required to enable contraction and relaxation of muscle in a living muscle (Ebashi et al., 1969). Following death of the animal, blood, oxygen and nutrient supply are cut to the muscle and this process triggers the pre-rigor phase in the animal after slaughter. During this phase muscle remain excitable as intracellular ATP can be used and regenerated by reaction between creatine phosphate (CP) and adenosine diphosphate (ADP) (Lawrie, 1998). Energy is released during muscle contraction by conversion of ATP to ADP to enable interaction between the myofibril proteins actin and myosin. This process will eventually deplete the amount of intracellular ATP causing failure of muscle to enter the relaxation state as ATP helps to disconnect the crossbridge between actin and myosin by binding with Mg^{2+} (Savell et al., 2005). The change from weak binding to a strong and irreversible binding between actin and myosin will results in a permanently shortened sarcomere length, thereby rigor develops (Koohmaraie, 1996).

1.4.2 Rigor phase

The rigor phase starts with the depletion of energy sources and subsequently ATP. During this phase, muscle attempts to maintain homeostasis and remains excitable by metabolising muscle glycogen via anaerobic glycolysis thus still providing continuity of ATP production by phosphorylation of ADP by CP. The depletion of ATP in the intracellular environment increases the concentration of free Ca^{2+} in the sarcoplasm. The sarcoplasmic reticulum is responsible for removing Ca^{2+} from the sarcoplasm by translocation of Ca^{2+} across membrane utilising the calcium ATPase pump and is dependent on ATP for this active process (Robbins et al., 2003). In the development of meat, lactic acid is produced from anaerobic glycolysis (needed to generate ATP) leading to a major reduction in intracellular pH. However as this process continues, CP will eventually become depleted resulting in a decline of ATP and thereby the availability of substrate required to maintain the contractile proteins actin/myosin in the relaxation state. Actin and myosin will form irreversible cross bridges and rigor mortis occurs in the tissue. During the conversion process, the muscle reaches its maximum toughness as the consequence of the shortening of the sarcomere (Goll et al., 1995). The time taken for development of rigor phase is highly variable and dependent on the animal species, chilling condition and the type of muscle (Sentandreu et al., 2002). For example, rigor development is found to be longer in beef and lamb (6-12 hours) than pork (1-6 hours) (Epley, 1992).

1.4.3 Post-rigor tenderization phase

In general the tenderization phase of meat conditioning (the gradual decrease in meat toughness) starts at 24 hours post-mortem storage, beginning after the muscle reaches the peak of toughness (Wheeler and Koohmaraie, 1994). The rate of tenderization during this phase, from 24 hours to 14 days, was demonstrated by Wheeler & Koohmaraie in (1994) as not being related to the rigor implying that other factors affect development

of tenderness. It was the variability of the process in this phase which reflects inconsistencies observed in meat tenderness. The rate of change which occurs during post-rigor is highly variable and most of the changes are primarily from the weakening of myofibrillar structure as the result of initiation of proteolysis. The weakening of muscle cells post-mortem does not depend on a single myofibrillar or cytoskeletal protein but are the result of collective disruption of structural protein within the muscle (Huff Lonergan et al., 2010). These structural proteins include vinculin, titin, dystrophin, nebulin, troponin-T, desmin and filamin which are part of the inter and intra-myofibrillar links (Hopkins and Taylor, 2002; Jiang, 1998; Taylor et al., 1995a). Completion of the tenderization phase varies between species with beef taking approximately (14-21 days), pork (4-10 days) and lamb (7-14 days) (Warriss, 2000).

1.5 Factors affecting conversion of muscle to meat

Among the micro environmental factors identified within muscle that can play a major role in the conversion process of muscle to meat are pH, temperature and tissue types.

1.5.1 Muscle pH

After slaughter of the animal, muscle tissue continues to undergo anaerobic respiration in order to maintain homeostasis accompanied with regeneration of ATP through CP; although the amount of produced ATP per unit glucose is less than that generated during aerobic respiration. During anaerobic respiration stored glycogen is metabolised into pyruvate which is then converted to lactic acid, which is the by-product from the anaerobic process of glycolysis. Lactic acid accumulates resulting in a gradual decline in intracellular pH and finally reaches an ultimate pH of about 5.4-5.7, 24 hours post-mortem (Maltin et al., 2003). Gradual decrease in pH value eventually stops with depletion of glycolytic substrate. In general, the rate of pH declination during post-mortem varies between species, glycogen level, rate of ATP turnover and the metabolic characteristic of the muscle (Lawrie,

1998). Meat with high ultimate pH often ($\text{pH} > 7.5$) is typically dark with higher vulnerability to bacterial spoilage and has reduced flavour characteristics (Watanabe et al., 1996). This condition occurs when animals have lower than normal muscle glycogen at slaughter, consequently producing meat with lower amounts of accumulated lactate. This dark, firm and dry (DFD) meat is also associated with variable tenderness as lower availability of glycogen substrate in meat causes rapid depletion of ATP and early rigor (Watanabe et al., 1996).

In contrast to DFD, meat defined as pale, soft exudative (PSE) meat is generally regarded to be of a poorer eating quality as the enzymes involved in post-mortem tenderization are inhibited by the lower ultimate pH environment as well as high drip loss ($\text{pH} < 6.0$) (Maltin et al., 2003). This condition occurs as a result of accelerated rate of post-mortem glycolysis leading to a low muscle pH while meat temperature remains high causing protein denaturation (Bowker et al., 2000).

1.5.2 Temperature

The role of temperature has been debated as being a factor significantly affecting the rate of meat tenderization during the post-mortem period. Temperature during pre-rigor and post-rigor phase can have a profound effect on the metabolism of the muscle and the rate of shortening (Hertzman et al., 1993). The increase of temperature at the early rigor can make the development of meat tenderisation prone to rapid pH decline, shortening of sarcomere length and also increase meat toughness (Bruce and Ball, 1990). Accelerated decline of muscle temperatures lead to an increase of muscle shortening. This occurs because of reduced calcium sequestering ability by the sarcoplasmic reticulum as a result of the depletion of energy compounds which in turn causes the muscle to contract and increase the toughness of meat (Huff Lonergan et al., 2010), so called cold shortening (Hannula and Puolanne, 2004). The ideal rigor temperature has been determined as being 15°C as it seems to optimize rate of tenderization and reduces rigor

shortening without contributing to any detrimental effect on water holding capacity or colour of the meat (Geesink et al., 2000).

1.5.3 Muscle fibre types

As indicated above the most basic classification of muscle type is define by the proportion of the slow-twitch oxidative (Type I), fast-twitch oxidative glycolytic (Type IIA) and the fast-twitch glycolytic (Type IIB) fibres within the muscle (Maltin et al., 2003). The fast-twitch fibres have higher amounts of glycogen, more efficient sarcoplasmic reticulum and Ca^{2+} activated myosin ATPase than the slow-twitch muscle. Therefore, given the relationship between metabolic substrate availability, anaerobic metabolism and thereby lactic acid production, it was proposed that the fibre type composition may influence post-mortem changes during conversion of muscle (Klont et al., 1998). Indeed higher frequency of glycogen depletion in Type IIB in meat has an adverse effect on meat quality after slaughter (Karlsson et al., 1999). However, Type I have also been reported to be beneficial to meat quality as an increase in its proportions was correlated to increases in tenderness and juiciness in cattle (Maltin et al., 1998).

1.6 Post-mortem proteolysis

Development of tenderness in meat during post-mortem is caused by proteolytic activity where alteration is made to the muscle structural and associated proteins (Hopkins and Thompson, 2002). Proteolysis in meat is initiated by a protease system(s) after rigor phase where meat gradually becomes tender after reaching the highest point of toughness during post-mortem. During this phase, vital proteins such as desmin, titin which are associated with cytoskeleton maintaining the myofibril's structure as well as those associated with structures maintaining the myofibril interaction with the sarcolemma (the costameres) are cleaved by the protease system(s) (Kemp et al., 2010)(Figure 1.4).

The basic criteria defining a protease system which could be involved in this tenderisation process is that it (or they) must be endogenous to skeletal muscle cells, able to induce post-mortem changes *in vitro* under the optimal conditions similar to those seen *in situ* and have access to the myofibril (Koochmaraie, 1988). A number of enzyme systems had been described as being associated with the proteolytic activity seen post-mortem these include the calpain system, cathepsin lysosomal system, caspase system and finally the ATP-dependent proteasome system (Kemp et al., 2010).

The role of each of these proteolytic systems in muscle post-mortem proteolysis has been the subject of a much research and associated debate. Essential there are three models which have been postulated as the proteolytic processes which are responsible for meat tenderisation. The first is only focused on the calpains as the main protease responsible in meat tenderization, the second suggests cathepsin and calpain are both involved in the process and the third suggesting a multienzymatic process which includes proteasomes, caspases, calpains, and cathepsins during post-mortem (Ouali et al., 2006). The majority of studies have agreed that the calpain system plays a major role in post-mortem tenderization (Boehm et al., 1998; Koochmaraie, 1992b; Taylor et al., 1995a).

It is doubtful that the cathepsin system takes part in post-mortem tenderization as its incubation with myofibrillar proteins results in a different degradation pattern than during the post-mortem period *in situ*. In addition it is uncertain that these proteases are released from the lysosomes (Koochmaraie, 1988).

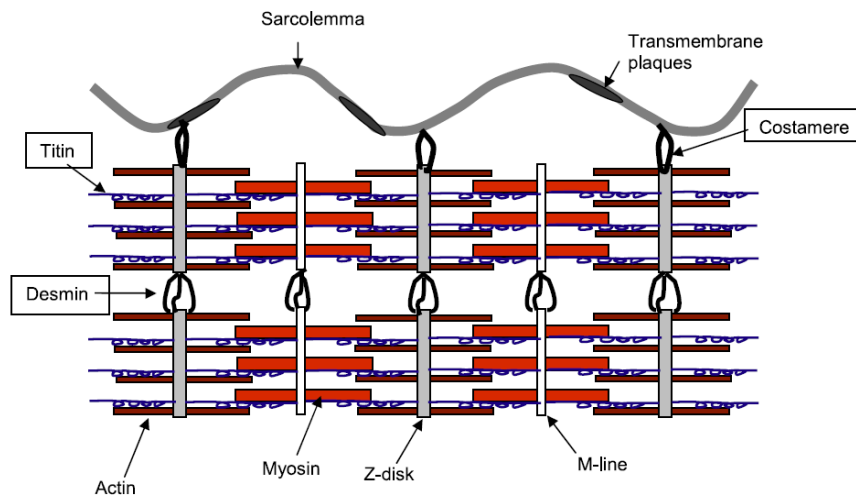


Figure 1.4 The key myofibril and associated proteins susceptible to proteolysis during post-mortem conversion of meat to skeletal muscle. Boxes indicate proteins that are cleaved during post-mortem tenderisation. Diagram of muscle myofibrillar proteins adapted from Kemp et. al (2010)

1.6.1 Cathepsin

The cathepsins are designated peptidases located in the lysosomes and are active in acidic conditions (Turk et al., 1999). They are categorized as a complex group of enzymes which originate from exo- and endo- peptidases belonging to cysteine (cathepsins B, H, L and X), aspartic (cathepsin D and E) and serine (cathepsin G) proteinases and are thought to be responsible for degradation of the myofibril when it is released from the myofibril and enters the lysosome (Sentandreu et al., 2002; Whipple and Koohmaraie, 1991).

There have been numerous debates on the role of cathepsin in myofibrillar degradation during post-mortem. For this to occur, cathepsins have to be released from the lysosomal membrane during post-mortem. It was proposed that the condition during post-mortem such as the failure of the calcium ion pump and low pH level will assist the released of cathepsin from lysosomes (Hopkins and Taylor, 2002). Most scholars have agreed that cathepsin may not directly contribute to post-mortem tenderization based on some of the studies described below.

Study on cathepsin proteolytic activity suggests that there was little effect of cathepsin on actin and myosin during normal post-mortem degradation *in situ* (Koohmaraie et al., 1991b) although incubation of cathepsin with myofibril *in vitro* had proven that these protein can be degraded (Matsukura et al., 1981) Another argument to support their lack of involvement in post-mortem proteolysis is that cathepsins are required to be released from lysosomes in order to access the myofibrillar proteins. It appears post-mortem that these enzymes are enclosed in the lysosome (Koohmaraie, 1996).

Despite the negative evidence against cathepsin's role post-mortem, there are reports of correlation of meat tenderness with the activity of cathepsins B and L (O'Halloran et al., 1997). A study using a histochemical method also found that as the ageing period proceeded, disruption of lysosomes continued gradually until day 14 where almost complete lysosome breakdown was achieved (Zeece, 1992). The view of cathepsins' role in post-mortem proteolysis has been described in several studies but the compiled evidence on cathepsins' involvement is against the idea of its role in tenderisation (Sentandreu et al., 2002). This argument was further strengthened by the evidence that a cathepsin inhibitor is not able to suppress proteolysis during the post-mortem period (Hopkins, 2000).

1.6.2 Caspase

Caspases are cysteine peptidases with highly specific cleavage of protein substrates and are known to be responsible for programmed cell death through cell apoptosis. The specificity of caspases is described by its capability to recognise tetra-peptide motif in a target protein, cleaving the peptide bond C-terminal to aspartic acid residues (Roy and Nicholson, 2000). To date, 14 members of the caspases family have been identified and caspases involved in apoptosis are further divided into initiator caspases (caspases 8, 9, 10) and effector caspases (caspases 3, 6, 7) (Earnshaw et al.,

1999). The involvement of caspases in cell apoptosis is supported by observations that caspase inhibitors prevent apoptosis and caspases have been identified as cleaving the key proteins degraded during cell apoptosis (Chang and Yang, 2000).

It has been proposed that post-mortem the gradual increase in calcium concentration in the cytoplasm, as a result of its release from sarcoplasmic reticulum, will trigger the apoptotic pathway in cells, as the process of apoptosis is calcium dependent (Herrera-Mendez et al., 2006). It has been suggested that cell death is the first step in the conversion from muscle to meat, as cells and tissues were deprived of nutrients and oxygen after slaughter and are therefore geared towards apoptosis (Herrera-Mendez et al., 2006). A recent study described an association between changes in caspase activity during the conditioning time and shear force; as there was a significant negative correlation of shear force value and the activity of caspase (Kemp et al., 2006). This suggests an association between caspase activity and development of tender meat but not a direct link.

Although caspase is known as being part of the central apoptotic machinery, skeletal muscle is still far from being characterised as a site for apoptosis and less is known about its potential role in post-mortem tenderisation. However, a few studies manage to suggest a role of caspases through apoptosis in post-mortem proteolysis (Huang et al., 2009; Ishida et al., 2003). There was also evidence that one of the primary substrates in caspase induce cells apoptosis are myosin and actin in the cytoskeleton environment (Taylor et al., 2008). The most recent evidence manages to provide the basis of argument for caspase involvement post-mortem, as muscle cell were observed to undergo apoptosis or cell death (Becila et al., 2010). However the role of caspase in post-mortem proteolysis which influences meat tenderness is not proven. Caspase may not directly be involved in tenderisation-associated proteolysis as its mechanisms of action is through a specific role in the cell process of apoptosis. Recent evidence suggested that caspase-3 is not involved in post-mortem tenderisation of beef muscle (Underwood et al., 2008).

1.6.3 Proteasomes

The ubiquitin proteasomes is an ATP dependent system which is a large multicatalytic protease complex and is thought to play a role in degradation of myofibrillar and skeletal muscle protein in a basal and catabolic state (Lecker et al., 1999). It has been proposed that the ubiquitin proteasomes could act as an additional endogenous proteolytic system responsible for the process of meat tenderization (Sentandreu et al., 2002). In order to initiate proteolysis a polyubiquitin degradation signal is required, substrates are tagged by ubiquitin at their lysine residues and are then recognized by the 26s proteasome, followed by degradation into peptides (Attaix et al., 2001). The catalytic core of these proteasome complexes is the 20s proteasome, which are regarded as highly conserved across organisms from yeast to human and were also found in prokaryotes and archaea (Dutaud et al., 2006).

The proteasomes are highly abundant in mammals and are found in multiple tissues and cells investigated (Brooks et al., 2000), a feature which suggest its importance as a proteolytic system. Earlier studies of proteasome activity post-mortem have suggested that the proteasome complexes are not involved in the early stages of destabilization of myofibrils (Koochmaraie, 1992a). However, Taylor et al. (1995b) manage to provide evidence of myofibril degradation without prior activation and the protein degradation observed displayed specificity for actin, myosin and desmin. This was strengthened by the fact that the level of proteasome protein remained constant for seven days of ageing period and decrease in its proteolytic activity during this period is proposed to be as the result of denaturation of the proteasome (Lamare et al., 2002). Furthermore, a study has also shown that myofibril incubated with purified 20s proteasome exhibited structural modifications of myofibril which have not been observed in cathepsin and calpain treated fibres including loss of Z-disk density and fragmentation of I-band at normal pH (Dutaud et al., 2006). The role of 20s proteasome in post-mortem degradation is still controversial as it has been shown that the pattern of degradation in myofibrillar proteins incubated with 20s

proteasome is different compared to the pattern during post-mortem *in situ* (Koohmaraie and Geesink, 2006).

1.7 The calpain-calpastatin enzyme system

During the post-mortem period, toughness in meat decreases parallel with the decline of myofibrillar protein structural integrity (Harper, 1999). This process is observed to take place directly after the process of *rigor mortis*. Through research it has been clearly described that the component of muscle which shows significant changes post-mortem is the z disk, and there is associated selective degradation of troponin, titin, and desmin at specific sites within these proteins. The changes that gradually occur during this process are instigated by proteolytic activity (Koohmaraie, 1994).

Tenderness has consistently been shown to be the most important contributory factor to meat quality in various farm species. Tenderization results from the proteolytic activities of enzyme present influencing protein turnover in muscles. The calpain system which is ubiquitously expressed in muscle is believed to play a role in post-mortem tenderization of meat by the degradation of myofibrillar and associated protein (Koohmaraie, 1992b). The protease system has largely, if not solely, been regarded as the main contributor of proteolytic activity during the post-mortem period with some suggestion that its activity accounts for 95% of all the proteolytic activity during post-mortem (Delgado et al., 2001; Koohmaraie and Geesink, 2006; Sentandreu et al., 2002). Furthermore the existence of calpastatin, an endogenous specific inhibitor of calpain regulates the protease activity indicates that there is a regulatory mechanism which can modulate the activity of the enzyme.

1.7.1 Calpain

The best characterised protein degradation system contributing to post-mortem meat tenderization is the calpain proteolytic system. Calpains are classified as cysteine proteases that are dependent on calcium for their activity (Goll et al., 2003). The system is highly sensitive to fluctuating levels of calcium, temperature and pH all of which are prone to changes during the post-mortem period (Melloni et al., 1992). In the proteolytic system calpain exists in two forms m-calpain and μ -calpain. Both of these molecules are ubiquitously expressed and are dependent on calcium for activity. The nomenclature of the m-calpain and μ -calpain refers to the calcium concentration required for activation (Cong et al., 1989). In general, the m-calpain requires 300 μ M - 1000 μ M Ca^{2+} for half maximal activity while the μ -calpain requires lower amounts of Ca^{2+} at 5 μ M - 65 μ M for half maximal activity (Goll et al., 1992). These two isoforms of calpain had been observed to cleave the same myofibrillar protein targets but do not significantly degrade myosin and actin (Dayton et al., 1976b; Koohmaraie, 1992b).

The first purification of calpain was done on porcine skeletal muscle and it was assumed at that time that the enzyme was potentially involved in the turnover of myofibrillar protein (Dayton et al., 1976a). Following this finding, partial characterizations of the protein were done to determine its properties. Dayton et al. (1976a) concluded from migration of the two polypeptide chains in SDS-PAGE, that calpain existed as a two sub unit heterodimer with 80kDA and 30kDA subunits. From this study, it was found that purified calpain had the capacity to disrupt or remove the Z disk, partly disrupt the M line as well as having some effect on troponin-T and troponin-I but not the entirety of the ultramuscular structure (Dayton et al., 1976b). Further studies on calpain substrates showed that many of the proteins associated with muscle structure, Z-disk protein and costamere were cleaved post-mortem (Taylor et al., 1995a). In addition, incubation of myofibrils with calpains degrades the key myofibrillar proteins including titin, nebulin, troponin-T and desmin (Huff Lonergan et al., 1996).

The importance of calpains especially, μ -calpain in post-mortem tenderization had been shown in a μ -calpain knockout mice, as post-mortem proteolysis in these animal was significantly inhibited (Geesink et al., 2006). Western blot analysis from these knockout animals shows that the degradation level of myofibrillar structural proteins such as desmin nebulin, dystrophin, vinculin and troponin are much lower compared to the control animals. Evidence from studies has also suggested that the two calpain isoforms behave differently during post-mortem. Data from bovine myofibrils suggest that μ -calpain is activated at the early stage of post-mortem (first 3 days) (Taylor et al., 1995a). Whilst data from post-mortem of porcine longissimus dorsi (LD), suggest that m-calpain is more stable than μ -calpain, however it is not activated during early post-mortem (1-8hours after slaughter) (Sensky et al., 1996). Furthermore, the concentration of Ca^{2+} which is limited during post-mortem may prevent the activation of m-calpain for the proteolytic activity to occur (Boehm et al., 1998).

1.7.1.1 Calpain structure

Both m-calpain and μ -calpain exists as heterodimers which are composed from two different subunits the catalytic, 80kDa subunit and a regulatory, 28kDa subunit. The small 28kDa is identical in both m-calpain and μ -calpain. The bigger 80kDa subunit is only 60% similar in amino acid sequence between the isoforms (Sorimachi et al., 1997). The 80kda catalytic subunit has four structural domains (domain I, II, III and IV) as shown in Figure 1.5. Domain I or the NH₂ terminal domain is known as the autolytic domain as it is autolysed upon calpain activation. Domain II is the cysteine catalytic site, which acts as the proteolytic domain and is similar to other cysteine proteases (Goll et al., 2003). Domain III in the large subunit contains the characteristic C2 domain and is said to be involved in structural changes during binding of calcium (Storr et al., 2011). Domain IV containing five EF hands is responsible for 'calmodulin like' binding capacity of calcium. The regulatory (small) subunit contains Domain V and Domain VI. Domain V in the 28kDa subunit is referred as a hydrophobic domain and is reported to bind phospholipids (Fernandez et al., 2006). Domain VI is highly similar to Domain IV in the catalytic subunit as it also contains four EF hands homologous to domain IV (Suzuki et al., 2004).

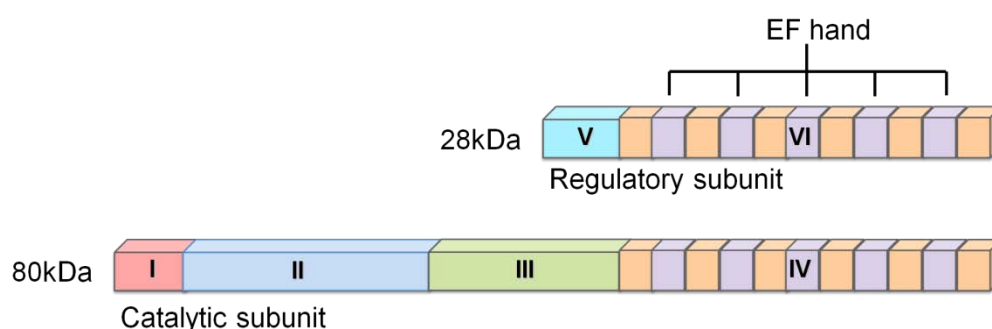


Figure 1.5 Schematic structures of calpain enzymes adapted from Sentandreu et al. (2002). Calpain enzymes are represented by two regulatory and catalytic subunit of 28kDa and 80kDa protein. Domain I: autolytic domain, Domain II: cysteine catalytic domain, Domain III: C2 like domain, Domain IV & Domain VI: calmodulin like domain, Domain V: phospholipids binding domain. The EF hands represented as five subdomains in domain IV and IV act as binding site to 'calmodulin like calcium'.

1.7.1.2 Calpain isolation

Calpain is found in many cell types in mammals with the majority of the characterisation of its function being associated with myofibrils in skeletal muscle. The earliest study on calpain (previously known as calcium activated protease) was in 1981 when it was purified from the proteins from rabbit skeletal muscle (Tsuji and Imahori, 1981). Characterization of the protein found that calpain consists of two subunits of ~80kDa and ~30kDa when identified by SDS PAGE. At around the same time calcium activated protease with the same molecular weight was also discovered in rat heart using gel filtration chromatography (Croall and Demartino, 1983). In human, purification of calpain was made from the cytosol fraction of homogenized human platelet using a combination of gel filtration chromatography and affinity chromatography. This yielded two different polypeptide of 80 kDa and 27 kDa (Yoshida et al., 1983). Other research in human tissues by Suzuki et al. (1979), purified the calpain from skeletal muscle and identified it as a hetero-dimer consisting of 78 kDa and 28kDa proteins. A comparison study revealed that calpain purified from porcine and bovine brain had almost the same molecular weight with both of them being heterodimers composed of 75kDa and 29kDa subunits (Kubota et al., 1986). Therefore the molecular weights of calpain molecules across different species are similar.

1.7.2 Calpastatin

Calpastatin is encoded by single gene (CAST gene) and is an endogenous specific inhibitor for calpain proteinase. It has been characterised as a pivotal gene candidate associated with meat traits in livestock (Casas et al., 2006). The activity of calpain is thought to be controlled not only by the free calcium concentration but also by the presence of calpastatin inhibitor. Calpastatin was initially found and identified following the observation that calpain activity could not be recorded in crude porcine muscle homogenates even though calpain could be purified from this tissue. Calpastatin was subsequently isolated from the tissue after the precipitation of the calpain at pH 6.2 (Dayton et al., 1976a). Furthermore, it was difficult to assess and relate certain calpastatin isoform to its molecular weight on a SDS PAGE. This was demonstrated with human-erythrocytes calpastatin at the molecular weight of 46.35kDa extracted using DEAE-cellulose purification method. The homogeneity of the final product was demonstrated on SDS PAGE which produced a single protein band at the calculated size of 70kDa (Takano and Murachi, 1982). This was due to the native behaviour of calpastatin migration in SDS PAGE where its migration was estimated to be approximately 40-50% slower than their true molecular weight (Maki et al., 1991).

1.7.2.1 Calpastatin structure

Early studies had found and characterized calpastatin as a polypeptide which contained a NH₂-terminal domain, named domain L followed by four repeating inhibitory domains of approximately 140 amino acids, each of these being 23-36% homologous. Within the four repetitive inhibitory domains is an additional repetitive shorter repeat found at 60-70 amino acid intervals (Takano et al., 1988). The predicted mass of calpastatin ranges from 68 to 78kDa the variation being dependent of the length of the L domain. However, migration of calpastatin in SDS-PAGE is anomalous and the relative molecular mass ranges from 107kDa to 172kDa (Goll et al., 2003).

Besides having an L domain, the calpastatin gene also encodes an XL region which was first described in studies on bovine cardiac muscle. The XL domain is N terminal to the L domain and an initiation start site was found to be at a position 204 bases before the protein translation initiation found in the L domain. This XL and L domain sequences does not share any homology with domain I-IV and do not have an inhibitory activity (Cong et al., 1998b) (Figure 1.6). The differences in the N terminal of calpastatin, (either a N terminus for the XL or L domain) might be responsible for differences found in its physiological function and subcellular localization. Primary transcripts of the calpastatin gene have been characterised as being alternatively spliced therefore the molecular diversity of the calpastatin polypeptide might occur because of exon skipping, thereby resulting in the production of a variety of proteins from a single gene. The functional consequences for this variation in calpastatin are not known however, it is likely that they have a functional consequence given calpastatin's regulatory role in reducing calpain activity (Takano et al., 1999).

1.7.2.2 Calpastatin gene

Previously completed sequencing of the calpastatin gene in mouse revealed 114kb of genomic DNA containing 34 exons (Takano et al., 2000) whilst the more recent study of the gene in bovine through sequencing revealed 130kb of genomic DNA containing 35 exons (Raynaud et al., 2005).

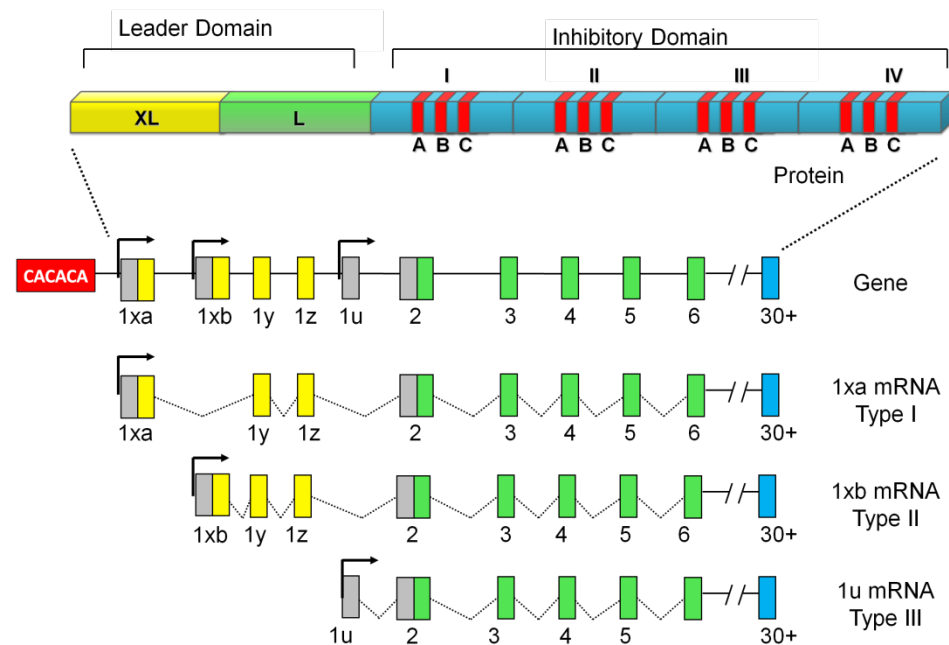


Figure 1.6 Structure of calpastatin protein, gene and its three types of mRNA which encodes three different isoforms, adapted from Parr et al. (2001) and Goll et al. (2003). Calpastatin protein is composed of leader and inhibitory domain. The leader domain contains XL and L region and the inhibitory domain contains repetitive inhibitory domain I-IV while the subdomains of A, B and C are responsible for binding of calpain. Each colour depicted on the mRNA transcripts represents the region of protein translated. CA tandem repeat region is indicated by the redbox labelled with CACACA. Arrows represent transcriptional start site for the transcripts.

1.7.2.3 Isolation of calpastatin

The first calpastatin purification from human-erythrocytes led to other discoveries and purification of calpastatin protein from different tissue and organ sources from different vertebrates. Purification of a calpain inhibitor from rabbit skeletal muscle revealed a 210kDa protein (Nakamura et al., 1985). Other purification from pig erythrocytes and pig heart muscle identified two sizes of calpastatin 68kDa and 107kDa respectively indicating differences between tissues in the same animal. However this research was unable to conclude whether the small protein was derived from the 107kDa protein or was synthesised *de novo* from a specific RNA (Takano et al., 1986). In addition, calpastatin purified from bovine cardiac muscle was determined as being a 115kDa protein (Otsuka and Goll, 1987). Therefore each purification of calpastatin from different sources demonstrated the heterogeneity of the inhibitor.

1.7.2.4 Heterogeneity of calpastatin expression

Calpastatin extraction utilising various techniques demonstrated that the protein exhibits an anomalous behaviour when migrating in SDS PAGE. This behaviour was confirmed when recombinant calpastatin protein expressed in *E. coli* were detected using a specific anti-calpastatin antibody, as the migration rate was describe as slow compared to the value calculated from the amino acid sequence (Takano et al., 1988). An early explanation why calpastatin behaves anomalously in its migration was that previous purification technique was done using the old method that disrupts the polypeptide sequence because of the denaturing effect of the technique. Thus, it increased the possibility of damaging the isolated protein. Another possibility was that the isolated calpastatin was produced from the same gene as result of splicing of the calpastatin gene mRNA (Wendt et al., 2004).

However, the most likely explanation is that calpastatin protein appears to be a protein which undergoes randomly conformational coiling. This was verified by circular dichroism (Konno et al., 1997). Other studies observed that calpastatin isolated using size exclusion chromatography turns out to have a bigger molecular weight than that observed on SDS PAGE which suggested that calpastatin exists as a dimer or tetramer in a non-denaturing solvent (Goll et al., 2003).

Various studies have been carried out on the calpastatin polypeptide to determine the relationship between the structure and function of the inhibitory domains. These studies were done by cloning calpastatin cDNA into a vector which allowed recombinant protein to be produced. Subcloning of calpastatin cDNA fragment encoding each individual inhibitory domain allowed subsequent calpain inhibition assays to be done on the crude cell extract of *E. coli* transformants expressing each domain in the calpastatin. From these studies all four repetitive regions predicted to have inhibitory activity possessed the capability to inhibit calpain (Maki et al., 1987). This

suggested that one calpastatin molecule had the capacity to inhibit four calpains.

The inhibitory role of calpastatin and its significance in affecting meat tenderness was shown in callipyge lambs as high level of calpastatin were observed with reduced proteolytic activity post-mortem resulting in a decrease in meat tenderness (Geesink and Koohmaraie, 1999). It was hypothesized from this experiment that calpastatin played an inhibitory role in protein degradation allowing efficient protein accretion resulting in muscle hypertrophy in the callipyge lambs. The calpastatin binds to the 'calmodulin like' domains of the catalytic unit (Domain IV) and the regulatory unit of Domain (VI) in calpain (Wang and Yuen, 1999). The three sub domain (A,B,C) located within the inhibitory domain of I,II,III and IV are responsible for binding to calpain and inhibiting its activity. The binding of calpastatin to calpain are discussed in a review that describes how it requires Ca^{2+} , is reversible and the concentration of Ca^{2+} required for the binding to occur is much less than the concentration required for half maximal activity of m-calpain (Wendt et al., 2004). The role of calpastatin as an inhibitor of calpain was strengthened as mice overexpressing calpastatin showed decreased in proteolytic activity and also substrate degradation such as desmin and troponin-T although there was no affect observed on the isolated activity of m-calpain and μ -calpain (Kent et al., 2004).

1.8 Calpastatin and meat tenderness

The existence of calpastatin in meat and findings from research suggests that it influences calpain by inhibiting its activity. The initial 24 hour period after slaughter is an important time to predict tenderness in meat and as a result biochemical factors regulating tenderness can be measured which predict tenderness. Accordingly Whipple et al. (1990) demonstrated calpastatin was a marker for predicting tenderness in meat. They found that calpastatin activity in beef at 24 hours was highly correlated to shear force value after 14th day of slaughter. It showed that an early event during the post-mortem period could be predictive of ultimate shear force, as the lower calpastatin activity recorded 24 hour after slaughter reflected a lower shear force value recorded in the 14th day.

The observed finding was repeated in pork where a higher level of calpastatin in the first few hours after slaughter (2 hours post-slaughter) was associated with increased incidence of toughness in meat (Parr et al., 1999; Sensky et al., 1998)(Figure 1.7). This finding was in agreement with Koohmaraie et al. (1991b) which concluded that calpastatin activity was responsible for variation of meat tenderness in beef, lamb and pork carcasses through findings which indicated the differences in proteolytic rate of these animals depending on the activity of calpastatin.

This calpastatin based marker has been identified as the best indicator for meat toughness and subsequent studies have identified calpastatin gene markers, in the form of polymorphisms, as a base for predicting tenderness in various production animal breeds (Casas et al., 2006; Ciobanu et al., 2004) which have become part of a commercialized genetic test (GeneSTAR Molecular Value Predictions, Pfizer). This test has been derived from studies assessing degree of meat tenderness and its association with single nucleotide polymorphisms in calpastatin and calpain sequences. Therefore, the calpastatin gene can be considered as an eligible candidate for predicting tenderness and understanding the consequences of the variability in gene

sequence will aid to build a basis of understanding of its role in meat tenderness variability.

Various studies have demonstrated that the level of calpastatin in different breeds of animal is associated with variable rates of meat tenderisation. One particular example is callipyge sheep which demonstrate an increased level of meat toughness after slaughter compared with other breeds. This breed has an inherited gene which confines muscle hypertrophy which is associated with an increase in the level of calpastatin activity (Duckett et al., 2000). The administrations of the growth promoter β -adrenergic agonist to porcine have been shown to significantly increase the expression of calpastatin in the skeletal muscle (Parr et al., 2001). Furthermore, this effect was also reported in bovine treated with β -adrenergic agonist as its skeletal muscle exhibited a significant increase in muscle accretion which leads to a condition of hypertrophic growth in the animal (Wheeler and Koohmaraie, 1992). A more complex study on the heritability of calpastatin activity in bovine intraspecies performed by Shackleford et al. (1994) established a correlation between both calpastatin level and meat toughness and the possibility of using these for selection purposed to improve meat quality.

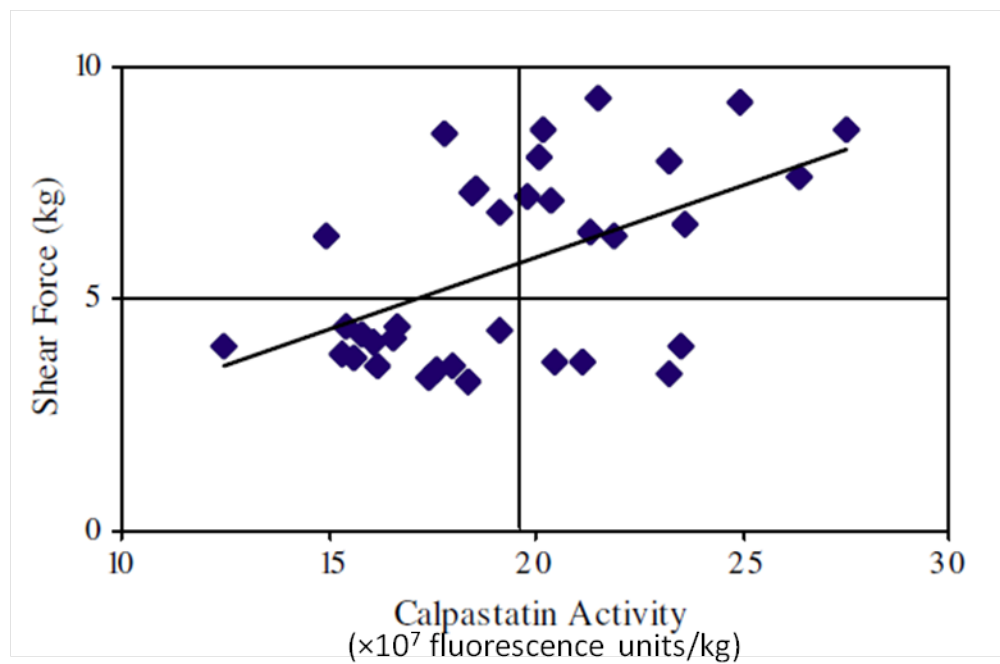


Figure 1.7 Relationship between level of calpastatin activity ($\times 10^7$ fluorescence unit/kg) 2 hours after post-mortem in pig and the shear force value of the meat at day 8th. Calpastatin activity was assessed in samples obtained from longissimus dorsi muscle according to the procedures described by Sensky et al. (1996). From the same carcass chops of longissimus dorsi were conditioned for 8 days at 4°C and then assessed for shear force using the Warner-Bratzler method as described by Parr et al. (1998). Adapted from Sensky et al. (1998)

1.9 Calpastatin gene regulation

Although, calpastatin polypeptide appears to be translated by the same gene in different species (Goll et al., 2003), and in spite of its anomalous size behaviour, purification of the polypeptide indicated different sizes of protein which suggested that there were multiple calpastatin transcript variants suggesting either the gene was transcribed from different promoters in the gene or transcripts were subjected to mechanisms of alternative splicing. Expression of calpastatin protein is regulated at the post-transcriptional (Parr et al., 2004; Raynaud et al., 2005; Takano et al., 2000) and post-translational level (Cong et al., 1998b; Salamino et al., 1997), thereby creating heterogeneity in its expression and therefore is a means of regulating calpain in different tissue types and physiological conditions.

The early study of calpastatin promoter was first established by Cong et al. (1998b) who identified a new N-terminal region (XL region) in the isolated bovine calpastatin cDNA which in its conceptual translation produced an additional 68 additional N-terminal amino acid. In addition, the study also found potential CRE sites in the promoter region which demonstrates the possibility of calpastatin gene regulation through the activation of cAMP dependent protein kinase activity in the promoter region of exon 1xa (later found to be 1xb promoter region by Parr et al., (2001)). However, the promoter studies performed by Cong et al. (1998b) were not complete as they failed to discover other promoter regions in the gene as only one promoter was considered as initiating gene transcription.

Further characterisation of the calpastatin gene promoter by two groups managed to identify promoter region which were predicted to be responsible for the initiation of transcription to create the Type I, II, III and Type IV transcripts (Parr et al., 2001; Raynaud et al., 2005). These mRNA transcripts of Type I, II and III and Type IV calpastatin mRNA were expressed in various tissues including skeletal muscle and these mRNA transcripts with differing 5' end were found to be expressed in cattle, mouse and pig (Parr et al., 2001;

Raynaud et al., 2005; Takano et al., 2000). Type IV mRNA transcripts were found to be tissue specific and was only expressed in testis (Raynaud et al., 2005; Takano et al., 2000).

Calpastatin mRNA transcripts of Type I, II, III and IV have been characterised as being transcribed from four different transcription start sites in the gene (Figure 1.6). These four transcripts have different translation start sites which potentially generate calpastatin isoforms with different N-terminal sequences. This observation was first reported in mouse cDNA sequences where Type I, II, III and Type IV calpastatin transcripts were proposed to be the results of transcription which starts at exon 1xa, 1xb, 1u and exon 14t respectively in calpastatin gene (Takano et al., 2000). The calpastatin mRNA transcripts from porcine heart and skeletal muscle indicate the phenomenon of exon skipping (Parr et al., 2001). This was shown as in Type I mRNA, gene transcription starts from 1xa promoter excluding 1xb and 1u exon sequences. In Type II mRNA, transcription starts at the 1xb promoter excluding 1u exon, whilst in Type III transcription starts at the 1u promoter. Furthermore, multiple transcription factor binding sites were also identified in the four promoter regions signifying the importance of the promoter sequences which is potential for gene regulation (Raynaud et al., 2005). For the bovine gene, ATG translation start codons were found in exons 1xa, 1xb and 2 and 14t which were similar to those that had been observed in mouse (Raynaud et al., 2005; Takano et al., 2000). However, there is lack of TATA boxes or CAAT sequences in the putative promoter regions of the porcine and bovine calpastatin genes indicating the importance of another regulatory element which may influence transcriptional activity of the gene. However it must be noted that variance in calpastatin isoforms could also possibly occur as the result of post-translational modifications as calpastatin is susceptible to proteolytic cleavage (Takano et al., 1988).

The functional aspect of promoter regions of the calpastatin gene responsible for type I, II and III calpastatin transcripts were confirmed in transient transfection assays by generating a series of porcine promoter constructs

using an expression vector that has a reporter gene (Parr et al., 2004). These observations were later, confirmed with the testing of all the possible bovine promoter sequence responsible in synthesizing type I, II, III and IV calpastatin transcripts in a similar experiment (Raynaud et al., 2005).

For bovine, the calpastatin gene was located and mapped in chromosome 7 (Bishop et al., 1993) with relative position 117.8 cM using RFLP (Kappes et al., 1997). From sequencing, the bovine calpastatin was identified as having 35 exons in ~130kb of sequence (Raynaud et al., 2005). The sequences that was predicted to have promoter activity in the bovine calpastatin gene were cloned into a vector pEGFP-1 that has a reporter gene (Figure 1.8). Activity of these promoters was investigated and was concluded that all four promoters in calpastatin gene were functional and its direct expression of the gene.

There were many interesting gDNA sequence elements found in the study of calpastatin promoter activity. Exon and intron boundaries between bovine, human, pig and mouse calpastatin genes appears to be conserved (Takano et al., 2000). Multiple motifs that are generally known as transcriptional activators, such as two GATA-1, SP1 and one NF κ B, are present near to the 5' sequence before exon 1xa. Deletion analysis on this area which includes the 'CA' tandem repeat sequence shows a significant decrease in promoter activity through reporter gene (Raynaud et al., 2005). This could indicate the potential of the CA tandem repeat in the regulational control of the gene transcription. However, this result was in contrast to that obtained with porcine. Deletion on the CA repeat sequence from the promoter region in porcine calpastatin shows an increase of promoter activity in both HEK 293 and L6G8 cells (Parr et al., 2004). CA repeat sequence deletion through this studies proved it might have played a role in transcriptional regulation of the gene as there was also evidence of CA tandem repeat length polymorphism characterised in calpastatin promoter region of different Hereford backcross cattle which may be useful as a marker for determining the tenderness trait in meat quality (Nonneman et al., 1999).

In the earliest studies examining the responsiveness of the calpastatin promoter Cong et al. (1998b) found at least one cAMP responsive element (CRE) which resided at -102 before the initiation start site in bovine calpastatin (later confirmed as 1xb promoter region by Parr et al. (2001)). This finding implied that cAMP dependent protein kinase might be responsible in influencing transcriptional efficiency of the calpastatin gene. This was an indication that the sequence 5' before the 1xa promoter region is influencing the transcriptional activity of calpastatin especially in bovine. In addition multiple CRE sites are also identified in porcine calpastatin promoter region signifying the importance of the site (Parr et al., 2001).

These observations went some way to explain previous experimental observations which had shown that the steady state level of mRNA and the level of calpastatin activity in bovine skeletal muscle are both stimulated by administration of β -adrenergic agonists (Killefer and Koohmaraie, 1994; Parr et al., 1992). The inclusion of a β -adrenergic agonist in animal feed stimulates skeletal muscle hypertrophy in treated animals. These findings suggest that the transcriptional activity of the calpastatin gene was induced by the secondary messenger cascade stimulated via the β -adrenergic receptor with β -adrenergic agonist activating adeneylyl cyclase, followed by activation of cAMP dependent protein kinase (PKA) and the subsequent phosphorylation of cAMP response element binding protein (CREBP) and which was suggested to interact with sequence elements with the calpastatin gene promoter and ultimately influencing calpastatin protein expression and calpastatin activity (Parr et al., 2001; Sato et al., 2011).

Furthermore, calpastatin also had been observed to be phosphorylated *in vitro* by protein kinase A (PKA) and protein kinase C (PKC) at the post-translational level and that its phosphorylation alters its ability to inhibit calpain (Salamino et al., 1997). This result were further clarified by Cong et al. (1998b) as the XL region of calpastatin in bovine contains at least three PKA phosphorylation site and *in vitro* incubation shows the site in expressed

calpastatin can be phosphorylated with purified PKA. It is not clear what the functional role of these modifications is.

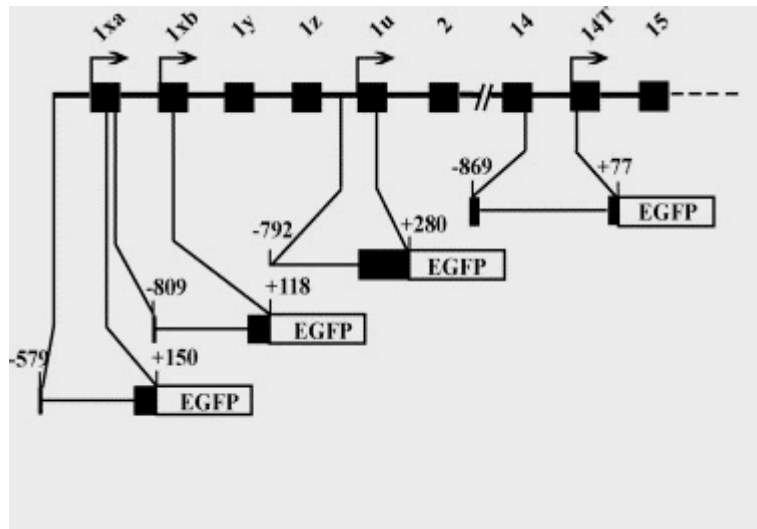


Figure 1.8 Characterisation of bovine calpastatin promoter expression by cloning the 5' upstream promoter region into pEGFP promoter expression vector. Adapted from Raynaud et al. (2005).

1.10 Summary

Calpain enzyme activity has been demonstrated as being involved in the development of meat tenderness. This meat tenderness attribute is highly variable and a commercially important factor determining consumers choice. Therefore factors that influence the meat tenderness attribute need to be understood not only to instigate actions that need to be undertaken in order to improve consumer satisfaction but also to reduce waste involved in generating carcasses of unacceptable quality. Studies have shown the role of calpastatin as an endogenous inhibitor of calpain activity. Therefore it is vital to understand the mechanisms of regulation of calpastatin gene expression and its effect on meat tenderness so that potential interventions or selection markers can be used to improve meat quality. Although, it was known that the nature and complexity of genetic selection to be made as they are affected by many factors along the line, identification of genetic markers could potentially help to improve tenderness of the meat as selection process started before animal slaughter. Patented genetic test reflects the demands

of livestock industry to seek the ability to predict meat tenderness based on genetic markers of the animals which could reduce cost and storage time required for meat conditioning.

1.11 Study scope

‘CA’ tandem repeat polymorphism is positioned in the 5’ upstream promoter region in bovine calpastatin gene (Figure 1.9). The CA tandem repeat, (CA) n polymorphism in the bovine calpastatin gene was firstly characterized in a Hereford backcross family using autoradiography (Nonneman et al., 1999). This tandem repeat is adjacent to exon 1xa which directs the transcription of Type I bovine mRNA calpastatin (Parr et al., 2004).

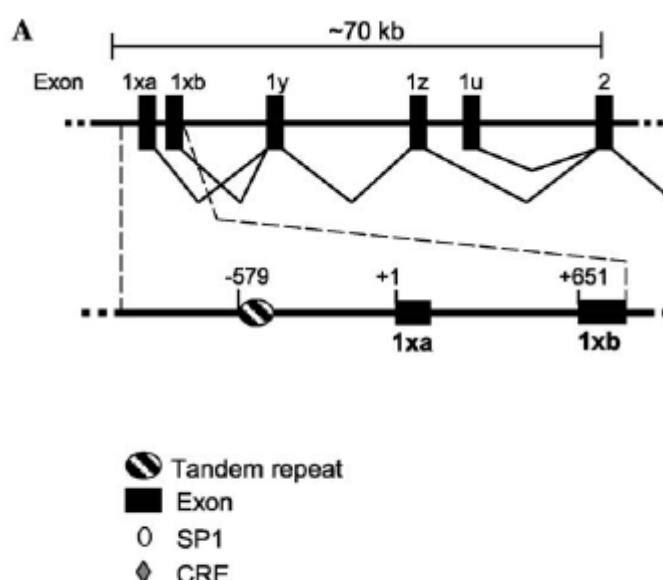


Figure 1.9 Location of the short CA tandem repeat sequence 5’ upstream exon 1xa promoter. Adapted from Parr et al. (2004).

The locations of the ‘CA’ tandem repeat near the 1xa promoter region is predicted to have an influence on regulating the transcriptional activity of calpastatin mRNA. Consequently, the level of calpastatin mRNA could be affected as well as its translation to protein and as a result could potentially regulate the calpain activity in meat. Calpastatin’s transcripts level was associated with the increased of its protein by β -adrenergic stimulation. This indicated that increasing the transcription of calpastatin mRNA influences the level of the protein and subsequently the activity of the inhibitor (Parr et al.,

1992). Therefore, this study is intended to examine polymorphisms in the calpastatin gene which might influence the expression of the gene and thereby could potentially influence the tenderness trait in meat.

1.12 Hypothesis

Using the evidence from previous research and based on the main hypothesis that the calpain-calpastatin system are accountable for most of the variation produced in meat tenderness, a series of hypothesis were built and tested for this study:

1. Amplification of the short CA tandem repeat sequence located in the 5' upstream region of the 1xa promoter region of calpastatin from different cattle with varying meat tenderness will yield different length of PCR product as the results of length polymorphisms of the CA repeat sequence.
2. Changing the length of the short CA tandem repeat will alter transcriptional activity of the calpastatin gene.
3. The length of short CA tandem repeat sequence (genotype) in individual cattle will determine the meat tenderness (phenotype) based on its shear force value.
4. Administration of anabolic agents will affect transcriptional activity of the calpastatin promoter

1.13 Aims and objective

The main aim of this thesis was to characterise the short CA tandem repeat region located in the 5' promoter sequence in the exon 1xa of the bovine calpastatin gene in relation to meat tenderness.

The specific objectives were to:

1. To determine the association of CA tandem repeat polymorphisms and meat tenderness by developing a PCR based technique to amplify the region in order to differentiate length of the amplified region between cattle from different individual in a breed and meat tenderness.
2. Develop a method for cloning the short CA tandem repeat region in a promoter expression vector.
3. Characterise expression from the three main promoters of the bovine CAST gene in various cell lines in order to ascertain the effect of differing host line on the transcriptional activity of the promoter.
4. To define the relationship between the length of CA tandem repeat sequence with the transcriptional activity of CAST Type I promoter.

Chapter 2: Materials and Methods

2.1 Materials used in this study

- Microcentrifuge with refrigeration (Volumes below 2ml) - Microfuge 22R Centrifuge, Beckman Coulter
- Swinging bucket rotor Centrifuge (Volumes over 2ml and cell culture) - Centaur 2, MSE
- Basic PCR Thermocycler – XP Cyclor, Bioer
- Gradient PCR Thermocycler – Mastercycler Gradient, Eppendorf
- Incubator with shaker – Lab-Therm, Kühner Switzerland
- Spectrophotometer - Nanodrop 1000 Spectrophotometer, Thermo Fisher Scientific
- Agarose gel electrophoresis tank – SubGel GT tanks, BioRad
- Electrophoresis Power Supply – Power Pac 300, Biorad
- Non-denaturing acrylamide gel electrophoresis tank – Mini Protean II, BioRad
- DNA gel imager – Gel Doc 2000, BioRad
- Band analysis software – Quantity One Software Version 4.6.8, BioRad
- Quantitative real time PCR machine – LightCycler 480, Roche Diagnostics
- Quantitative real time PCR software – LightCycler 480 SW Version 1.5
- Fluorescence imager - Typhoon Trio, GE Healthcare
- Fluorescence quantification software – Image Quant TL
- Plater Reader - Fluostar Optima, BMG Labtech
- Inverted Fluorescence Microscope – Leica DFC420C

All laboratory reagents used in this study were obtained from Sigma Aldrich (Poole, UK), Fisher Scientific (Loughborough, UK) and Promega (Southampton, UK). Water used for buffer solution was glass distilled or column purified using the MiliQ Elix purification system (Milipore, Watford, UK) and RNase/DNase free water was used for nucleic acid work which was purchased from Sigma Aldrich.

2.2 Bovine calpastatin BAC clones

In the process of developing a technique to differentiate different lengths of the short CA tandem repeat sequence in the bovine calpastatin promoter gene sequence, a viable bacterial artificial chromosome (BAC) bovine genomic clone containing the gene insert was used as a positive control. The main purpose of the BAC bovine genomic clone was to use it as a positive control in PCR experiment and also to test it with designated primers designed specifically for amplification of the short CA tandem repeat region in calpastatin promoter sequence. The BAC clones construct originated from a library array produced by Children's Hospital Oakland Research Institute. The clones' library was derived from a bovine male and named CHORI 240 (Moore et al., 2003). Various length of genomic DNA ranged from 153Kbp to 180Kbp were cloned into BAC vector pTARBAC 1.3 and gridded into 22×22 cm nylon high density membrane filters. Using a specific probe for the 5' end of bovine Type III calpastatin cDNA the membrane filters were probed and two clones were detected on the nylon filters. Two identified clones CH240 - 8011 and CH240 - 7N18 were then ordered from Children's Hospital Oakland Research Institute. The inserts of these clones were verified by PCR and partial sequencing of the region spanning the 1xa exon which included the associate promoter region. DNA from the BAC clones was isolated and purified for use as a positive control in this study.

2.3 Tissue collection

Tissue collection from meat was made previously in Tendercheck Shear Force 2009 program. Two to three cm thickness of steak was cut from the same loin area of each of 30 cattle of different ages and breed at approximately 24 hour post slaughter with the carcass have been held at 4°C overnight. The steaks were aged at 4 °C for 10 days and shear force value was measured and later frozen at -20°C until analysis could be performed. All the procedure was done by third party and meat samples with shear force data was used with permission in this thesis.

2.4 Amplification of nucleic acids

2.4.1 Primer design

Primer pairs of 18-22bp length were designed using Primer Express Software (V1.5, Applied Biosystems) and Primer Blast Program available from NCBI website. Designed primers were purified using High Purity Salt Free (HPSF) (Purity level >70%) and High Performance Liquid Chromatography (HPLC) (Purity Level >95%) method were acquired from Eurofins MWG Operon and Sigma.

2.4.2 Polymerase Chain Reaction (PCR)

General PCR reaction carried out in this study used AmpliTaq Gold Enzyme (Applied Biosystems) and was carried out in a thin-walled 200µl microcentrifuge tube (Fisherbrand). For the template, 10-50ng of DNA sample in 5µl RNAase/DNAase free water was used in each PCR reaction. Mixture for a PCR reaction contained 2.5µl of forward 5' and reverse 3' primer (10pmol/µl), 3µl of Magnesium Chloride (25mM), 5 µl of 10X PCR Gold Buffer, 1µl of dNTP (2mM dATP, 2mM dTTP, 2mM dCTP, 2mM dGTP), 0.2µl of AmpliTaq Gold enzyme (1.5units) and the final volume being made up to a total 50µl per reaction. The reaction was incubated in a standard PCR thermocycler at 94°C for 10 minutes to activate the AmpliTaq Gold enzyme before moving into amplification cycles. The amplification cycle contained three steps: denaturation of DNA strand at 94°C for 30 seconds, annealing of primers at temperatures 5°C below the primers T_m for 30 seconds and elongation by polymerase enzyme at 72°C for 45 seconds (elongation time depends on the size of amplified product- at least 60seconds for 1kb extension). The amplification cycle was repeated for 35 times, followed with the final extension of polymerase enzyme at 72°C for 5 minutes and, finally, cooling of the amplified product to 4°C at which temperature it was held indefinitely.

2.4.3 Gradient PCR

The gradient PCR was largely used in this study to optimise annealing temperatures for primer binding as well as to study the effect of different annealing temperatures on amplification of products which were giving apparent nonspecific amplicons; for example CAST promoter short CA tandem repeat region. PCR reaction was prepared as described in section 2.3.2. The gradient PCR thermocycler (eppendorf) was set to run with a different annealing temperature across the wells. Range of temperature for annealing in the gradient PCR thermocycler were set at $\pm 10^{\circ}\text{C}$ while the general cycling conditions were the same. Therefore, the annealing temperatures from left to the right slot in the gradient PCR thermocycler block had a range of temperatures which was a maximum of 20°C . After completion of the PCR, the amplicons were electrophoresed on non-denaturing polyacrylamide gel as described in section 2.9.1.

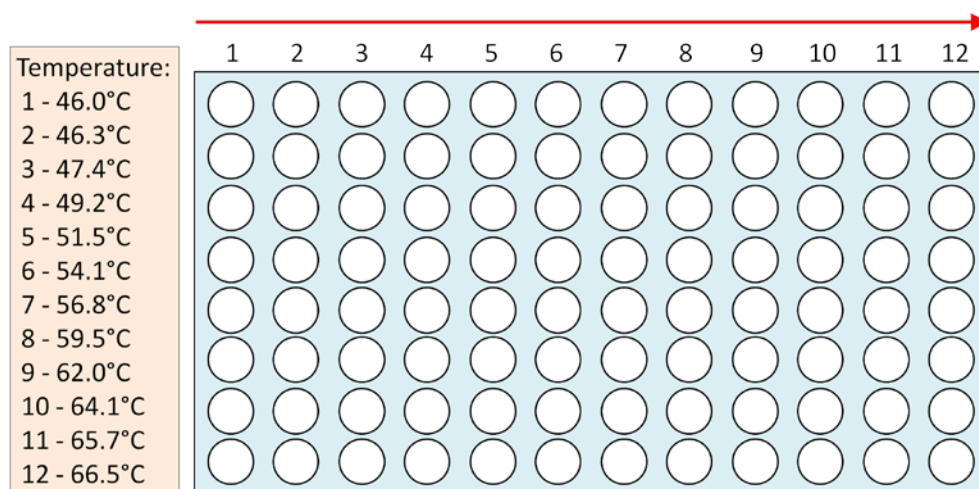


Figure 2.1 Typical construction of 96 wells gradient block in a gradient PCR thermocycler. Annealing temperatures for PCR reaction can be set up to vary across the block during PCR reaction. Shown in the image was gradient annealing temperature increase and decrease of $\pm 10^{\circ}\text{C}$ from its original setup temperature at 55°C .

2.5 DNA purification

2.5.1 Wizard Genomic DNA Purification Kit (Promega)

Genomic DNA was extracted from bovine tissues using Genomic DNA Purification Kit (Promega). Bovine tissue snaps frozen in liquid nitrogen and stored at -70°C, subsequently it was crushed to a powder. For extraction of DNA, 10-20mg of tissue was homogenized for 10 seconds with 600µl of chilled nuclei lysis solution in a 1.5ml microcentrifuge tube. The homogenate was incubated for 30 minutes at 65°C before 17.5µl of Proteinase K (20mg/ml) was added and the sample was incubated for 3 hours at 55°C with vortexing once per hour for 30 seconds. Then, 3µl of RNase solution (4mg/ml) was added to the homogenate which was then mixed by inverting the tube 2-5 times. The tissue homogenate was incubated again at 37°C for 30 minutes then cooled to room temperature. To the tissue homogenate solution was added with 200µl of protein precipitation solution, followed by vortexing for 20 seconds and then chilled on ice for 5 minutes. The tube was then centrifuged at 13 000 x *g* for 4 minutes at room temperature. Supernatant was transferred to a fresh 1.5ml microcentrifuge tube containing 600µl of isopropanol at room temperature. The tube was gently mixed by inversion and was centrifuged at 13 000 x *g* for 1 minute at room temperature. The supernatant was removed from the tube and 600µl of room temperature 70% (v/v) ethanol added with the contents then mixed together by gentle inversion of the tube. Finally, tube was spun at 13 000 x *g* for 1 minute at room temperature and the supernatant was removed. The remaining DNA pellet was air dried for 15 minutes and resuspended in 50µl DNAase and RNAase free water. Extracted DNA was stored at 2-8°C temperature.

2.5.2 DNeasy Blood & Tissue Kit (Qiagen)

An alternative method used in this study to extract DNA was DNeasy Blood & Tissue kit. This alternative method of DNA extraction using spin column was considered in order to maximise DNA quality and yield. 25mg of tissues crushed in liquid nitrogen were placed in a 1.5ml microcentrifuge tube and

was added with 180µl of ATL buffer. To lyse the tissue, 20µl of proteinase K (20mg/ml) was added and tube was incubated at 56°C until the tissue was completely lysed. The tube was vortexed occasionally during the incubation period to disperse the sample. Four µl of RNase A (100mg/ml) was added, mixed by vortexing and incubated for 2 minutes at room temperatures. The tube was vortexed again for 15 seconds before adding 200µl of buffer AL, followed by vortexing to mix the sample. Then, 200µl of 100% (v/v) ethanol was added it was mixed thoroughly by vortexing. Content from the tube was transferred into DNeasy mini spin column with collection tube and spun at 6000 x *g* for 1 minute at room temperature. Flow through was discarded with the collection tube. The spin column was transferred into a new 2ml collection tube, 500µl of buffer AW1 added then centrifuged at 6000 x *g* for 1 minute at room temperature. Flow through liquid and collection tube was discarded. The spin column was then placed in a new 2ml collection tube, 500µl of buffer AW2 added followed by centrifugation at 20 000 x *g* for 3 minutes at room temperature to remove impurities. Flow through liquid along with the collection tube was discarded. To elute DNA, spin column was transferred into a fresh 1.5ml microcentrifuge tube, 50 µl DNase and RNase free water added and finally spun down at 6000 x *g* for 1 minute at room temperature. Eluted DNA was stored at 2-8°C.

2.6 Preparation of plasmid DNA

2.6.1 Plasmid mini prep procedure

Extraction of plasmid from bacteria clones was done using Gen-Elute Plasmid Miniprep Kit (Sigma-Aldrich). 3ml of an overnight culture is spun down in a centrifuge tube at 12 000 x *g* for 1 minute at room temperature and supernatant is discarded. The cell pellet was resuspended using 200µl of resuspension solution followed by 200µl of lysis solution which was mixed gently by 6-8 times inversion or until the solution became clear and viscous. Through the addition of 350µl neutralization solution and inversion of the tube for 4-6 times cell debris was precipitated. This mixture was then spun at

12000 x *g* for 10 minutes at room temperature. Supernatant was transferred to a prepared Gen-Elute Miniprep Binding Column® that was initially washed with 500µl column preparation solution and spun down at 12 000 x *g* for 1 minute at room temperature. The loaded supernatant was spun at 12 000 x *g* for 1 minute at room temperature and flow through liquid was discarded. Column was washed with diluted wash solution (20% v/v) and centrifuged for 1 minute at 12000 x *g* at room temperature. Excess of washing liquid was removed by another spin at 12 000 x *g* for 1 minute at room temperature before the DNA was eluted by adding 100µl of RNAase and DNAase free water followed by another spin at 12 000 x *g* for 1 minute at room temperature.

2.6.2 Endotoxin free plasmid midi prep procedure

Extraction of plasmid at a higher yield and free from endotoxin was carried out using GenElute Endotoxin-free Plasmid Midiprep Kit (Sigma-Aldrich). The higher plasmid yield that was free from endotoxin was required for transfection experiments as the endotoxin present in the conventionally prepared plasmid reduces transfection efficiency in mammalian cell culture. 40ml of overnight grown *E. coli* bacterial clones in a 50ml conical-ended tube were spun at 5000 x *g* for 10 minutes at room temperature and supernatant was discarded. The cell pellet was resuspended with 1.2ml of resuspension solution that has been added with 1% (v/v) amount of RNase A. The resuspended cells were then lysed at room temperature by adding 1.2ml of lysis solution and the contents were gently inverted for 6-8 times until the mixture became clear and viscous. Care were taken not to allow the lysis procedure to exceed more than 5 minutes, therefore reducing the possibility of bacterial chromosomal DNA contamination in the final recovered plasmid. After adding the lyse solution the mixture was neutralized by adding 0.8ml of neutralization/binding solution and the tubes were inverted 4-6 times. Cell debris, proteins, lipid and chromosomal DNA denatured by SDS will form a viscous and cloudy precipitate by addition of neutralization/binding solution.

The mixture was then centrifuge at 15 000 x *g* for 15 minutes at 2-8°C. The clear lysate was transferred into a 15ml conical tube.

To remove endotoxin, 300µl of endotoxin removal solution was added to the lysate which was mixed thoroughly by inversion for 1 minute. The conical tube was chilled on ice for 5 minutes and was inverted 1-2 times during this incubation. The tube was warmed in the water bath at 37°C for 5 minutes until the solution turned cloudy. To separate the phases, the conical tube was centrifuged at 5000 x *g* in a swinging bucket rotor for 5 minutes at room temperature. The clear upper phase containing plasmid DNA was carefully transferred into a new 15 ml conical tube and the blue lower phase, which contains endotoxin, was discarded. This endotoxin removal step was repeated again. To the endotoxin free lysate, 0.8ml of DNA binding solution was added, the contents mixed thoroughly through inversion and vortexing. Meanwhile, the supplied GenElute MidiPrep Binding column was prepared by inserting it into a collection tube. Three ml of column preparation solution was added to each column which was spun at 5000 x *g* for 1-2 minutes at room temperature. Flow through liquid was discarded after the centrifugation step. Cleared lysate from the previous step of endotoxin removal was transferred into the prepared column which was already seated in the collection tube and subsequently centrifuged in a swinging bucket rotor at 5000 x *g* for 2 minutes at room temperature. Flow through liquid was discarded after the centrifugation. After the addition of 3ml wash solution the column was centrifuged again at 5000 x *g* for 2 minutes at room temperature. Before proceeding to the next step, it was made sure that the column was free from remaining wash solution by additional spin. Finally, 1ml of endotoxin free water was added to the column and it was spun at 5000 x *g* for 5 minutes at room temperature to elute the DNA. The eluted DNA was stored at -20°C to prolong the plasmid DNA integrity.

2.7 DNA sequencing

Plasmid constructs containing inserts were sequenced by GeneService and Plant Sciences Sequencing facility (University of Nottingham) using primers specially designed for sequencing the plasmids. The primers used for the sequencing were purified using (HPSF) at the purity level of >70%. At least 1µg of purified plasmid DNA was required for sequencing purpose.

2.8 Quantification of DNA/RNA

DNA and RNA concentration was quantified using the Nanodrop ND1000 Spectrophotometer at the ratio of absorbance at 260nm and 280nm. Samples measured at this ratio of absorbance at 260nm and 280nm will determined the level of purity from extracted DNA/RNA. For DNA a ratio of ~1.8 is acceptable as pure whilst for RNA the ratio of ~2.0 is acceptable as pure. The ratio value which is lower in either DNA or RNA usually indicates the presence of protein contaminants in the sample. Mass or concentration of DNA or RNA was calculated by the software basically using the Beer-Lambert equation based on the recorded value of absorbance at 260nm. The ratio of absorbance recorded from the extracted DNA is in the range of 1.8-2.0.

2.9 Non Denaturing Agarose Gels

2.9.1 Agarose gel electrophoresis

Agarose powder at the weight of 1.2g was added to 120ml of 1X TAE Buffer (0.04M Tris acetate, 0.001M EDTA, and pH 8.0) for preparing 1% (w/v) gels. The mixture was then heated in microwave for 3 minutes and was left to cool before being poured into a gel casting module (Biorad). A gel comb was placed on to the gel to before it solidifies. The solidified gel was placed in the gel tank and 1X TAE buffer was used to fill the tanks as running buffer. In order to electrophorese DNA samples, 1µl of 5X loading dye (Promega) was mixed with 5µl DNA sample before the mixture was being loaded into a well. Five µl of 100bp DNA ladder (Promega) was used as a marker for DNA size.

The loaded DNA sample was electrophoresed at 100 volt for 45 minutes. The time used in electrophoresis of samples depends on agarose concentration of prepared gel. For 2% gel concentration, running time of 100 minutes was required for electrophoresis.

2.9.2 Metaphor agarose gel

Metaphor agarose powder at the weight of 3.6g (Lonza) was added to 120ml of 1X TAE for preparing a gel with a 3% (w/v) concentration. The mixture was heated below boiling point and stirred on a hot plate for about 15 minutes before being heated again in a microwave for 3 minutes at the highest power setting. The heated mixture was then poured into a gel cast and was left to solidify and the used of electrophoresis of DNA.

2.10 Non Denaturing Poly-acrylamide Gel

2.10.1 Non denaturing polyacrylamide gel electrophoresis

For preparation of non-denaturing acrylamide gel, 2.5ml of 30% (w/v) stock acrylamide (37.5:1, acrylamide: bis acrylamide Bio-Rad) was added mixed with 3.0ml 5X TBE (360mM Tris, 360mM Boric acid, 8mM EDTA, adjusted to pH8.3) and 9.5ml water to produce 15ml of 7.5% (w/v) gel solution. Immediately before gel was poured, 5 μ l of Tetramethylethylenediamine (TEMED) and 10 μ l of 10% (w/v) ammonium persulphate (APS) was added to assist polymerization of gel. The gel solution was poured into a vertical Bio-Rad Protean II mini gel apparatus with 1mm width spaces and left to set for 45 minutes at room temperature. 1X TBE running buffer, which was diluted from 5X TBE, was used to fill up the lower and higher reservoir of (Bio-Rad) vertical electrophoresis apparatus. Gels were set to pre-run for 15 minutes at 100V at room temperature. For loading of DNA samples, 1 μ l of 6X blue orange loading dye (Promega) was mixed with 5 μ l of PCR product and samples were loaded into the well.

2.11 Visualization of Gels

All types of gel which have been electrophoresed including non-denaturing acrylamide, agarose and metaphor gel were stained with ethidium bromide solution (0.5µg/ml) for 30 minutes at room temperature, rinsed in distilled water, and finally visualized under UV light using Gel Doc System (Bio-Rad). Gel pictures were documented using the same software. The band size from amplified PCR product were determined using Quantity One software (Bio-Rad)

2.12 Cloning of DNA insert

2.12.1 Purification of PCR product

Purification of PCR product was done using PCR purification kit (Qiagen). In general, the PCR product from replicate reactions were pooled together in order to produce a concentrate product for purification. Five volumes of PB buffer were mixed with one volume of PCR product. The mixture was then transferred into a Qiaquick Spin Column® placed in 2ml collection tube and centrifuged at 17900 x *g* for 1 minute at room temperature. Flow through was discarded and the column was washed with 0.75ml PE buffer which has been diluted with ethanol and spun for at 17900 x *g* for 1 minutes at room temperature. After the centrifugation step the flow through liquid was discarded. The column was placed into the same collection tube and to remove remaining wash solution, the tube was spun again at 17900 x *g* for 1 minute at room temperature. The column was placed into a clean 1.5ml microcentrifuge tube and DNA was eluted by adding 50µl of DNase/RNase free water to the column's membrane and the DNA was collected by a centrifugation at 17900 x *g* for 1 minute at room temperature.

Estimation on the concentration of the purified PCR product was made by running the purified PCR product alongside Lambda HindIII marker (Promega) as a standard on a 1% agarose gel (appendix 10). Qualitative measurement of DNA quantity from purified PCR product was done by comparing the intensity of electrophoresed purified amplicons band with several fragments of

lambda HindIII DNA band electrophoresed along with the purified PCR amplicons. Apart from the qualitative measurement, DNA amount was also determined using Nanodrop Spectrophotometer as described in section 2.7.

2.12.2 Cloning and ligation of DNA insert into cloning vector

PCR using AmpliTaq Gold enzyme generates a product with an additional 3' adenine (A) overhang on the amplicons. Therefore, cloning vector such as pGEM®-T easy (Promega) can be used as it incorporates a complementary 5' thymine (T) overhang which are compatible with PCR product amplified by AmpliTaq Gold polymerase enzyme. Ligation between amplified PCR product and pGEM-T easy cloning vector was done using the T4 DNA ligase enzyme. This vector was used to generate recombinant plasmids, which have an ampicillin resistance gene that can be used as a selection marker for screening bacteria containing the plasmid plus DNA insert. Ligation reactions were carried out using an optimized quantity ratio between insert DNA and cloning vector. For normal ligation between DNA insert and cloning vector, a ratio of 1:1 was used. It was crucial to make sure that the quantity ratio was accurate between both insert and cloning vector. Therefore, calculation was made using the formula below to determine the quantity of insert DNA and cloning vector used during ligation:

$$\text{Quantity of vector (ng)} \times \text{Size of insert (kb)} = \text{Quantity of insert (ng)}$$

Cloning Vector size (kb)

Ligation reaction was carried out in a 10µl reaction mixture which contained 5µl of 2X Rapid Ligation Buffer (Promega), 1µl of linearised pGEM®-T easy vector (50ng), 1µl of T4 DNA ligase, the calculated amount of insert DNA and nuclease free water added to a final volume of 10µl. Finally the reaction was mixed thoroughly using a pipette and the ligation reaction was incubated overnight at 15°C.

2.12.3 Transformation of *E. Coli*

Ligated vector-insert was transformed into a highly competent ($>10^8$ cfu/ μ g) JM109 cells (Promega). From -70°C storage, the competent cell was placed on ice until it was just thawed. Subsequently, 2 μ l of ligated vector-insert reaction (section 2.11.2) was carefully mixed with 50 μ l of competent cells then incubated it on ice for 20 minutes. The cells were then heat shocked in a water-bath at 42°C for 40-50 seconds to facilitate transformation with the ligated vector. Immediately, the tube was put on ice for 2 minutes. 950 μ l of Luria Broth (LB) Media (0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl) was added to the tube and it was incubated for 1.5 hours at 37°C with shaking (150 rpm). The short 1.5 hours incubation period allows the cells to express the antibiotic resistance gene and to produce protein related in order to make cells resistance.

After incubation cells were spun down at 9000 x g for 5 minutes. Eight hundred μ l of supernatant and cell were resuspended using remaining 200 μ l supernatant in the tube. The resuspended cells were split equally and pipetted on to two prepared media agar plates (each with 100 μ l containing 1.5% (w/v) agarose, 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 100 μ g/ml Ampicillin) and spread. Finally, the inverted plates were incubated overnight at 37°C (approximately 18 hours).

2.12.4 Bacterial colony selection

Overnight grown colonies on the media plate were picked and grown overnight in a universal bottle which contains 5ml Luria Broth media with 100 μ g/ml ampicillin or 50 μ g/ml kanamycin (depending on the type of antibiotic resistance conveyed by the plasmid vector) for a minimum of 8 hours at 37°C . Plasmid extraction was done on 3ml of grown cells as described in section 2.5.1 Glycerol stock was made from 800 μ l of the remaining cell suspension by adding 200 μ l of sterile (autoclaved) 50% (w/v) glycerol solution, mixing then storing at -80°C .

2.12.5 Confirmation of insert in purified plasmid

Extracted DNA plasmids from different colonies of overnight grown bacterial clones were tested to confirm for its availability of the cloned DNA insert with a restriction-digest method. The type of restriction enzyme used to digest the plasmid in order to release the cloned DNA insert depends on the type plasmid vector. For pGEMT-easy plasmid vector, EcoRI enzyme was used to release the cloned insert DNA.

2.13 Restriction endonuclease digest of DNA

Restriction endonuclease (RE) digest was used on purified plasmid DNA and PCR amplicons. The reaction mixture as described in Table 2.1 was prepared for a general digestion of plasmid vector to release the ligated DNA insert from the vector. Digestion mixture was for 1 hour at 37°C. The results from digestion of plasmid were determined by electrophoresing 5µl of digestion product on non-denaturing 1% (w/v) agarose gel as described in section 2.8.1.

Table 2.1 Formulation of a general restriction digest reaction

	Volume
Purified plasmid DNA (1µg)	10µl (Total 1µg of DNA)
Buffer H 10X*	2µl
Bovine Serum Albumin (BSA) 10µg/µl	0.2µl
EcoRI restriction enzyme*	1 µl
Nuclease free water	6.8µl
Final volume	20µl

*Restriction enzyme and compatible buffer used depends on the type of target DNA. Similar procedure was used for other types of plasmid/amplicons.

2.14 Development of a cloning vector

2.14.1 BovProt1/1 clone

The BovProt 1/1 clone was made available by (Raynaud et al., 2005) and served the purpose as a backbone vector to be developed as a cloning medium for the CA tandem repeat region. The clone was developed based on the now obsolete Green Fluorescence Protein (GFP) vector system from Clontech. The GFP reporter vector contains 5' 1xa promoter sequences from bovine calpastatin gene cloned into its multiple cloning sites. The cloned region also contains short CA tandem repeat sequences located at the 5' to exon 1xa CAST gene promoter sequences which are valuable for this study. BovProt1/1 promoter construct was intensively used in this study as the base vector for the cloning of amplified short CA tandem repeat sequences into the 1xa CAST promoter which was readily available inside the vector. It was also used to compare length of short CA tandem repeat from exon 1xa CAST promoter.

2.14.2 Site directed mutagenesis

In order to clone variable short CA tandem repeat region in BovProt1/1 vector, a minor base modification have to be made on the vector sequences. The minor modification was required to introduce a novel HindIII restriction site in BovProt1/1 vector sequences and this was performed on the vector using site directed mutagenesis. The experiment was carried out using Quikchange II™ site directed mutagenesis kit (Stratagene). Mutagenic primers with the size of 25-45bp and annealing temperature of $\geq 78^{\circ}\text{C}$ were design specifically with the desired mutation for the site directed mutagenesis experiment (Table 2.2). The forward primer was designed with the mutation site located in the middle of the primer sequences. The accompanying reverse primer was based on the reverse complementary sequence of the forward primer itself. Primers were purified using HPLC method to increase mutation efficiency.

Table 2.2 Primers designed for site directed mutagenesis experiment, highlighted nucleotides indicate those which were substituted

Primers	Sequence 5' to 3'	Length	Purification	T _m °C
SDM F	CGGGCCATCTGAGTTGGAAGC	40bp	HPLC	74.6*
CAST 1xa	TTCTCCTCCTAGTTAACTC			
SDM R	GAGTTAACTAGGAGGAGAAGC	40bp	HPLC	74.6*
CAST 1xa	TTCCAACCTCAGATGGCCCCG			

*Primer T_m was determined using Quikchange™ T_m calculator available from Stratagene website.

2.14.2.1 Mutant strand synthesis reaction

The newer mutant copy of BovProt1/1 plasmid was synthesized using the specially designed primer pair (Table 2.2). BovProtI plasmid at the amount of 50ng (original construct) was used as template in 5μl nuclease free water. Reaction mixture for the mutant strand synthesis reaction contains 1.25μl forward and reverse primers (100ng/μl), 1μl of dNTP, 5μl of 10X reaction buffer and 37μl of water to total up for 50μl reaction. A control reaction was done with the supplied 2μl of 10ng pWhitescript plasmid as template and 1.25μl forward and reverse control primers (100ng/μl). Amplification of the mutant strand was facilitated with 1μl (2.5u/μl) of *Pfu* Ultra High Fidelity DNA polymerase. Thermal cycler parameters for the amplification of mutant strand was set-up as on Table 2.3.

Table 2.3 Thermal cycler temperature used for the amplification of mutant strand to generate site specific variation in nucleotide sequence.

Temperature	Time	Cycles	Description
95°C	30s	1	Preheat
95°C	30s	*Repeat 16 times	Melting
55°C	1 minute		Annealing
68°C	*6 minutes		Extension
4°C	∞	1	Storage

*Extension times and cycles were determined by the length of construct and number of desired mutation point according to QuikchangeII™ manual.

Directly after the amplification step, 1µl of DpnI (10u/µl) restriction enzyme was added to each of the amplification reaction and incubated at 37°C for 1 hour to digest the original non-mutated parental copies of BovProt1/1 plasmid construct. The mutated plasmid construct was transformed into XL-1 Blue Supercompetent cells using the method as described in section 2.11.3. The tube containing transformed cells with mutated plasmid construct (BovProt1HindIII) was then added with 500µl of prepared NZY+ broth media (Appendix 9) and incubated at 37°C for 1 hour with shaking at 225rpm. This was followed by plating of the transformed cell suspension onto LB agar (0.5% yeast extract, 1% tryptone, 1% NaCl, 2% agar) with 50µg/ml Kanamycin overnight at 37°C as the base plasmid vector for BovProt1/1 contains Kanamycin resistance gene. Using 250µl of cell suspension duplicate plates were spread with the cells. The next day, single grown colony was picked from the plate using a sterile pipette tip and incubated overnight in a 5ml LB broth media containing 50µg/ml Kanamycin at 37°C with shaking at 225rpm. Finally, mutated plasmids were extracted as described in section 2.5.1. Plasmid which had been successfully altered by mutagenesis were identified by HindIII restriction digest.

2.15 Purification of DNA band from gel

The electrophoresed gel was placed on the transilluminator in order to visualize DNA bands. The expected size of DNA band was cut from the gel for extraction using Gen Elute Gel Extraction Kit (Sigma). Gel fragments were weighed and 3 gel volumes of gel solubilisation solution were added to the gel in a 2ml centrifuge tube. The mixture was then incubated at 55°C for 10 minutes until the gel is completely dissolved. The binding column was prepared by spinning it down with 500µl of column preparation solution at 12 000 x *g* for 1 minute at room temperature. One gel volume of 100% ethanol was added to the solubilised gel and it was mixed until homogenous. The mixture was added to the prepared binding column which was spun at 12 000 x *g* for 1 minute at room temperature and the flow-through was discarded from the collection tubes. To the binding column was added 700µl of wash solution and it was then spun at room temperature for 1 minute at 12 000 x *g*. Flow-through liquid was discarded after the centrifugation step. To remove excess ethanol the column was re-spin at room temperature for 1 minute at 12 000 x *g*. Finally, to elute DNA, 50µl of nuclease free water was added to the column membrane placed in a clean 1.5ml microcentrifuge tube. This was incubated for 1 minute followed by centrifugation at 12 000 x *g* for 1 minute at room temperature.

2.16 Measurement of gene expression

2.16.1 Extraction of total RNA from mammalian cell lines

Total RNA for RT PCR analysis was extracted from mammalian cells lines in culture using High Pure RNA isolation kit (Roche). Media was removed from cells which were then resuspended in 200µl of sterile filtered phosphate buffer saline (PBS) and frozen on dry ice. Four hundred µl of lysis buffer was added to the frozen content and then vortex for 15 seconds. Samples were transferred into a High Pure Filter Tube with collection tube and were spun at 8000 x *g* for 15 seconds at room temperature. After centrifugation the flow through liquid was discarded and the same filter tube was reused. A mixture of 90µl DNAase incubation buffer and 10µl DNAase I (10 000units) was pipetted into the upper reservoir of the filter and incubated for 15 minutes at room temperature. This was done to remove contamination of genomic DNA in extracted total RNA. Five hundred µl of wash Buffer I was added to the upper reservoir of the filter and it was then spun at 8000 x *g* for 15s at room temperature. Flow through liquid was discarded and 500µl Wash Buffer II was added to the filter which was spun at 8000 x *g* for 15 seconds at room temperature. Flow through liquid was discarded and finally 200µl of Wash Buffer II was added and filter tube was spun at 13000 x *g* for 2 minutes at room temperature. Collection tube was discarded and filter tube was then placed on a new clean and sterile 1.5mls micro centrifuge tube. For RNA elution, 50µl of elution buffer was added to the filter and it was spun at 8000 x *g* for 1 minute at room temperature. RNA was stored at -80°C for later analysis. Concentration of eluted RNA was measured as described in section 2.7. For the purpose of reverse transcription of mRNA, total extracted RNA was diluted to 100ng/µl. Integrity of extracted total RNA was checked by running 5µl of the diluted RNA with 1µl 6X blue orange loading dye (Promega) on 1% (w/v) non denaturing agarose gel.

2.16.2 TRIzol total RNA extraction method

Another alternative method to extract RNA used in this study was the TRIzol method. TRIzol reagent (Invitrogen) (1 ml) was added to each well and cells were scraped from the well surface transferred into a 1ml microcentrifuge tube frozen on dry ice and stored at -80°C until the day of extraction. For extraction 200 µl of chloroform was added per ml of thawed TRIzol and tubes were mixed by shaking for 15 seconds, followed by incubation for 3 minutes at room temperature and centrifugation at 12 000 x g for 15 minutes at 2-8°C. The upper layer colourless aqueous solution, which contains total RNA from extracted cells, was transferred into a sterile 1.5ml microcentrifuge tube and mixed with 500µl isopropanol per ml TRIzol. Tubes were incubated for 10 minutes at room temperature and then spun at 12 000 x g for 15 minutes at 2-8°C. Supernatant was discarded and the RNA pellet was washed with 1ml 75% (v/v) ethanol per ml TRIzol, vortexed and centrifuged at 7500 x g for 5 minutes at 2-8°C. Finally, supernatants were carefully removed and pellet was air dried followed by resuspension in 50µl of RNAase free water then stored in -80°C.

2.16.3 Reverse-Transcription (RT) of mRNA

Endogenous gene expression in cells can be calculated by quantifying the level of a specific mRNA in the extracted total RNA. This was done by reverse transcription of extracted total RNA into cDNA followed by PCR. For each reaction 10µl of RNA (1µg) was added into a microfuge tube containing 2µl (60µM) of Random Hexamer Primer (Roche) and molecular grade water (Sigma) to a final volume of 13µl. The template primer mixture was incubated for 10 minutes at 65°C to ensure denaturation of RNA secondary structure, followed by immediate cooling on ice for a few minutes, to allow primers to anneal. To this mixture, 4µl Transcriptor Reverse Transcriptase Reaction Buffer (1X; Roche), 0.5µl Protector RNase inhibitor (20 Units; Roche), 2µl Deoxynucleotide mix (1mM; Roche) and 0.5µl Transcriptor Reverse Transcriptase (10 units; Roche) were added before incubation in the thermocycler for 10 minutes at 25°C, 30 minutes at 55°C, 5 minutes at 85°C and finally cooled down to 4°C for an indefinite period. The final volume of 20ul first strand cDNA mixture was diluted 1:5 to 100µl with molecular grade water (Sigma) and stored as cDNA stock at -20°C.

2.16.4 Quantitative PCR

Before starting, the synthesized first strand cDNA was tested with primers that will target the house keeping gene of the sample source. Samples were measured in triplicates on a 384-well plate using volume and reaction as shown in (Table 2.4). To determine the specificity of the amplified PCR product, a melting curve analysis was done on all the samples from the raw data of this experiment. From the raw data, average value plus or minus two standard deviations was calculated. All samples falling within this range are considered acceptable to be pooled to be used as a standard curve for the quantitative RT PCR experiment. Any sample falling outside of this range was run but not used as part of the pool (appendix 12).

Table 2.4 Volume for reagents for quantitative PCR and thermal cycler parameters for LightCycler®480 Real-Time PCR System

Quantitative PCR LightCycler®480 System		
Reagents	Volume (µl)	Final Concentration (µM)
cDNA template	5	
Mastermix	7.5	
Forward primer	0.45	0.045
Reverse primer	0.45	0.045
Water	1.6	
Total Volume	15	
Cycling Conditions	Temperature °C	Duration (seconds)
Preincubation	95	300
Amplification	95	10
	60	15
	72	15
Repeated for 45 cycles		
Melting Curve	95	5
	65	60
	97	Continuous
Cooling	40	10

For quantitative PCR reaction, quantification of the relative level of a transcription was determined using a standard curve which was generated by pooling of cDNA generated from the same source. To generate a standard curve, serial dilutions were made from the pool of cDNA as shown in (Table 2.5). All cDNA samples were diluted to 1:10 for measuring gene to ensure samples were quantified against the linear phase of standard curve.

Table 2.4 summarized the volume of each reagent in one reaction of quantitative RT-PCR and mouse calpastatin primers were used in the reaction (appendix 6). Samples were measured in triplicates reaction in a 384-well plate. Using the Roche LightCycler®480 SW V 1.5 software, the standard curve was plotted from the raw data and samples were compared to this to generate calpastatin gene expression levels.

Table 2.5 Dilutions of pooled cDNA for generating a serial diluted standard curve

Standard Curve	
Dilution Factor	Relative Quantity
1	1
1:2	0.5
1:4	0.125
1:16	0.0625
1:32	0.03125
1:64	0.015625
1:128	0.007813
Dilution 1:2 (20µl standard curve +20 µl water)	

2.17 Cell culture method

The final experiment in this study involved in transfection of fluorescence based reporter vector construct made into compatible mammalian cell lines of varying types. To achieve this, two types of cell line were propagated in a controlled environment enriched with growth media in order for these cells to be viable for the transfection of the fluorescence based reporter vector construct. Two different cell lines were used in order to investigate the effect of different host cells on the transcriptional activity of the promoter. Experiment was carried out with 3 independent replicates for each type of promoter construct as well as for the treatment and control.

2.17.1 Propagation, quantification and seeding of cells

Frozen mammalian cell lines thawed at 37°C immediately after removed from cryostorage. The thawed contents was then transferred into a sterile T25 flask (Nunc) containing 7ml DMEM+Glutamax (GIBCO) growth media with supplemented 10% (v/v) fetal calf serum (FCS) and 1%(v/v) Penicillin-Streptomycin (GIBCO) at 37°C. The cell solutions were gently mixed and evenly dispersed in a T25 flask for the cells to adhere. These cells were left in

the 37°C incubator supplied with 5% (v/v) Carbon dioxide (CO₂). Media was changed every 48 hours to allow growth.

At 90% confluency the cells were passaged to ensure cells were maintained in the exponential growth phase. The media was removed and cells were gently washed with 10 ml of pre-warmed PBS and detached from the base of the T75 flask with 2 ml of Trypsin-EDTA (Gibco) with 5 minutes incubation at 37°C. In order to neutralize the Trypsin-EDTA enzyme, 8ml growth media at 37°C was transferred into the flask. Cell suspension was transferred into a universal tube and centrifuge for 4 minutes at 100 x *g* at room temperature. Supernatant was removed and the cell pellet was resuspended in a 10ml fresh growth media. 20µl of this cell suspension was transferred into chambers of haemocytometer to quantify number of cells whilst 1ml was transferred into a new T75 flask containing 14ml growth media for a ratio of 1:15. Cells were incubated in 37°C incubator supplied with 5% (v/v) CO₂. It was essential to quantify the as the appropriate cell density was essential for transfection. Required amount of cells were counted using the formula below:

$$\frac{\text{Desired cell number}}{\text{Total cell number}} \times \text{Total volume of cell suspension} = \text{Cells volume}$$

Prior to transfection, optimized number of cells for seeding in a 24 well plate was used to ensure that the seeded cells achieved 70-80% of confluence on the day of transfection. Each cell type was seeded in a 24 well plates with the cell density as shown in Table 2.6.

Table 2.6 Optimized density of seeded cells in each well of a 24 well cell culture plate*.

Cell Type	Cell Origins	Cells Density in each well (cells/well)
C2C12	Mouse (myoblast)	2×10^4
COS7	Monkey kidney (fibroblast)	4×10^4
HEK 293	Human embryonic kidney (epithelial)	5×10^4

*Number of cells seeded for 6 well plates were scaled up according to the surface area in order to have the same density of cells in each well.

C2C12 mouse myoblast cells were seeded at density of 4×10^4 for each well of a 6 well cell culture plate. Cells were incubated at 37°C incubator in 5% (v/v) CO₂. The following day, media was discarded from the well and were replaced with 3ml cell differentiation media for each well (DMEM+Glutamax, 2% horse serum and 1% Penicillin-Streptomycin) to induce differentiation of the cells.

2.17.2 Transient transfection of promoter construct

Cells transfection were done on cells lines seeded on 24 well plate on the day after cells seeding. The lipid base transfection reagent FuGENE HD (Roche) was used to transfect each plasmid vector into cell lines in triplicates well. To remove variability caused by varying transfection efficiency co-transfection with a control vector containing CMV promoters expressing DsRed fluorescence was carried out (pDsRed Express N-1). Each well was transfected with 25µl of transfection complex containing 200ng of plasmid DNA, 5ng of co-transfection vector (pDsRed Express N-1) and FuGENEHD transfection reagent at the ratio of 3:1 to transfected DNA. Before transfection, all the mixture from the transfection complex was added to a reduced serum media Opti-MEM I (Gibco) placed in a sterile 1.5mls tube. The tube contents were vigorously mixed by vortexing and incubated at room temperature for 30

minutes. The transfection complex solution was then added to each well and cells were incubated in a 37°C incubator supplied with 5% (v/v) CO₂ for 24-48 hour to allow cells transfection before the expression was observed from transfected promoter construct.

2.17.3 Treatment of transfected cells with anabolic agent

Treatment using anabolic agents on cell lines transfected with promoter construct was performed on the next day after transfection. Growth media was removed from each well of the 24 well cell culture plates and was replaced with a 500µl fresh growth media containing anabolic agents at a final concentration of each of the agents as indicated in Table 2.7:

Table 2.7 The final concentration of agents in prepared cell growth media

Anabolic Agents	Dibutyryl cAMP	Forskolin	Calcium Ionophore
Final concentration	2mM	10µM	1µM
Solute	Water	DMSO	DMSO
Molecular weight	491.4	410.5	523.6
Initial stock concentration	1M	10mM	1mM

Treatments of promoter construct transfected cells with agent were performed on triplicates well.

2.18 Quantification of promoter expression

Promoter activity from transfected CAST promoter was measured 48 hours after transfection as fluorescence initially using FluoSTAR OPTIMA plate reader (BMG Labtech) and later was replaced with more sensitive fluorescence based detection equipment Typhoon Trio (GE Healthcare).

2.18.1 FluoSTAR OPTIMA plate reader

Cell growth media was removed from each well of the 24 well plates. Cells were scraped from the bottom of the well using sterile tips and were resuspended with 100µl of PBS solution. The cells suspension was transferred into a 24 well non-transparent white plate and fluorescence was measured using FluoSTAR Optima fluorescence plate reader. Separate fluorescence reading were performed using the plate reader, firstly on the main transfected reporter promoter of GFP or ZsGreen fluorescence expression followed by fluorescence reading from the co-transfection marker, pDsRed Express N-1. Table 2.8 summarised excitation and emission filter used for detecting fluorescence.

Table 2.8 Fluorescence filters used in FluoSTAR Optima plate reader

Filter	Detected fluorescence	Excitation (nm)	Emission (nm)	Gain setting
1	GFP/ZsGreen	485	520	1600/800
2	DsRed	520	584	2000

2.18.2 Typhoon Trio

Fluorescence expression from transfected promoter construct was measured using Typhoon Trio Fluorescence Imager (appendix 13), 48 hours after transfection. The whole 24 well plate containing living transfected cells was inserted into the exposure cassette and scanned using two available fluorescence filter in order to detect ZsGreen based promoter expression vector and the co-transfected marker pDsRed Express N-1 (appendix 14). Two types of filters with differing excitation and emission values were used to detect fluorescence signal from both ZsGreen and DsRed fluorescence Table 2.9. The duration of time taken to scan the cell culture plates depends on the size of the scanning area and the pixel size chosen for the final output of the scan. In normal scan, it will take 90 minutes to scan six 24 well plates using 100 microns for the pixel size setting.

Table 2.9 Fluorescence filters used in Typhoon Trio fluorescence imager

Filter	Detected fluorescence	Excitation (nm)	Emission (nm)	Sensitivity setting
1	ZsGreen	488	520	Medium
2	DsRed	532	580	Medium

Raw data from the fluorescence scanning were analysed using ImageQuant TL software and the relative fluorescence value was calculated by division of ZsGreen by dsRed Express N-1 expression. Expression was expressed normalized to the average value of positive control (pZsGreen 1-N1) expression (100%). The formula for determining relative fluorescence value was shown as below:

$$\frac{\text{ZsGreen raw value}}{\text{DsRed Express N-1 raw value}} = \text{Corrected value}$$

$$\text{Relative fluorescence value} = \frac{\text{Corrected value}}{\text{Average of corrected ZsGreen positive control value}} \times 100$$

2.19 Statistical analysis

Data produced from the transfection experiments involving effect of co-transfection of pDsRed vector were analysed by one-way ANOVA using SPSS 19 software. Analysis of promoter activity from bovine CAST promoter deletion construct was done using two-way ANOVA ($P < 0.05$) followed by post hoc Dunnett's test and to determine promoter deletion construct which have significant increase in its activity compared to the control vector and Bonferroni's test. For analysis of promoter response from anabolic agent treatment, data were analysed by paired T-tests. For forskolin dose response experiment on CAST promoter 1xb, one-way ANOVA ($P < 0.05$) was used followed by Dunnett's test to determine significance relative to the control. Data accumulated from promoter expression in the variable CA repeat length CAST promoter construct were analysed using one-way ANOVA ($P < 0.05$) followed by Dunnett's test to determine significance relative to the control. For data generated by quantitative RT-PCR experiment gene expression was analysed using either one-way or two-way ANOVA.

Chapter 3: Variation in CA repeat Length of Calpastatin Promoter

3.1 Introduction

Calpastatin is the specific endogenous inhibitor to calpain. The protein is translated from a single gene (CAST) in the genomic DNA which contains 35 exons that are distributed over 130kb on bovine genomic DNA (Raynaud et al., 2005). Although originating from a single gene, the protein is expressed as four isoforms found in various bovine tissues. Variants of calpastatin are generated by transcription being initiated at different sites in the gene or by splicing of different exons during transcription (Lee et al., 1992).

Calpastatin isoforms are produced by differential transcription from the four different promoters (Raynaud et al., 2005). The four functional promoters are located 5' to exon 1xa, 1xb, 1u and lastly exon 14t which are responsible for initiating the transcription of mRNAs encoding Type I, Type II, Type III and Type IV calpastatin (Figure 3.1). Type IV is found to have a tissue specific expression where it is only expressed in testis (Takano et al., 2000). Whilst in other tissues, such as skeletal muscle Type I, II and III are expressed and are the focus of gene promoter studies in relationship to impacts on meat tenderness. Alongside a number of transcription factor binding sites that have been identified as influencing gene expression in various species (Cong et al., 1998a; Parr et al., 2004). Other motifs or promoter element such as short CA tandem repeat in these promoter region are also considered to be influencing the expression of CAST. In addition there are various post-transcriptional mechanisms which regulate gene expression which include alternative splicing of transcripts originating from the 3 principle promoters. There are also three sites in the XL domain of bovine calpastatin where it was demonstrated that phosphorylation could be performed *in vitro* (Cong et al., 1998b) indicating post-translational regulation.

There is a possibility that the transcription of all the these transcripts are driven from only a single point at the extreme 5' end of the gene associated with the promoter 5' to exon 1xa and all other transcript variants are a result

of alternative splicing. Variations in exon 1xa promoter sequence, associated with polymorphisms in the CA repeat, had been reported by Nonneman et al. (1999) and it was suggested that variations in the length of this sequence could act as potential candidate marker in determining meat quality. Therefore the overall aim of this study was to investigate and identify genotype variation in CAST promoter region in influencing expression of calpastatin.

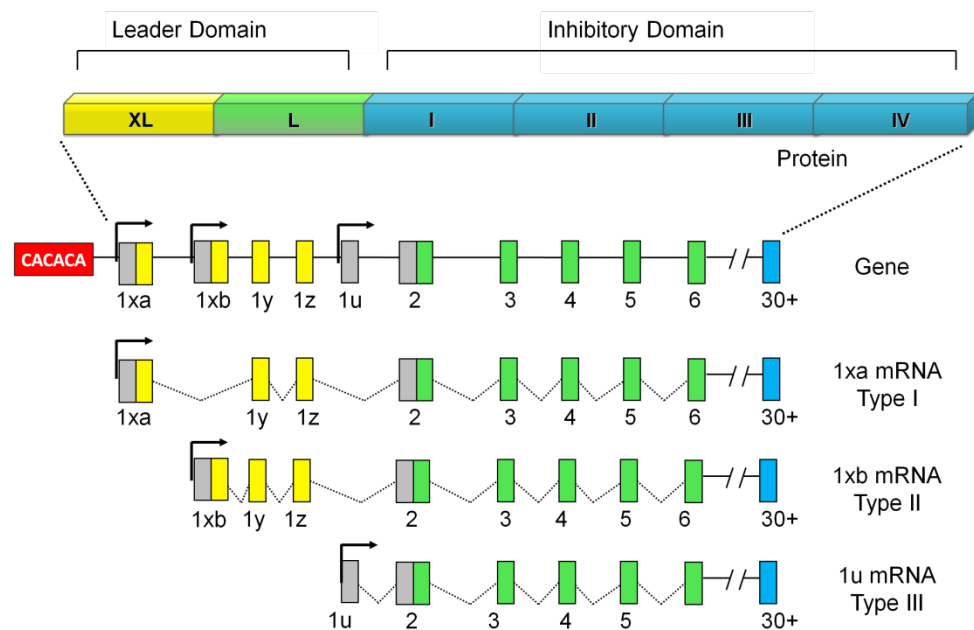


Figure 3.1 The structure of bovine calpastatin gene and the three types of CAST mRNA transcript produced from three different promoters located at the 5' end of the gene. Each promoter is responsible for the transcription of Type I, Type II, Type III variants of calpastatin isoforms.

3.2 Characterization of the short CA tandem repeat in the bovine 1xa promoter

At the time when this study started, there was a lack of knowledge on the properties of the promoter region 5' to exon 1xa of CAST gene Figure (3.1). This region might be important as it could regulate transcriptional activity of the gene eventually affecting the level of the calpastatin protein. Nonneman et al. (1999) described that the region which contained polymorphisms of cytosine-adenine (CA) repeat was identified in bovine population, and showed breed associated inheritance. This CA tandem repeat is located at approximately 400 base pair, 5' to exon 1xa. The reported microsatellite was considered to be very highly informative and could be linked to quantitative trait loci involved in meat quality and muscle growth. Sequencing of this region unveiled 67bp short CA tandem repeat region located 410bp before the transcriptional start site of 1xa promoter (accession number: NW_001495281). Based on the limited information regarding the area, in the study described in this thesis an initial experiment was set up to characterize the length of the short CA tandem repeat region in different animals using PCR. The ultimate objective was to distinguish differences of length in the tandem CA repeat sequences from bovine meat samples of varying quality and determine whether there was an association with meat tenderness.

3.3 Variations in short CA tandem repeat sequence repeat length using PCR based techniques

The main objective of this part of the study was to establish a reliable working PCR-based method which will allow identification of the CA-tandem repeat located in the promoter region of exon 1xa and establish variations in its length. Once the amplification technique was been established, further experiments were to be carried out to clone the sequence length variants region into a suitable cloning vector so that ultimately their influence or gene expression could be determined.

The sequence for bovine CAST gene was available in the NCBI database. This gene sequence contained the short CA tandem repeat region located 410bp

5' to the transcriptional start site for Type I CAST (Figure 3.2). This sequence (accession number: NW_001495281), published in 2008, was an early version of bovine CAST gene sequence which was subsequently strengthened by further sequencing and annotation of the bovine genome. The locus for the sequence and definition are shown in details in (Figure 3.2). The primary objective in obtaining this sequence was design suitable primers to amplify varying length of short CA repeat sequence in bovine CAST gene.

LOCUS	NW_001495281	3121 bp	DNA	linear	CON 29-JUL-2008
DEFINITION	Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.				
ACCESSION	NW_001495281 REGION: 770041..773161				
VERSION	NW_001495281.2 GI:194668375				
KEYWORDS	WGS.				
SOURCE	Bos taurus (cattle)				

1021	tgccatggca	gggttaacag	gttttcttct	gtcagatctt	cacaaactgg	cagcaaccac
1081	aggcagacaa	tcactgcaga	ggaggagcct	tttctagtct	ggcttataac	atttcacttc
1141	aagcttggtta	taattcacag	ccccaaacca	ggaagtgtct	tttaagatga	accaaagaca
1201	aaagcaatct	ggcctcctcg	caaacgaaat	tcaacagcct	cctgaaaggc	aatgggggttt
1261	aaatttagga	ctgttcttcg	gttttgattg	gaaccaaga	cttgttccac	atgaaacatc
1321	attgcacttt	caaacaagt	aaagccgcac	aaaacacacc	caggcccgtg	tgtgta caca
1381	cacacacaca	cacacacaca	cacacacaca	cacacacaca	cacaatcaca	acacacggag
1441	tcacacacag	accaatttgt	ttctgcaatc	cgcttctctca	tccagagggt	ccaggccccg
1501	ggccatctga	gttggtaaat	tctcctccta	gttaactcag	agcagattgc	agaaatgctg
1561	tctaattctt	gagattctta	gggagtgggg	cttgagatgt	agacggggcc	acgccccggt
1621	tccgctctcc	ttgcacaact	gcaagccaag	tctaggcaag	tcggggggaa	aaccccccca
1681	tctccagccc	ctcctctctg	cgaccactg	gggcaccaag	gcgacctcgg	gtgggggtggg
1741	gtgtccctgg	gagaagatac	gggaccagg	gtgtgagttg	caaacaggga	gccccgggct
1801	gctgccgccc	gggcgctgcc	aactgcaggc	aggaagggga	gggccctgcc	cggcgaaggg
1861	gagctctcgc	gggtcggggc	tggttcggaa	aagctgcctc	acaggcgcgc	ccgccagccc
1921	ctccgcgccc	tcgctccctc	ccagcgctcc	ccggctccag	cctcccttcc	aggctccgccc
1981	gcgcccgcgc	ggaggcagcg	ctcgcaaccg	cctcgccatg	tcccagccgg	gcccgaagcc
2041	cgccgcctcc	ccgcggcccc	ggcgcgctgc	ccgccacacc	caggag gtga	gtggcgctcc
2101	taccgccggg	gttgagcgcg	gggaggatct	cggggccccc	cgaagcctcg	ggtccacctt
2161	cagaggaggg	ttagccttcc	tggccttctg	tccctggggc	ccacccctcc	caggccccga
2221	gaaacatctg	gagagagacg	ggtgtggact	cagaacctgc	ctctgggcag	gtgaccggag
2281	gggcccgcgg	atggccctgg	gtcccggcca	agttcactgg	gcgcggcgca	agccacattg

Figure 3.2 Bovine calpastatin exon 1xa promoter sequences retrieved from NCBI websites. Bolded red indicates the short CA tandem repeat region which span across 67bp in length. Bolded blue indicates sequences of exon 1xa from calpastatin gene.

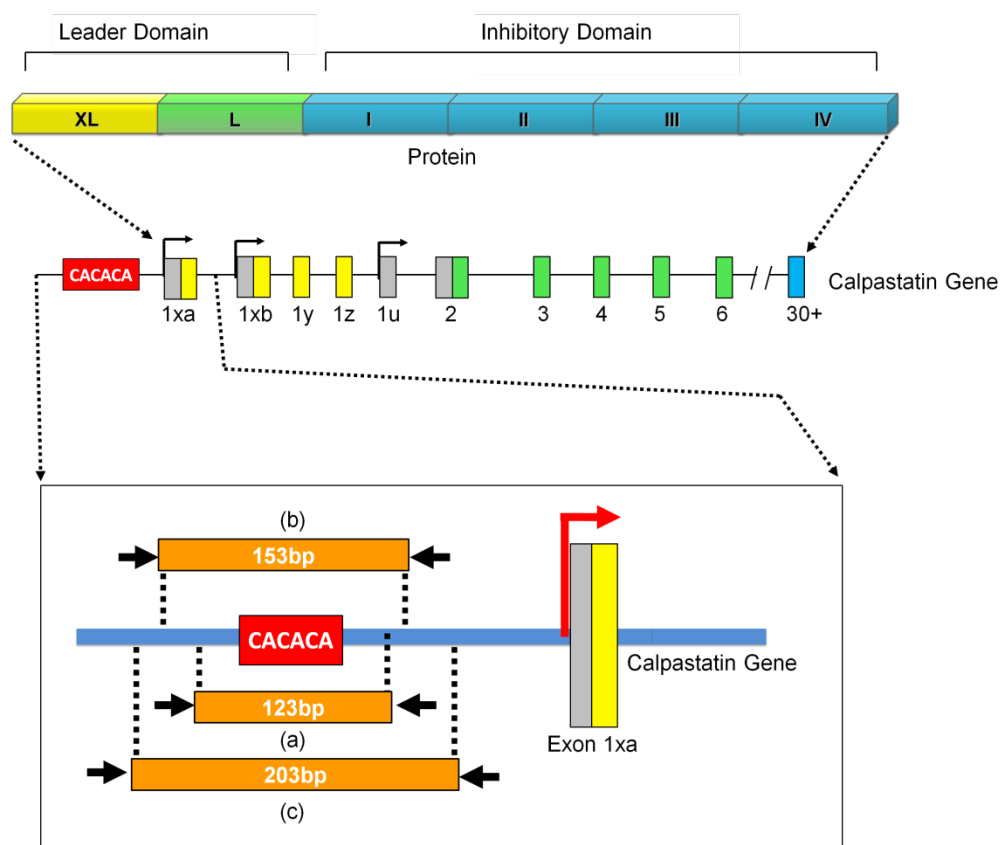


Figure 3.3 Amplification of the short CA tandem repeat region in the promoter sequences of 1xa calpastatin gene. Amplicon of 123bp, 153bp and 203bp length containing the CA tandem repeats were amplified using three different primer pairs (a) 1xaCASTtand1 (b) 1xaCASTtand2 and (c) 1xaCASTtand3.

The first three primers were designed to span the CA repeat region (Appendix 1, 2 & 3). The size of the CA repeat region was in the range of 70bp (however this length varied due to suggested polymorphisms in this region). The different positions of the primers spanning this region were predicted to give a three differing amplicon sizes, 123bp, 153bp and 203bp (Figure 3.3). The aim was to develop the most suitable primer sets that reliably generated amplicons across CA repeat region. Amplicons generated from the short CA tandem repeat region from a template of a bovine genomic DNA BAC clone (7N18) containing CAST gene (section 2.2) or bovine genomic DNA isolated from Holstein-Limousin cross was shown to produce multiple bands when it was run on a native 1% agarose gel (Figure 3.4).

Furthermore, these results on gel electrophoresis suggest that the primers bind to the correct target as the amplicon's size correspond to the theoretical size for amplicons generated calpastatin gene sequence as indicated in Figure 3.4. However, as can be seen in Figure 3.4 multiple bands were observed with the lowest band being the predicted size product whilst there were associated bands seen as multiple bands above the “correct” size band, often appearing as a very indiscrete form of fuzzy band. These results were observed from all the utilised primers. Gel resolution was poor due to the properties of the non denaturing gel and the low concentration of agarose use in this experiment.

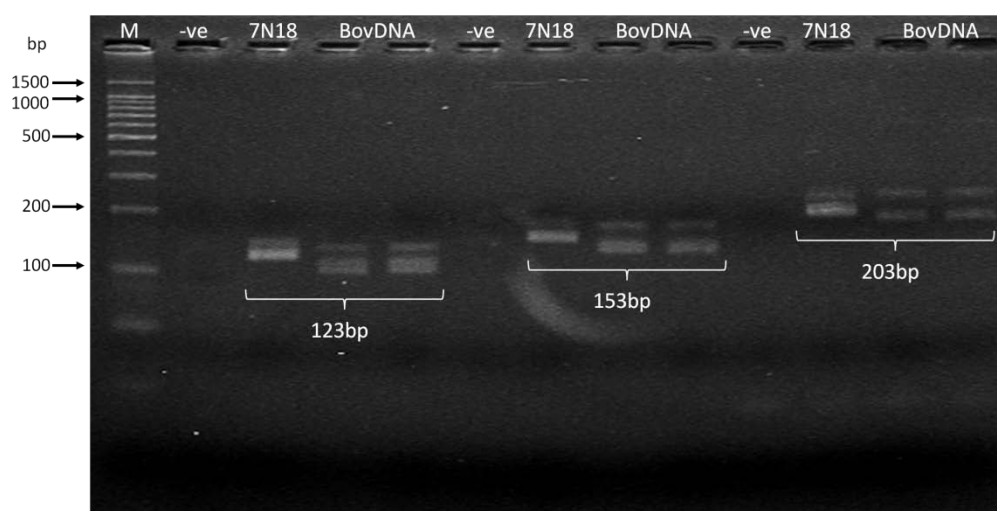


Figure 3.4 Various amplicon length generated across short CA tandem repeat region in the 5' exon 1xa region of CAST from bovine gDNA (two independent samples) and and bovine BAC clone containing the CAST gene. Electrophoresis of amplicons on non-denaturing gel with 1% (w/v) agarose. Bovine BAC clone containing calpastatin gDNA (7N18), bovine genomic DNA (BovDNA).100bp markers are indicated (M) and negative control water as (-ve).Theoretical size for the amplicons were shown as 123bp, 153bp and 203bp.

Given that the three templates used for the PCR originated from different animals it was proposed that short CA tandem repeat sequence in bovine CAST 5' exon 1xa region varied in length between animals. As can be seen in Figure 3.4 the lowest band in each lane clearly shows obvious size differences when comparing amplicons originating from the BAC clone are compared to those amplicons produced from the two bovine genomic DNA samples (originating from two Holstein-Limousin cattle). This amplicon size differential was maintained even when the size of the amplicons was increased through the selection of primers that spanned a longer region containing the CA repeat. Given the heterogeneous nature of the bands seen, further investigations were then carried out to determine whether the variation in size of amplicons originating from different template sources was affected by gel types and concentrations or whether the differing sizes produced were in fact because of the variation in the length of the CA tandem repeat.

3.4 Analysis of short CA tandem repeat sequence in the promoter region of CAST exon

3.4.1 Effect of increasing gel concentration on DNA band migration

PCR amplicons of CAST 5' exon 1xa CA repeat region both from bovine BAC clones (7N18) and bovine genomic DNA give multiple apparent amplicons which appeared with a smeared band characteristic on native non-denaturing agarose gel. It was thought that the smeared band was possibly caused by the properties of the agarose gel. In theory, the resolution of nucleic acid separation can be increase in the non-denaturing agarose gel by increasing the concentration of agarose. For smaller DNA fragments with size in the range of 0.1-0.3kb, a high concentration of agarose gel is necessary to provide more reslution in terms of nucleic acid migration. Therefore, in this experiment, gel concentration was tested as a parameter to increase gel resolution and clarity of the amplicons band by increasing the concentration of agarose.

In this experiment, a single primer was used (1xaCASTtand1) on BAC clones (7N18) template appendix 1. In this experiment the same PCR product was tested on all the gels of differing agarose concentration. The amplicons from PCR of BAC clone (7N18) using primer set 1xaCASTtand1 (predicted size of 123bp (Figure 3.3)) were electrophoresed on native non-denaturing agarose gel with differing concentration from 1%, 1.5%, and 2% (w/v) (Figure 3.5). Separation and clarity of DNA band in non-denaturing agarose gel was shown to be significantly enhanced when the concentration of gel was increased from 1% to 1.5% (w/v). The trend continues when a 2% agarose gel was used for electrophoresis of the amplicons.

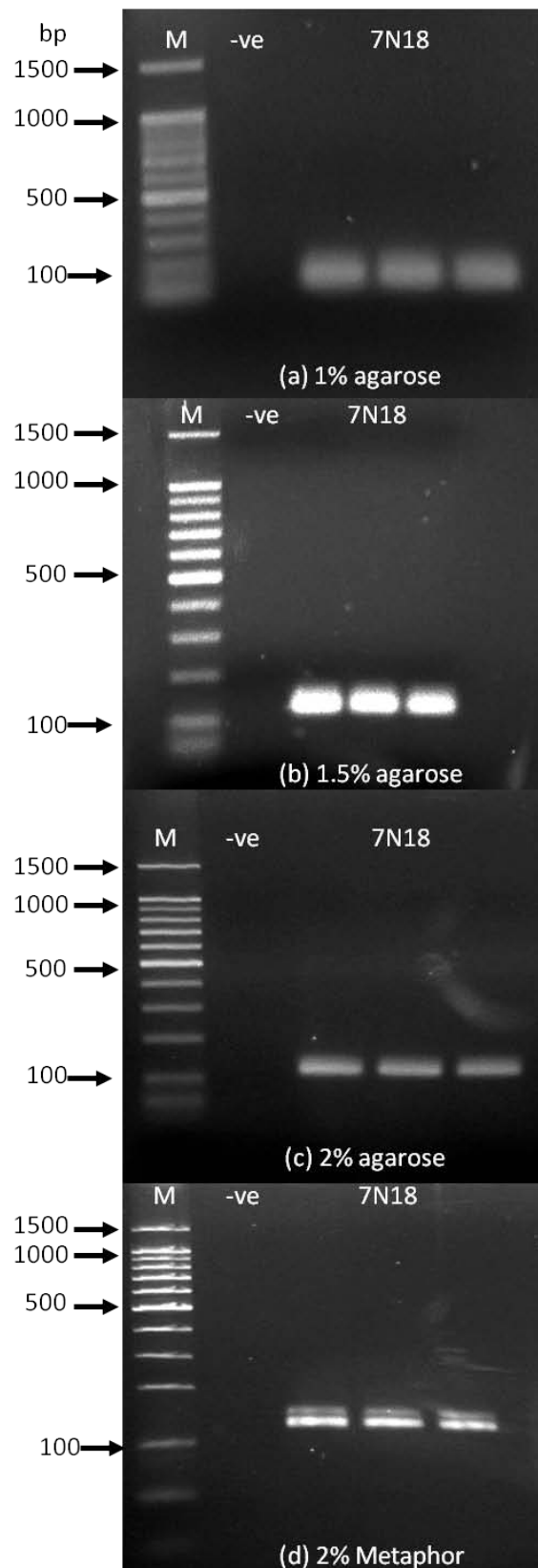


Figure 3.5 Migration of CA repeat amplicons generated from bovine calpastatin gene 1xa promoter in agarose gel with differing concentration. 123bp short CA tandem repeat amplicons from calpastatin gene in bovine BAC clones (7N18) was electrophoresed with (M) 100bp marker, and water (-ve) as negative control on (a) 1% (w/v) agarose, (b) 1.5% (w/v) agarose, (c) 2% (w/v) agarose and (d) 2% (w/v) Metaphor agarose.

The pace of DNA band migration was reduced when using a much higher concentration of agarose gel. At 2% agarose gel concentration, clarity and resolution of the gel was vastly improved compared to the original concentration of 1% (w/v). The amplicons band of 123bp in size appeared to be much more clearer on the 2% (w/v) agarose gel as well as the clarity of DNA fragments migrating from 100bp DNA ladder. In order to confirm the results, a new and improved agarose MetaPhor agarose (Lonza) was used as it have twice the resolution capabilities of normal agarose as claimed by the manufacturer. Gel comparison was made between amplicons electrophoresed on normal 2% (w/v) agarose and 2% (w/v) MetaPhor agarose (Figure 3.5). Metaphor agarose was shown to have a better resolving capabilities compared to the normal agarose. A single and thick band that appeared on the normal 2% (w/v) agarose was represented as a double individual band when electrophoresed on a 2% (w/v) MetaPhor agarose. It was concluded that native agarose gel might have contributed to the effect of smearing and lower resolution observed in Figure 3.4. The increased of agarose gel concentration to 2% (w/v) managed to vastly improve the clarity and gel resolution. However, when compared to Metaphor agarose, the resolving capability of normal agarose gel was still low. As the aims of the study was to develop a method of differentiating between variable sizes of CA repeat in different cattle, a more reliable and cost effective gel system was required. The inclusion of Metaphor agarose was a starting point in offering a more effective viewing medium for migration of amplicons. However, the cost of using Metaphor agarose was high therefore more cost effective gel system need to be considered in the method development. Therefore the study proceeded to investigate another alternative reliable and cost-saving method to determine short CA tandem repeat amplicons sizes.

3.4.2 Effect of differing gels system on DNA band migration

The use of non-denaturing agarose gel in lower concentration proved to be less effective for resolving PCR products with small size differences. To increase visibility and clarity on amplified region, an alternative gel system was considered. Non-denaturing acrylamide gel is known to be effective in giving better separation in nucleic acid migration than agarose (Savov et al., 1992). The resolving power of acrylamide is excellent as it can separate molecules of DNA with length differing by only 0.1% (Sambrook and Russell, 2001). Therefore, in this experiment a comparison of amplicon's migration was made between three types of non-denaturing gel systems, agarose, MetaPhor (agarose) and acrylamide.

Amplicons were synthesized from BAC clone (7N18) containing the promoter region 5' to exon 1xa using 1xaCASTand3 primers with theoretical product size of 203bp (appendix 3). These amplicons were then subjected to electrophoresis on the three types of gel system (Figure 3.6). The use of agarose and Metaphor at a high concentration, 2% and 3% (w/v) respectively gave a rather diffuse single band whilst 7.5% (w/v) acrylamide gel gave a pattern of five bands within a single lane, each band being sharply resolved (Figure 3.6). The result indicates the effectiveness of non-denaturing acrylamide gel in nucleic acid separation and resolution compared to MetaPhor and agarose. The explanation for the increased resolution is acrylamide gel is able to form a narrower mesh network compared to normal agarose and metaphor agarose which are built from more porous matrix, hence the former has a higher efficiency in separating smaller size of DNA fragments. Furthermore additional bands with size around 500bp was observed in acrylamide gel and this probably caused by non-specific amplification during PCR. Therefore, it was then decided that non-denaturing acrylamide gel at 7.5% concentration was being recognized as a proper and suitable medium for viewing the output from amplification across short CA tandem repeat region in CAST gene 5' to exon 1xa in the future experiment.

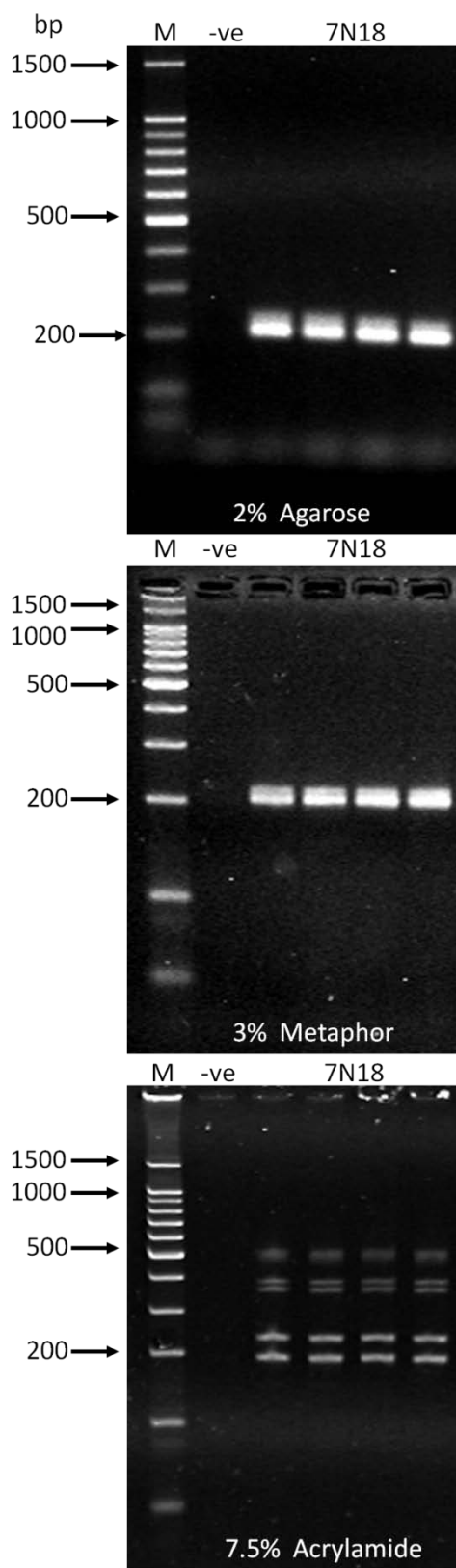


Figure 3.6 Effect of differing gel systems on migration 203bp CA repeat amplicons generated from bovine calpastatin gene 1xa promoter. Migration of 203bp short CA tandem repeat amplicons from calpastatin gene generated from bovine BAC clones (7N18) (in replicate) with (M) 100bp DNA marker and (-ve) as negative control on differing gel system; agarose, metaphor and acrylamide as indicated. Amplicons were synthesized using 1xaCASTand3 oligonucleotide (Appendix 3).

3.4.3 Effect of denaturing of PCR product on DNA band migration

An alternative method to non-denaturing acrylamide gel electrophoresis is the use of a denaturing system. Therefore, attempts were made to develop a method in this study using a denaturing gel system, which includes the use of urea in acrylamide gel combined with the use of formamide in the sample loading dye. However, it was difficult to obtain nucleic acid band separation using this method. The amplicons on the gel image were significantly smeared to the extent that it was not possible to distinguish amplicons size differences from the short CA tandem repeat region (these experiments are not included in this thesis). For these reasons, the denaturing method using denaturing urea polyacrylamide gel with the combination of denaturing formamide loading dye was not used in the future experiment. However to determine whether the separation of the amplicon could be improved by denaturing the DNA sample rather than running the sample through a denaturing gel, an experiment was set up to study the effect of nucleic acid band migration in non-denaturing acrylamide gel using amplicons that had been denatured using formamide loading dye. The use of such denaturing agent releases the hydrogen bond in single-stranded DNA (Weber and Osborn, 1969). In this experiment, formamide loading dye was used as denaturing agent coupled with the heating of amplicons at 95°C. Amplicons were synthesized from BAC clone (7N18) containing the promoter region 5' to exon 1xa using 1xaCASTtand3 primers (appendix 3). As can be seen in Figure 3.7, when changes were made in loading dye buffer composition combined with the denaturation of PCR amplicon, there was apparent distortion and retarded migration of nucleic acid in non-denaturing acrylamide gel when compared to amplicons that were electrophoresed using non denaturing dye. Formamide containing loading dye also appeared to affect migration of 100bp DNA marker as when it was added to the marker sample there was disruption of the bands (Figure 3.7), when compared to the non-denaturing sample preparation. In conclusion, there was no significant difference in terms of nucleic acid separation when amplicons were

denatured using formamide loading dye as size differences in amplified PCR product appear to be more difficult to assess as DNA bands and marker became distorted in appearance. As one of the objectives of this project was to distinguish size differences between short CA tandem repeat amplicons in different individual cattle it appeared, from these experiments, that subsequent experiments should be carried out using a non-denaturing loading dye for the separation of the PCR product amplified from the short CA tandem repeat region.

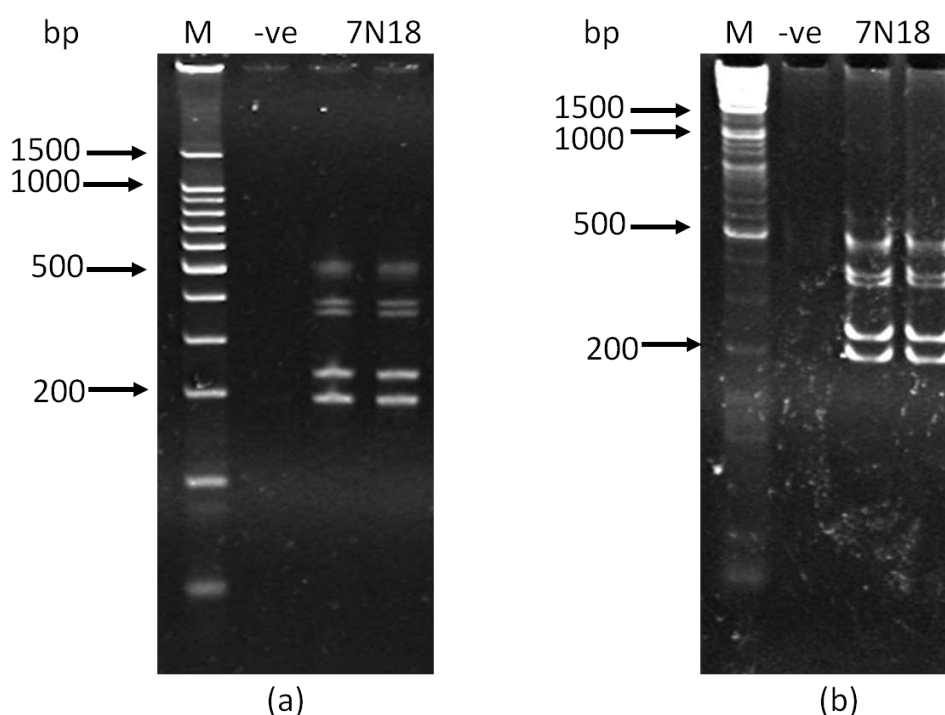


Figure 3.7 The effect of in differing loading dye mixture on the migration of 203bp size 1xa CAST tandem repeat amplicons migration in 7.5% (w/v) acrylamide gel. (a) PCR amplicons have been mix with Promega 6X Blue/Orange loading dye, (non denaturing treatment of the amplicon). (b) PCR amplicons were added to Formamide loading dye and denatured with heat at 95°C (denaturing treatment of the amplicon).

3.5 Approaches to enhance PCR specificity of the calpastatin promoter CA tandem repeat region.

As described above amplicons generated across the CA repeat region in CAST gene promoter associated with exon 1xa with relatively smaller base pair size gave multiple unspecific products when visualised on non-denaturing as well as denaturing electrophoresis (Figure 3.7). It was hypothesised that the short length of the amplicon was the reason why the amplicons are producing these results. Primer designed with a smaller predicted size amplicons will possibly have a tendency for non specific binding in other sequences of the genomic DNA during PCR as the templates contains repetitive CA tandem repeat sequences (appendix 3) which might cause slippage during PCR amplification (Murray et al., 1993). Therefore, it was propose that designing a new primer set with larger predicted size amplicons would reduce the chances of having a mismatch during primer annealing as a result of matching sequences from the repeating CA tandem repeat sequences.

In order to investigate the problem, primers were designed to generate longer amplicons across the CA repeat region (Figure 3.8). The objective in this experiment was to study whether increasing the distance between the primers (the amplicon length produced) on the short CA tandem repeat area would reduce the appearance of PCR artifacts on the gel. Primer sets of RE1xa and 555bp 5'1xa were designed to amplify longer amplicons of 295bp and 555bp respectively across the short CA tandem repeat region associated with exon 1xa promoter region (appendix 4 & 5). Both bovine BAC clone (7N18) and bovine genomic DNA were used as a template in this experiment to identify whether the problem only arises in either BAC clone or cattle genomic DNA. It was assumed that there might be a difference when performing PCR on these two sources of DNA as BAC clone contains homogenous set of DNA whilst cattle genomic DNA contains heterogenous set of DNA which will theoretically yield more nonspecific amplicons.

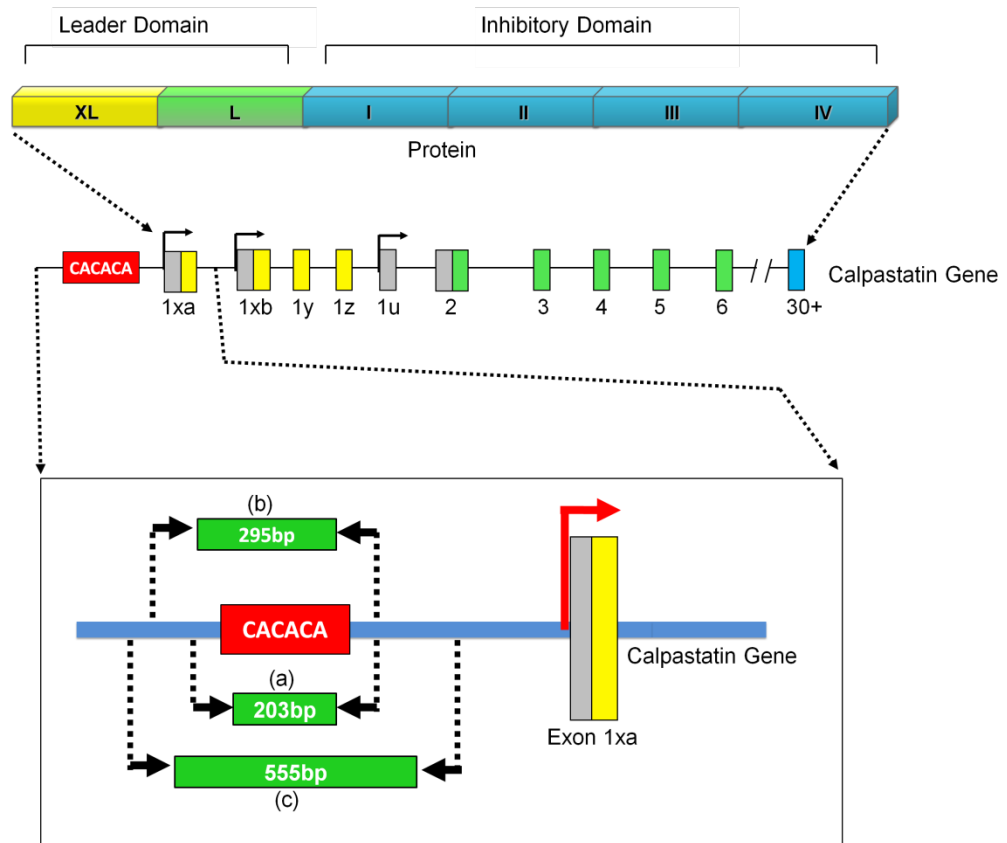


Figure 3.8 Longer amplification region of CA tandem repeat sequences from bovine CAST exon 1xa promoter. (a) 203bp amplicons generated from 1xaCASTand3 primer set (b) 292bp amplicons generated from RE1xa primer set (c) 555bp generated from 555bp 5'1xa primer set

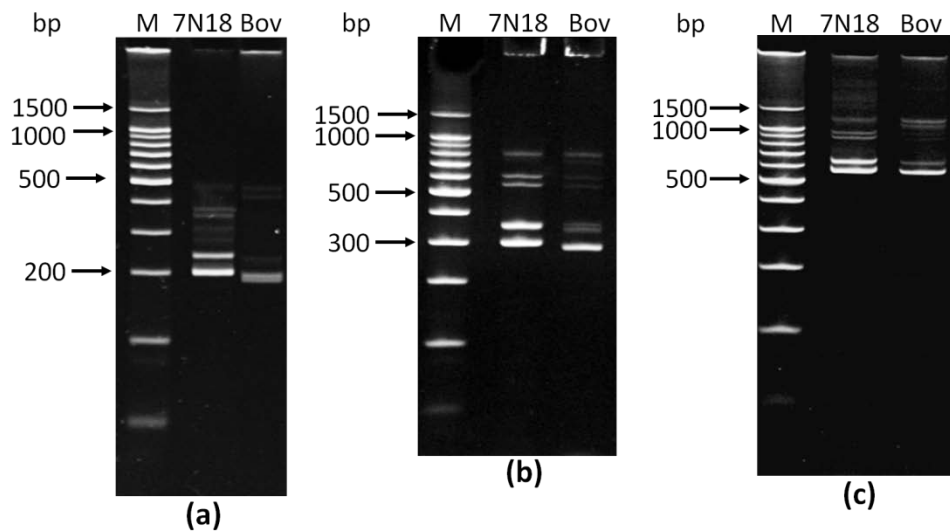


Figure 3.9 Amplicons generated with varying length on non denaturing acrylamide gel. Generation of differing amplicon sizes across the calpastatin gene promoter CA tandem repeat from bovine BAC clone containing calpastatin promoter (7N18) or bovine genomic DNA (Bov), 100bp markers are indicated (M). (a) 203bp product (b) 295bp product (c) 555bp product

Generated PCR amplicons with the predicted length of 203, 295 and 555bp were electrophoresed on 7.5% (w/v) non-denaturing acrylamide gel (Figure 3.9). Additional multiple bands were shown with all the amplicons. However, the apparent size of the band which migrated with the highest motility on the gel was similar to the theoretical amplicons length predicted (203bp, 295bp and 555bp) from the database sequence (accession no: NW_001495281). Given this observation, it was assumed that the primer pairs had annealed to the correct target in the sequence 5' and 3' relative to the short tandem CA repeat region but, during amplification, non-specific DNA fragments were produced. It was initially hypothesized that PCR artifacts were caused by smaller size of PCR product covering the short CA tandem repeat region.

The short CA tandem repeat in CAST is located at the 5' untranscribed region of the gene where it is rich with GC content (~60%). Amplification from region with high GC content usually results in multiple bands as secondary intramolecular structures were formed during PCR and amplification becomes unspecific (Frey et al., 2008). PCR Amplification efficiency was also found to be reduced in smaller size amplicons which have higher GC content (McDowell et al., 1998). Therefore it was hypothesised that by generating a longer amplicon containing the short CA tandem repeat region, the proportion that this region contributed to the overall length would be reduced thereby its predicted effect in preventing amplification of the correct target in the CAST gene sequence would be reduced. The use of PCR primers predicted to give 295bp and 555bp products still produced PCR artifacts when electrophoresed on non-denaturing polyacrylamide gel. It was not known whether the additional multiple bands amplified during PCR all contained the CA tandem repeat sequence from the CAST gene promoter region.

In conclusion, the amplification of longer PCR product, up to 500bp, spanning the short CA tandem repeat region within the promoter region of the calpastatin gene 1xa exon failed to produce products that appeared as a single band when observed on the non-denaturing acrylamide gel. As a result of these findings an experiment was carried out to investigate whether multiple bands produced from PCR that was predicted to give 203bp amplicons contained the short CA tandem repeat region from the CAST gene using nested PCR strategy.

3.5.1 Nested PCR strategy to confirm short CA tandem repeat region on amplified DNA band

Previous results indicate that when attempting to generate amplicons containing from CAST gene promoter CA tandem repeat region they had a tendency to produce multiple non-specific bands. However one of the amplicons generated were the same size as theoretical amplicon, based on database sequence, when compared to the size of 100bp DNA marker on gels and this was the DNA band with the greatest motility as estimated by relative comparison with the 100bp DNA marker. As can be seen in Figure 3.9, low motility bands of apparently high molecular weight were seen stacked together above the correct size band.

An experiment was carried out to enhance the specificity of the PCR to ensure that the amplicon contained the short CA tandem repeat region. In this experiment, a nested PCR was performed using primers that were predicted to anneal to sequence within the 203bp size amplicon generated from a first round of PCR that had been purified from a non-denaturing acrylamide gel (Figure 3.10a). The 203bp size amplicons were generated from bovine BAC clone using 1xaCASTtand3F/R primer. Amplicons from this reaction produced four distinctive multiple non-specific bands on non-denaturing acrylamide gel (Figure 3.10a). The four distinctive bands were extracted from the gel. The nested primers 1xaCASTtand1F/R (appendix 1) were used on extracted DNA from the individual band B1, B2, B3, B4 (indicated in Figure 3.10a) and also to the whole purified PCR product from the first PCR reaction.

In theory by performing nested PCR on the extracted amplicon (extracted band), it was expected that a single band with the same length and size of the will be amplified. However, nested PCR using 1xaCASTtand1F/R primers on the extracted bands seems to produce more non-specific product as indicated on the 7.5% (w/v) non-denaturing acrylamide gel (Figure 3.10b). Therefore, it was apparent that nested PCR strategy failed to increase specificity in amplification of the short CA tandem repeat region. It was also not confirmed through nested PCR reaction whether the extracted DNA bands from the amplicons contains the short CA tandem repeat.

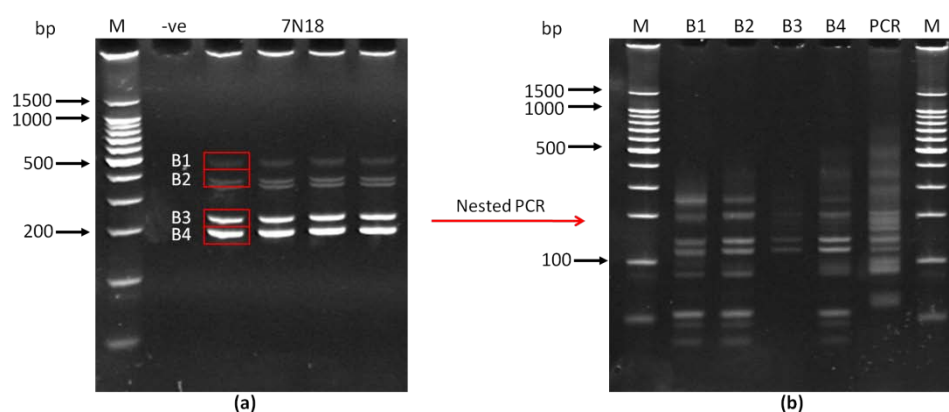


Figure 3.10 Nested PCR on extracted band (B1), (B2), (B3) and (B4) of amplicons with 203bp size generated from the short CA tandem repeat in Calpastatin 1xa promoter region. Nested PCR were generated using 1xaCASTtand1 primer. Negative control water as (-ve), (PCR) indicates template as whole 203bps size amplicons, (M) 100bp marker and (7N18) bovine BAC clone containing CAST gene. (a) DNA was extracted from individual band from 203bp size amplicons (b) Nested PCR on extracted amplicons band

3.5.2 Increasing annealing temperature effect on PCR product

The effect of different annealing temperature in increasing specificity of primer binding in PCR reaction was carried out. Amplification of short CA tandem repeat region in CAST gene using 1xCASTtand3F/R primers yielded amplicons with multiple bands of nonspecific product (previous section) most of which were assessed as being larger than the predicted size band when electrophoresed on non-denaturing acrylamide gel. It was hypothesised that the amplified PCR product with multiple bands was caused by un-optimized annealing temperature for primer binding.

Therefore the same primer set 1xCASTtand3 was used on bovine BAC clone (7N18), which contains the bovine calpastatin promoter, to study the effect of annealing temperature in improving primer specificity during PCR reaction. Amplification of short CA tandem repeat region using 1xCASTtand3F/R was originally done using an annealing temperature of 51°C.

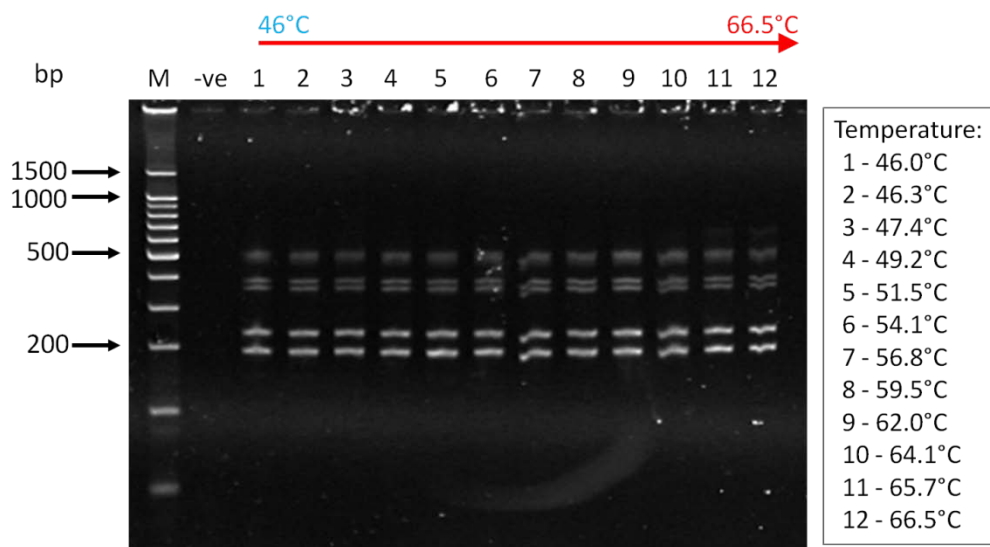


Figure 3.11 Non denaturing polyacrylamide gel electrophoresis of 203bp size amplicons using PCR carried out with differing annealing temperatures on bovine BAC clone containing CAST gene (7N18) on 7.5% acrylamide gel. (M)100bp DNA ladder, (-ve)Non template control, 1-12: Series of annealing temperature using gradient heating block PCR machine from 46°C - 66.5°C

Amplicons were generated from bovine BAC clone (7N18) using primer 1xaCASTtand3 across varying annealing temperature. The annealing temperatures for primers during PCR were tested in the range of $\pm 10^{\circ}\text{C}$ differences from a temperature set at 55°C (Figure 3.11). Amplified 203bp size short CA tandem repeat region yielded from different annealing temperature did not show any significant changes across the temperature range. Multiple non-specific bands were seen across increasing annealing temperature ranging from 46°C to 66.5°C as had been shown previously. The increase in annealing temperature did not seem to improve the specificity of amplified PCR product. Therefore, the original annealing temperature (51°C) for amplification of 203bp size amplicons from the short CA tandem repeat region was used for future experiment.

3.6 Verification of CAST short CA tandem repeat region amplified from BAC clones

As clearly indicated above PCR across CA tandem repeat region in bovine CAST promoter has been proved to be difficult as multiple PCR amplicons were visualized in the gels. In order to verify that these amplicons were actually amplified from the short CA tandem repeat region in CAST gene an experiment was carried out to clone and then sequence these amplicons.

PCR was performed on the CAST gene short CA tandem repeat region using 1xaCASTtand3 primer with bovine BAC clone (7N18) used as a template. The whole PCR amplicon with estimated size of 203bp was purified using (Qiagen) PCR purification kit (section 2.11.1).

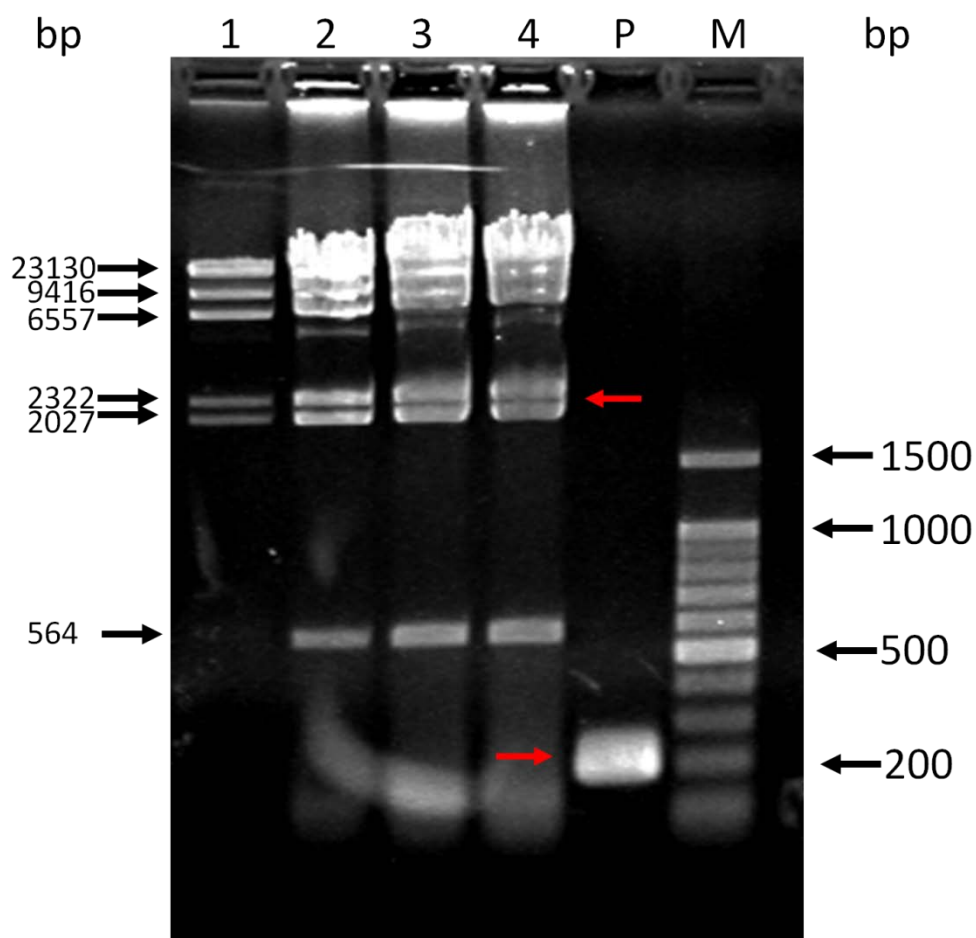


Figure 3.12 Semi-Quantitative estimation of purified PCR product of CA tandem repeat region of CAST gene promoter based on intensity and size of band in Lambda HindIII marker(500ng/ μ l) from horizontal non denaturing agarose gel electrophoresis. (P) Purified PCR product for CA tandem repeat region of the 1xa promoter region of CAST, (1) to (4) 500, 1500, 2500, 3500ng respectively of Lambda HindIII marker (M): 100bp DNA marker ladder. The doublet indicated by the arrow shown on lane (4) was estimated as having the same intensity as band for the PCR product as indicated by the arrow in lane P.

Figure 3.12 shows the electrophoresis of purified PCR product (P). A single band was observed on horizontal non denaturing agarose gel electrophoresis and estimated as being 203bp. The whole 203bp purified PCR amplicon was ligated into pGEM-T easy cloning vector system followed by JM109 competent cells being transformed with the ligated product.

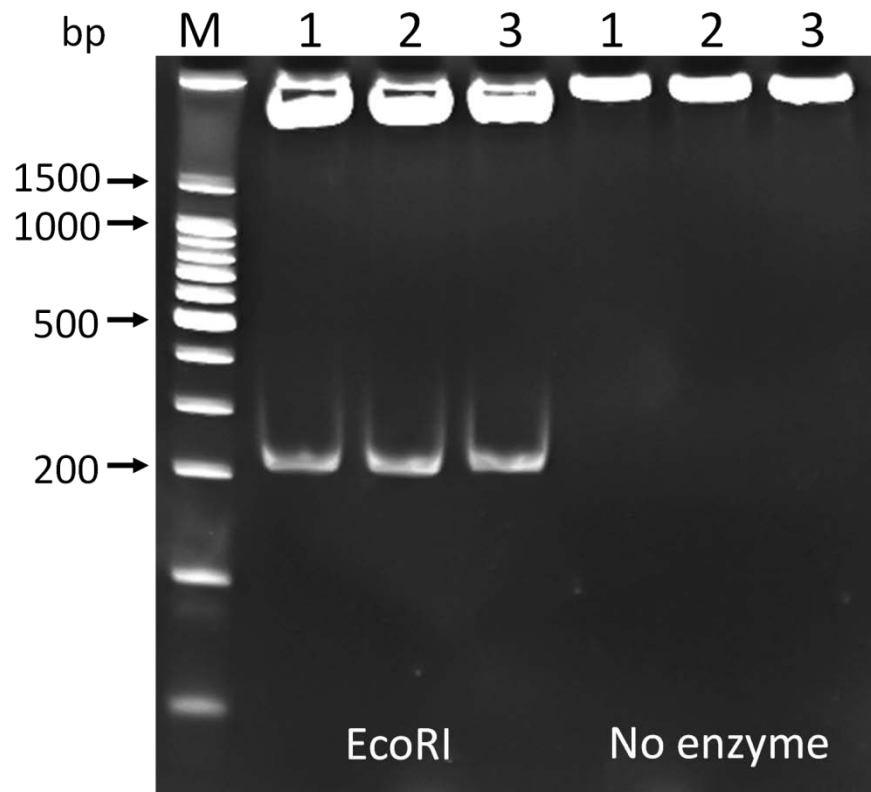


Figure 3.13 7.5% gel electrophoresis of EcoRI digest of construct made of pGEMT-Easy vector (Promega) (B1xaTAND5') with 203bp insert (tandem repeat region) made from bovine BAC clone (7N18) in exon 1xa promoter region.

Extracted plasmid of single grown colonies from transformation plates was digested using EcoRI enzyme to release the cloned short CA tandem repeat amplicons from the multiple cloning site in pGEMT-easy vector. This plasmid was named as B1xaTAND5' construct. Restriction endonuclease digestion of the plasmid construct isolated from separate clones indicated that a DNA band with the size of 203bp was released from the plasmid (Figure 3.13). The released DNA band was shown to be similar in size as the 203bp amplified PCR product of short CA tandem repeat when visualized using 7.5% acrylamide gel (data not shown). It should be noted that DNA bands which were larger than the 203bp band in the same PCR reaction (Figure 3.11) did not appear to be cloned into the vectors. This was inspite the fact that the clone insert originated from whole purified PCR product which contained additional non-specific bands (Figure 3.11). This raised the question why

these non-specific bands were not cloned into the plasmid. Possible explanations for this might be that the amplified non-specific bands, which had been previously observed as being larger than 203bp size band, did not contain any region of complementary 3' (A) overhang required for ligation. Thereby preventing cloning of these non specific bands into the pGEMT-easy cloning vector.

Verification of whether the clone region of the 203bp size bands in B1xaTAND5' construct contains the short CA tandem repeat region was done through sequencing (Figure 3.14). DNA sequencing of B1xaTAND5' construct reveals that the insert contains 203bp in total of short CA tandem repeat region from CAST gene (Figure 3.14). Therefore, the 1xaCASTtand3F/R primers used in amplification of short CA tandem repeat region in CAST gene was specific. As can be seen in the alignment shown in Figure 3.14, there was variation in length of short CA tandem repeat region compared to the sequence in the database. The latter being 10bp longer. The database sequence, BovProt1/1, is exon 1xa CAST gene sequence from Holstein cattle (Raynaud et al., 2005) whilst B1xaTAND5' contains CAST gene sequence from Hereford cattle. This suggests CA tandem repeat length varies between cattle (Figure 3.14) thereby strengthening our primary hypothesis that the short CA tandem repeat length in CAST gene is variable between cattle.

BovProt1/1	101	GAAACATCATTGCACTTTTCAAAACAAGTAAAGCCGCACAAAACACACCC	150
		.	
B1xaTAND5	1	-----GCATTGCAC-TTTCAAAACAAGTAAAGCCGCACAAAACACACCC	43
BovProt1/1	151	AGGGCCCGNNNGTGT-----ACACACACACACACACACACACG	190
B1xaTAND5'	44	A-GGCCCGTGTGTGTGTACACACACACACACACACACACACACA	92
BovProt1/1	191	CACACACACACACAATCACAACACACGAGTCACACACAGACCAATTTGT	240
B1xaTAND5'	93	CACACACACACACAATCACAACACACGAGTCACACACAGACCAATTTGT	142
BovProt1/1	241	TTCTGCAATCCGCTTCCTCATCCAGAAGGTCCAGGCCCGGGCCATCTGA	290
B1xaTAND5'	143	TTCTGCAATCCGCTTCCTCATCCAGAGGTCCAGGCCCGGGCCATCTGA	192
BovProt1/1	291	GTTGGTAAATTCTCCTCCTAGTTAACTCAGAGCAGATTGCAGAAATGCTG	340
B1xaTAND5'	193	GTTGGTAAATT-----	203

Figure 3.14 Alignment of nucleotide sequences from database bovine genomic DNA for the promoter exon of 1xa BovProt1/1 (AY834765) and B1xaTAND5' plasmid which contains tandem CA repeat region of Bovine CAST 1xa promoter from different cattle. Highlighted green indicates forward and reverse primer. Highlighted yellow indicates size differences in CA repeat region for B1xaTAND5' plasmid.

3.6.1 Amplification of short CA tandem repeat region from linearized plasmid

Amplification of CA repeat region from CAST gene promoter in cattle genomic DNA consistently produced a multiple banding pattern even though the template used was homogenous for the sequence to be amplified (a BAC clone containing the 5' end of the calpstatin gene, including the 1xa promoter). A possible explanation was that the vector was supercoiled form thereby giving the possibility of the production of a non-specific PCR product as a result of this physical interference. An experiment was conducted to test this hypothesis using previously generated clones B1xaTAND5' and the available BovProt1/1 (containing 723bp size of sequence 5' to 1xa promoter). Plasmid from clone B1xaTAND5' contains 203bp size insert spanning the CA repeat region while plasmid from clone BovProt1/1 contains the same CA repeat region except with more of the adjacent sequence so the insert is 739bp. Furthermore, the host plasmids of these two clones are different therefore the differing backbones give differing length, restriction endonuclease site and vector properties.

Amplifications of CA repeat region was done using 1xaCASTtand3 primers, using linearised vectors, which theoretically should yield a 203bp product on both constructs based on the database sequence. The amplicons were then electrophoresed on 7.5% non-denaturing polyacrylamide gel along with amplicons that is amplified from the same plasmid source which has not been linearized to observe the differences (Figure 3.15). Overall, PCR amplification on linearized plasmid when observed on non-denaturing 7.5% acrylamide gel did not show any significant improvement in terms of increase in specificity of produced amplicons. PCR product still produces multiple non-specific bands when compared to the amplicons produced from non-linearized plasmid.

Interestingly, B1xaTAND5' plasmid which have shortest possible insert for amplification still generated doublet and longer amplicons band above the prominent doublet band compared to the BovProt1/1 plasmid which have

larger insert. Therefore it was concluded that the amplification short CA tandem repeat on linearized plasmid does not increase specificity of amplicons produce during PCR reaction as the amplification of the short CA tandem repeat region was not affected by the conformity of the templates used. Band sizes from migrated amplicons in Figure 3.15 were determined by calibrating the gel using 100bp DNA marker. Band sizes were calculated from short CA tandem repeat amplicons made from linearized and non-linearized plasmid shown in (Table 3.1). There was relatively small base pair size differences between amplicons synthesized from B1xaTAND5' and BOVProt1/1 construct similar to what have been observed through sequencing of these plasmid (Figure 3.14).

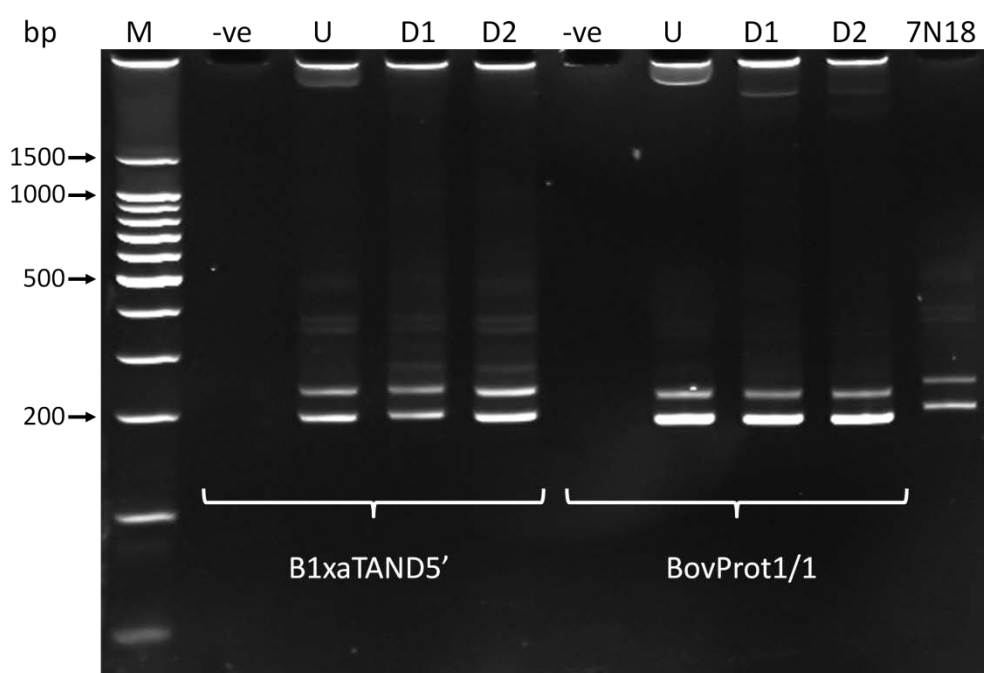


Figure 3.15 The effect of linearising plasmid containing the target template sequence, CA repeat region in CAST gene , on PCR amplicons produced. PCR amplicons of 1xa calpastatin CA tandem repeat region with predicted size of 203bp size generated from plasmid constructs B1xaTAND5'and BovProt1/1, as indicated. Both plasmid contains insert of the tandem repeat from the exon 1xa CAST Promoter from different cattle sources. Lanes contain; (-ve) negative control; (U) amplicons from undigested plasmid; (D1 & D2) amplicons from plasmids digested with PstI enzyme; (7N18) bovine BAC clone containing bovine CAST gene.

The size difference revealed through sequencing was 10bp. In contrast, the size difference determined using quantitative method was only 7bp in length (Table 3.1) as the size of the amplicons were 204bp band 5 in B1xaTAND5' (U) and 197bp band 2 BovProt1/1 (U). All size comparison in this experiment was made between the band that migrated the furthest from each amplicons as it was assumed that other bands were non-specific bands produced during amplification of the short CA tandem repeat region. These results indicate variation of short CA tandem repeat length from different cattle both by sequencing of plasmid and by estimating amplicon band sizes. The similarity in size was also observed on amplicon sizes both on band 5 (U) and band 6 (D) in B1xaTAND5' plasmid and band 2(U) and band 2(D) in BovProt1/1 plasmid, this being the slower migrating band. The size similarity was also observed on non-specific bands that migrated slower than the two predominant bands which migrated the furthest in PCR product amplified from linearized and non-linearized plasmid. B1xaTAND5' plasmid is derived amplicons containing CA repeat made from bovine BAC clone (7N18). Theoretically, the size of the furthest migrating band in (7N18) and B1xaTAND5'(U) should be similar sizes as both would be expected to contain the short CA tandem repeat with the same length. On the contrary, there was a size difference with the furthest migrating band derived from 7N18 at 220bp (band 2) whilst for B1xaTAND5' it was 204bp (band 5(U)). The 16bp size differences on the smallest amplicons band observed in clone B1xaTAND5' compared with the amplicon generated from its original template source (7N18) was hypothesized to occur as the results from cloning the entire PCR amplicons generated from 7N18. The amplicons from the short CA tandem repeat contains multiple bands that might have been ligated into the cloning vector, pGEM-T easy. Therefore an investigation was carried out to see what length variation there was in inserts of clones generated from the ligation of the PCR products generated from 7N18 CA tandem repeat region.

Table 3.1 Estimation of band size from short CA tandem repeat amplicons generated from linearized and non-linearized plasmid. The size of the bands from Figure 3.15. (U) Size of amplicons made from non-linearized plasmid and (D2) size of amplicons made from linearize plasmid both calculated using linear equation derived from graph of log base pair sizes versus band relative distances from the point of origin. The bands are those which were clearly identified from the top to bottom of the gel

Plasmid	distance from origin (cm)	band size bp (antilog)
B1xaTAND5' (U)		
Band 1	4	496
Band 2	4.6	397
Band 3	4.8	369
Band 4	5.9	246
Band 5	6.4	204
B1xaTAND5' (D2)		
Band 1	4.1	478
Band 2	4.6	397
Band 3	4.8	369
Band 4	5.5	285
Band 5	5.9	246
Band 6	6.4	204
BovProt 1/1 (U)		
Band1	6	237
Band2	6.5	197
BovProt 1/1 (D2)		
Band 1	6	237
Band 2	6.5	197
7N18		
Band 1	5.8	255
Band 2	6.2	220

3.6.2 Variation of insert length from cloning the short CA tandem repeat amplicon

The short CA tandem repeat amplicons had been successfully cloned into pGEMT-easy cloning vectors using 7N18 as a template to give the clone B1xaTAND5' which was shown to contain the short CA tandem repeat from CAST gene exon 1xa promoter region (section 3.6). However amplification of the short CA tandem repeat of B1xaTAND5' isolated from two independent clones using 1xaCASTtand3 primer revealed size differences when compared with amplicons made from the original source for the insert (7N18) bovine BAC clone. The objective of this experiment was to investigate whether the size variation between the amplicons from the clone (7N18) and B1xaTAND5' was caused by ligation of non-specific band from the 203bp size amplicons when B1xaTAND5' was generated.

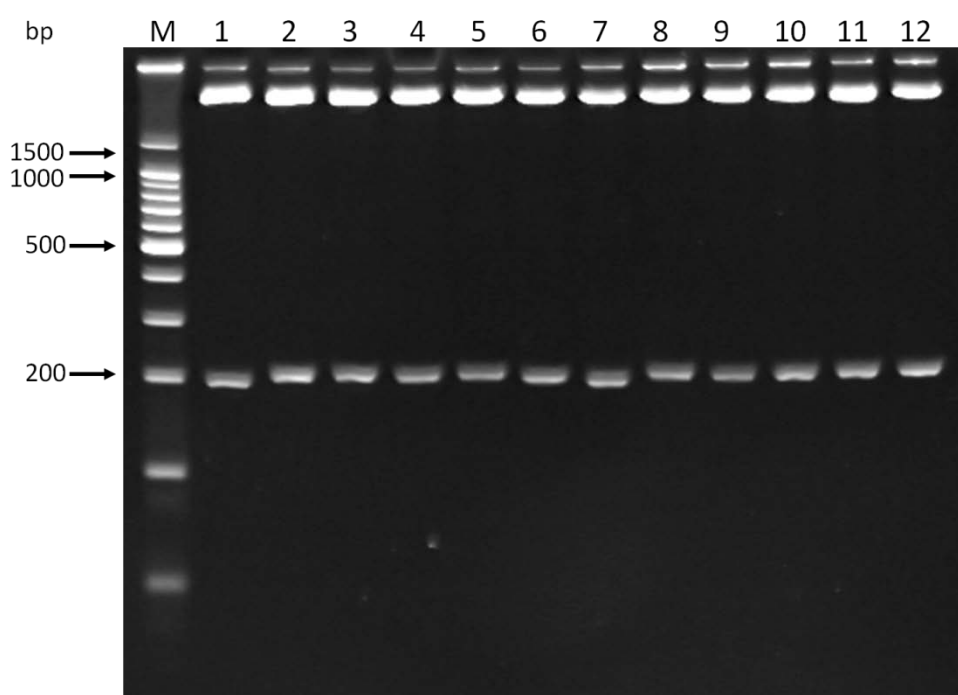


Figure 3.16 Variation of insert length from cloning 203 bp short CA tandem repeat amplicons. 12 independent clones generated by the insertion of the PCR amplicon made from a single original template containing bovine CAST short CA tandem repeat region into pGEMT easy (B1xaTAND5') were restriction endonuclease digested with EcoRI then run on a non-denaturing polyarylamide gel. (M) 100bp. (1) to (10) indicates plasmid of different colonies grown from transformation plate.

In this experiment the whole PCR reaction containing amplicons produced using 1xaCASTand3 primers were cloned into pGEMT-easy vector. The cloning was done from a single PCR reaction originated from a single template. 12 individual colonies were picked from the transformation plate and grown for plasmid extraction then digested with EcoRI enzyme to release cloned insert and electrophoresed on non-denaturing polyarylamide gel. Figure 3.16 shows restriction digest performed on 12 independent colonies of B1xaTAND5' plasmid. All 12 colonies from the clone contain insert. Size of the released DNA bands were calculated (Table 3.2). Slight size differences between independent colonies were apparent on the gel. However the size of the released bands from each colony was almost similar to the size of 203bp size amplicons with only a few base pair differences when they were compared to the predicted size. The smallest insert visualized on the gel was 193bp and the largest was 208bp in size. The size differences compared to the predicted size of insert varies around 5-10bp long.

The results shown from the individual clones originate from a single PCR reaction generated from a homogenous template. Variation in the amplicon size as determined from gel calibration with markers must be due to error in assessments from gel. However, the ranges of size of PCR amplicons within separated clones suggest that the PCR products generated from the original template were not completely homogenous. Variation in size could be potentially due to slippage across the CA repeat allowing the generation of amplicons which are shorter or longer than the original template. This variation is small but indicates the difficulties of discrete identification of the length of CA repeat in the calpastatin promoter region associated with 1xa. From this result, it was proposed that amplicons of varying sizes were cloned into pGEMT-easy vector as the result of cloning the whole purified amplicons of short CA tandem repeat.

In order to further verify the size differences plasmid from colony 1 and colony 2 were sequenced and results from sequencing were shown in Figure 3.17. Pairwise sequence alignment of both plasmid from colony 1 and colony 2 B1xaTAND5' sequences revealed 8bp size differences in the short CA tandem repeat region. Moreover, the base pair differences between both colonies were almost similar to the size calculated from the gel electrophoresis (Table 3.2) where the size difference was 8bp. This result indicates that the method of determining size of the CA tandem repeat through electrophoresis of released insert on non-denaturing acrylamide gel was accurate.

In conclusion, cloning of the whole purified amplicons of CAST short CA tandem repeat produced colonies which contain varying length of short CA tandem repeat sequences. Cloning of short CA tandem repeat amplicons originated from a single copy of bovine DNA revealed varying size of CA tandem repeat which suggest that the problem may possibly develop during PCR of the short CA tandem repeat region.

Table 3.2 Size of short CA tandem repeat insert released from independent B1xaTAND5' clones. The size of insert released using EcoRI restriction digest enzyme from B1xaTAND5' clone were calculated using (BioRad) Quantity One software.

B1xaTAND5' Plasmid	Band size (bp)
Colony 1	193
Colony 2	202
Colony 3	202
Colony 4	200
Colony 5	205
Colony 6	200
Colony 7	193
Colony 8	205
Colony 9	202
Colony 10	205
Colony 11	208
Colony 12	208

Chapter 4: Calpastatin Promoter Studies

4.1 Introduction

Study of calpastatin gene promoter activity had been done previously on pig and cattle. Type I, Type II and Type III calpastatin transcript had been characterised in porcine with the reports of three promoters directing the expression (Parr et al., 2004) while in bovine calpastatin transcripts including Type IV had been characterised with the reports of four functional promoters in the gene (Raynaud et al., 2005). There was however little knowledge of calpastatin promoter response towards agents such as calcium related stimuli and cyclic adenosine monophosphate (cAMP) as the study had only been performed on the porcine calpastatin promoters associated with exon 1xa, 1xb and 1u (Sensky et al., 2006). The study done by Sensky et al. (2006) have shown that transcriptional activity of Type III promoter in pigs was elevated with dbCAMP treatment and this was explained with the presence of CRE sites located 5' upstream promoter 1u. However treatment of dbcAMP in porcine Type I promoter significantly decrease its activity and Type II showed no significant difference with treatment. In bovine, promoter construct made from promoter 1xb had been shown to be responsive towards cAMP treatment (Cong et al., 1998b). However there has been no recent study of responsiveness of the bovine 1xa and 1u promoter region towards this treatment. Therefore, the objective of this study was to characterise expression of bovine CAST promoter and to study the responsiveness of the promoter towards these treatments.

Type I, Type II and III of CAST main promoter constructs for this study were made available from Raynaud et al. (2005). Bovine calpastatin gene promoter sequence from three main promoter regions 5' to exons 1xa, 1xb and 1u had been previously cloned into pEGFP reporter vector (clontech) by Raynaud et al. (2005) in order to study CAST promoter activity. In addition they had also generated a series of 5' deletion constructs for each promoter (Figure 4.1). These bovine CAST promoter constructs were used as a basis in this study to analyze the expression of CAST promoter activity in the

mammalian host cells C2C12 mouse myoblast cells, COS7 monkey kidney fibroblast cells and HEK 293 human embryonic kidney epithelial cells.

Various cell types were used in order to look at the efficiency of CAST promoter activity in different host cells. Raynaud et al. (2008) reported that transcriptional activity of CAST promoter is higher in COS7 cells than in C2C12 cells. There could be a lot of factors that generate this differences and one of them could be potentially caused by the differences in these cells lines. Differentiations of cells allow selected expression of certain genes and this is mediated by transcriptional factors (Reece and Campbell, 2011). Calpastatin gene is not a TATA box controlled genes and was shown with the absence of TATA boxes binding sequences in all three promoters suggesting constitutive expression of CAST gene in all cell lines (Meyers and Beever, 2008). Because the characteristic of gene expression is determined by the regulatory factor (transcriptional factors) which switch on or off the gene is specific to the cell types (Lewin et al., 2009). Therefore it is possible that transfection of CAST promoter construct in differing cells types is likely to give a variation in expression which is not the same as in the tissue where the organisms originated for examples in skeletal muscle of a cattle. Furthermore, all commercially available cell lines were generally designed to transform into an immortal cells which have potential to proliferate indefinitely in culture (Smith et al., 1992). Therefore this could also potentially leads to further diversion from the gene regulatory environment found in normal tissue. It was decided that the use of different types of cells lines in CAST promoter transfection will helps us to determine suitable cells lines that could be use as a model for assessing transcriptional activity of the gene.

4.2 Mouse myoblast cells differentiation

C2C12 cells are a mouse muscle cell line which are able to differentiate and fuse to form myotubes. They have endogenous expression of the calpastatin protein. As the principle candidate host cell for transfection of CAST promoter construct, it was important to investigate the expression of the endogenous CAST as the expression might interfere with expression of exogenously introduced CAST (transfected CAST promoter construct). The main objective of this experiment was to investigate whether the expression of the endogenous CAST from C2C12 mouse myoblast was affected as the cells start to differentiate as this the point at which the calpastatin constructs would be transfected into the cells.

Mouse myoblast, C2C12 cells were cultured for 9 consecutive days in a time-course experiment to measure their expression of endogenous CAST (Figure 4.2). On day 1, the day after the cells were seeded, C2C12 cells appear to be widely dispersed in the well and were still in the form of single myoblast cells. Differentiation media was supplied to the seeded cells on Day 2 (section 2.16.1) to induce cell differentiation. On Day 3, after 1 day in differentiation media cells were observed as almost confluent. Apparent differentiation of C2C12 cells started at day 3 post differentiation media, as cells were fusing to form myotubes. The formation of myotubes became more visible on day 7 as many short length myotubes were formed in the well. On day 9 of observation, the C2C12 cells morphology was completely changed with the formation of longer myotubes in the well as a result of cell differentiation.

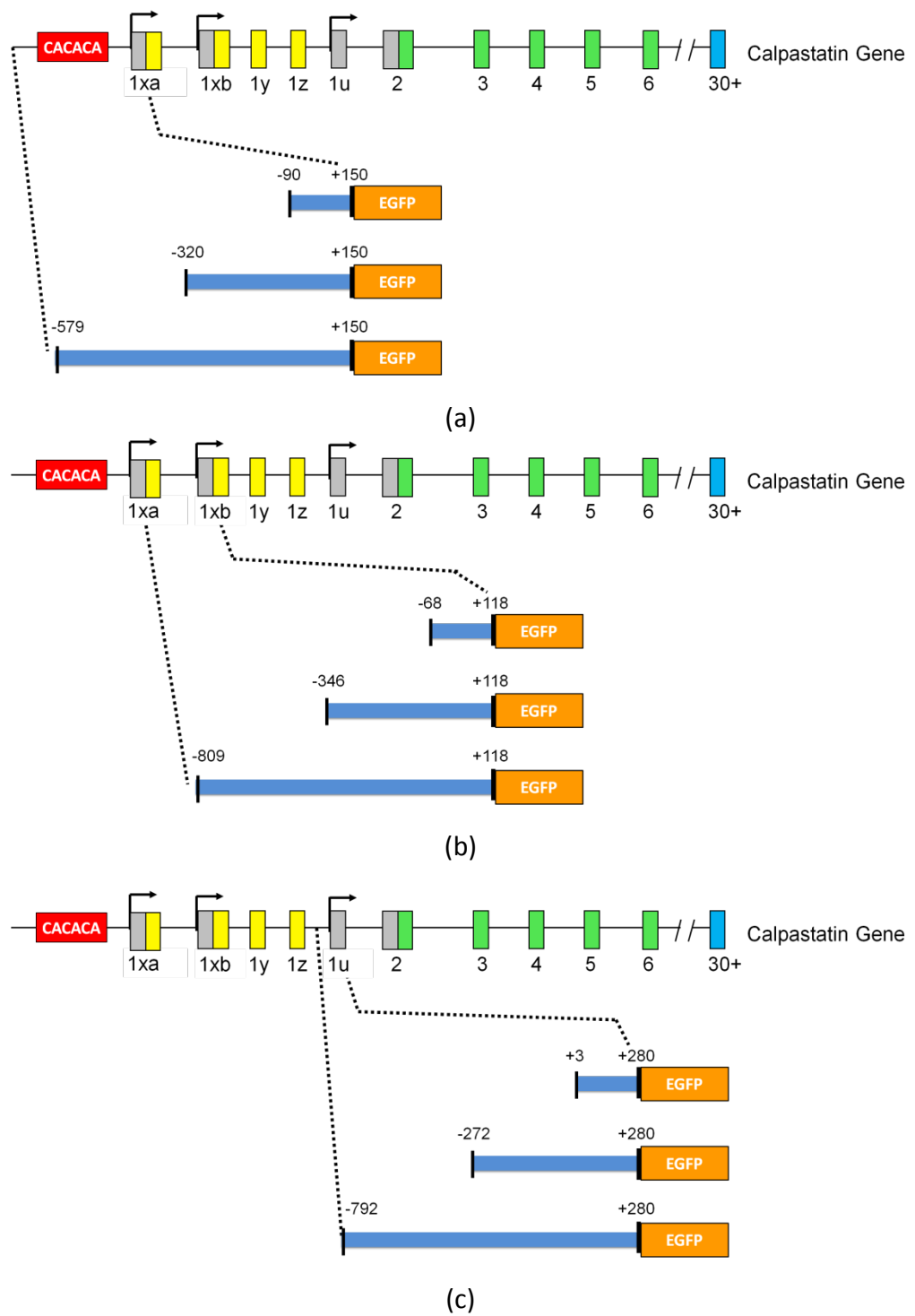


Figure 4.1 GFP based calpastatin promoter construct developed from bovine calpastatin gene developed by Raynaud et al. (2005). (a) Promoter construct of exon 1xa and its 5' deletion. (b) Promoter construct of exon 1xb and its 5' deletion. (c) Promoter construct of exon 1u and its 5' deletion

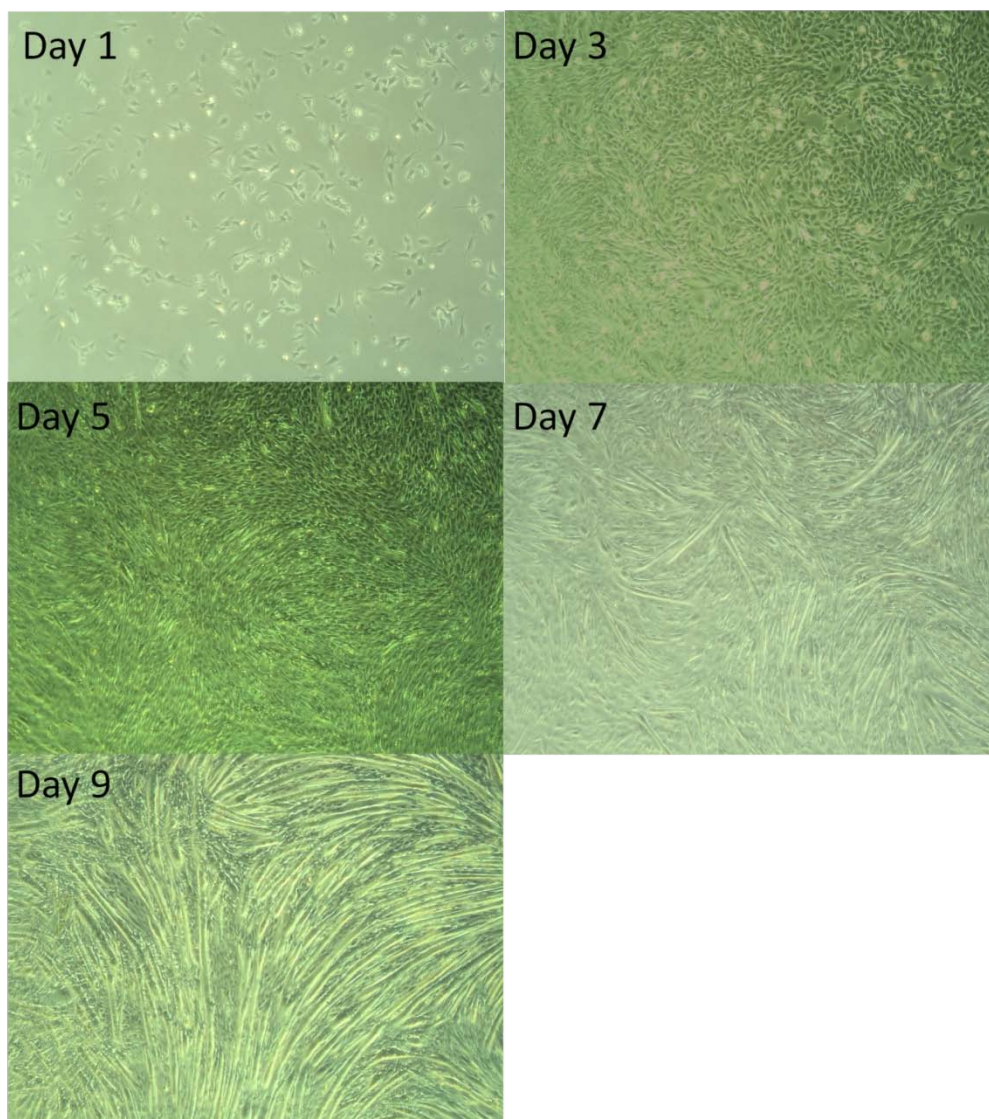


Figure 4.2 Differentiation of C2C12 mouse myoblast cells. Cells were induced to differentiate by adding differentiation media (low serum) starting from day 2 (seeded at density of 4×10^4 cells per well) until day 9. Cell differentiation was observed using inverted microscope at 20 \times magnification.

4.3 Expression of endogenous CAST in differentiating mouse myoblast cells

A suitable mammalian host cell line was required to study the effect of the bovine CAST promoter expression. The mouse muscle cell line was elected as it has the capacity to differentiate into myotubes, as identified above and thereby was applicable in relating expression of calpastatin in muscle cells. Previous reports had indicated that endogenous calpastatin expression varied during muscle cell line differentiation with expression reported to decline during differentiation of L8 rat myoblasts (Barnoy et al., 2000). Such changes may influence the expression from exogenous CAST reporter constructs inserted into mouse muscle C2C12 cell line. Therefore an examination of mouse CAST gene expression in differentiating C2C12 cells was carried out to quantify the expression of endogenous CAST in differentiating mouse myoblast cells.

Extracted total RNA from a triplicate of C2C12 cells on day 1, 3, 5, and day 7 during the time course experiment were electrophoresed on 1.5% normal agarose gel to checked for the integrity of extracted RNA (Figure 4.3) by assessment of 28s and 18s rRNA bands which found the RNA extracted from the cells showed no significant degradation. Single strand cDNA was generated from extracted C2C12 mRNA cells by reverse transcribing the mRNA using a reverse transcriptase enzyme. A glyceraldehydes 3-phosphate dehydrogenase (GAPDH) internal reference gene primer, kindly given by Miss Lucy Craggs (University of Nottingham) was used to test the newly synthesized cDNA. Amplification using this primer on the cDNA yielded 300bp theoretical size product (Figure 4.4).

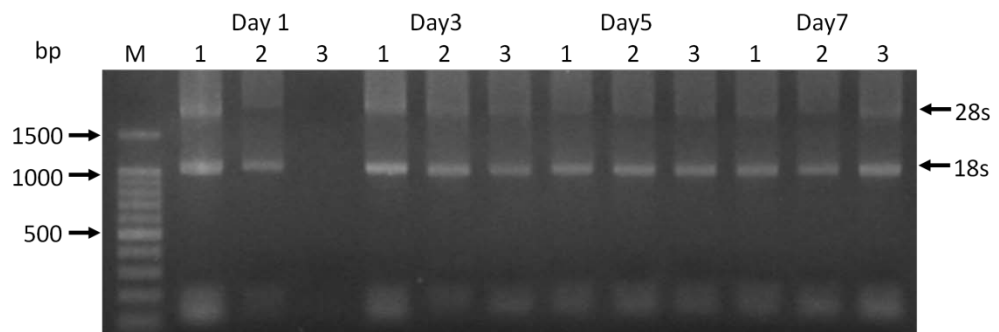


Figure 4.3 Extracted RNA from C2C12 cells time course experiment from day 1, 3, 5 and day 7. The quality of RNA extracted from the C2C12 cells in the mouse CAST time course experiment was checked on a non-denaturing 1.5% agarose gel along with 100bp DNA ladder (M). Visible 28s and 18s rRNA band on the agarose gel indicates the integrity of the extracted mRNA.

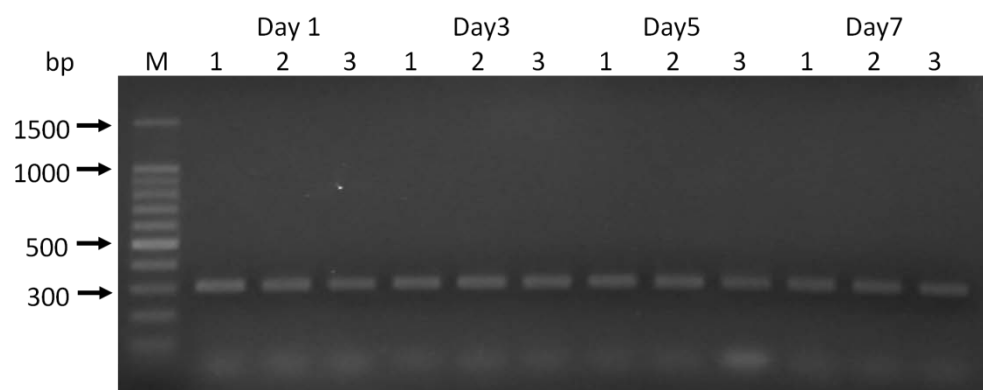


Figure 4.4 Gene expression of GAPDH over a mouse C2C12 muscle cell differentiation time course. Total RNA from C2C12 cells was prepared at day 1, 3, 5 and 7 incubation with cells being switched to myoblast differentiation media (low serum) at day 2 of incubation. Equal quantities of total RNA per sample (500ng) were utilised to synthesise first strand cDNA which was then subjected to PCR using primers for GAPDH. PCR samples were subjected to non denaturing agarose gel electrophoresis (1.5% w/v). Shown are the products from replicate cell incubation experiments (1-3). The position of 300bp GAPDH amplicon is indicated. 100bp DNA ladder (M).

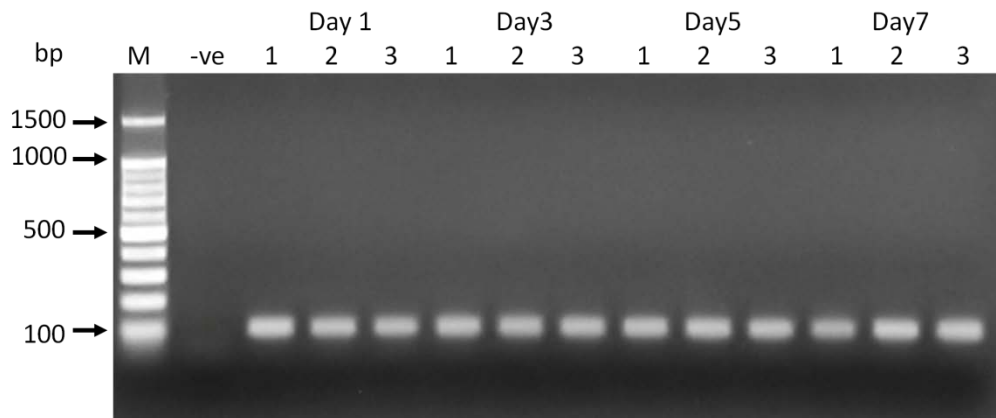


Figure 4.5 Gene expression of CAST over a mouse C2C12 muscle cell differentiation time course. Total RNA from C2C12 cells was prepared at day 1, 3, 5 and 7 incubation with cells being switched to myoblast differentiation media (low serum) at day 2 of incubation. Equal quantities of total RNA per sample (500ng) were utilised to synthesise first strand cDNA which was then subjected to PCR using primers for CAST. PCR samples were subjected to non denaturing agarose gel electrophoresis (1.5% w/v). Shown are the products from replicate cell incubation experiments (1-3). The position of 100bp musCAST amplicon is indicated. 100bp DNA ladder (M).

Quantification of endogenous CAST expression from mouse myoblast cells was done using a mouse spec designed primer and probe. The primer and probe from this experiment was designed based on mouse CAST gene sequence available from NCBI database (accession number: AB026997). In order to avoid differential amplification of a variant the primers were designed using the sequence encoding the inhibitor domains at the 3' end of the open reading frame as this is constitutively expressed in calpastatin mRNAs. The sequence of musCAST primers and amplification region within the mouse CAST cDNA sequence was described in (Appendix 6). Before using the primers for qPCR reaction, an end point (set number of cycles) PCR reaction was carried out and the amplification product was electrophoresed on a 1.5% non-denaturing agarose gel to assess the specificity of the designed primers. Figure 4.5 shows the theoretical size amplicons were produced from the amplification of first strand cDNA using the designed musCAST primers.

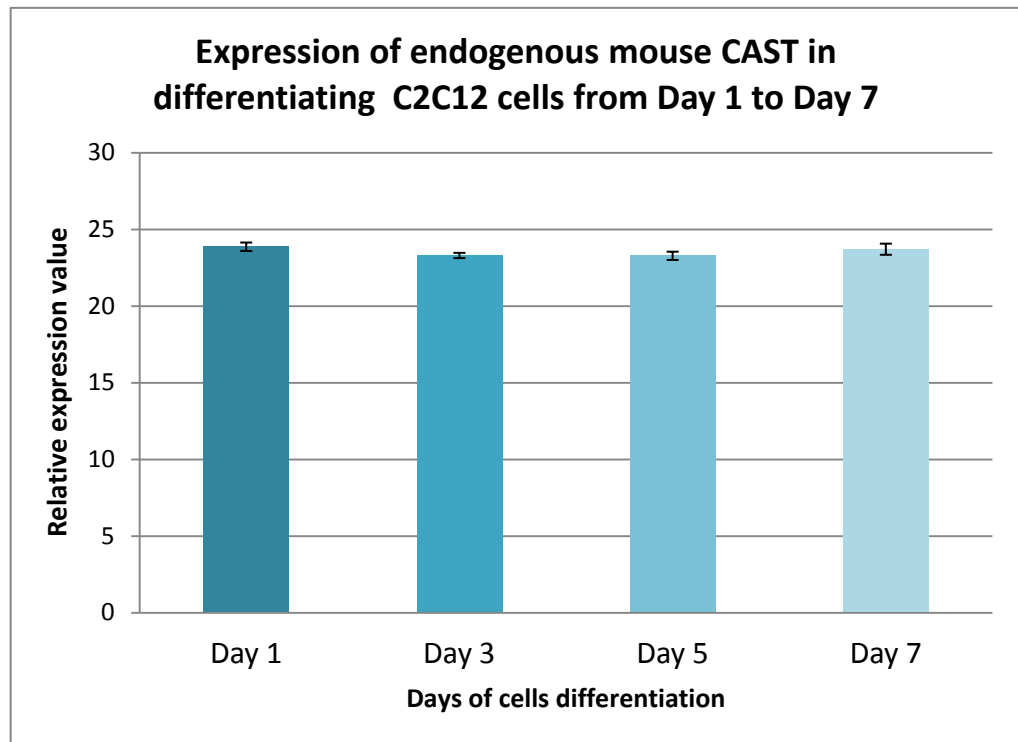


Figure 4.6 Quantitative RT-PCR assessment of CAST gene expression over a mouse C2C12 muscle cell differentiation time course. Total RNA from C2C12 cells was prepared at day 1, 3, 5 and 7 incubation with cells being switched to myoblast differentiation media (low serum) at day 2 of incubation. Equal quantities of total RNA per sample were utilised to synthesise first strand cDNA which was then subjected to PCR using primers for mouse CAST. Mean quantified value for mouse CAST expression relative to GAPDH gene were plotted from day 1, 3, 5, and day 7, error bars are SEM and n=3. ANOVA was used to analyse the data.

Relative expression value for endogenous mouse CAST expression during differentiation of mouse C2C12 myoblast cells was determined using quantitative RT-PCR using the musCAST primer (appendix 6). There was no significant difference in the expression of calpastatin mRNA and during post-differentiation period on day 3, 5 and 7 it was unchanged when compared to its expression on day 1 (prior to differentiation induction on day 2 using low serum media) (appendix 11). The report from this study was consistent with previous findings from (Hitomi et al., 2000) which reported unaltered expression of calpastatin in differentiating C2C12 mouse cell line myoblasts, although the quantification was made at the protein level. Data from their western blotting experiment indicates constant expression of calpastatin protein throughout C2C12 mouse myoblast cell differentiation. Another study measuring calpastatin mRNA levels (northern blot) was performed on differentiating L8 rat myoblast cell lines found that the level of calpastatin mRNA was significantly down regulated during L8 myoblast cell differentiation and regain back its initial level after fusion of myoblast cells (Barnoy et al., 2000). The result from this study was however arguable as northern blots have a lower sensitivity and accuracy when compared to qPCR. As for the study reported above, it was concluded that general calpastatin expression remain unchanged from its initial value despite differentiation of mouse myoblast cells.

4.4 pEGFP as base vector in the study of bovine CAST promoter activity

Study of bovine CAST promoter was previously done using pEGFP-1 as based vector (Raynaud et al., 2005). The basic promoter expression vector named pEGFP-1 (genbank accession no: U55761) contains enhance green fluorescence protein gene (EGFP). from the activity of a promoter can be quantified by cloning the putative promoter sequence into the multiple cloning site area in the vector then transfecting this plasmid into a suitable host vector and the expression from the promoter being measured using fluorescence based detection apparatus.

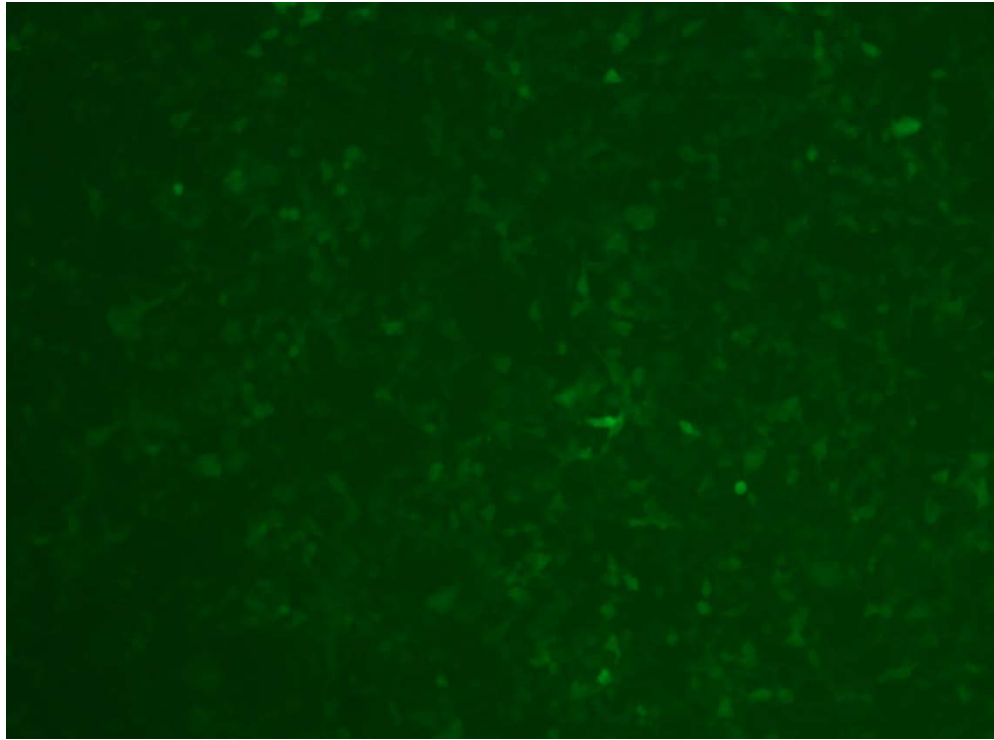
Study of CAST gene promoter was done previously on gene promoter construct made from pig and cattle CAST (Cong et al., 1998b; Parr et al., 2004; Raynaud et al., 2005). Study and characterization of CAST gene promoter in cell line was started by Cong et al. (1998b) who focused on the effect of administering dibutyl-CAMP on the expression of bovine CAST promoter in NIH3T3 mouse embryo fibroblast cells. In porcine, to be from the activity of three independent promoters associated with Type I, II and Type III was characterised (Parr et al., 2004) while the most recent study use differing types of anabolic agents to investigate its effect on CAST promoter expression (Sensky et al., 2006). Anabolic agents such as dibutyl- CAMP, calcium ionophore, forskolin and and cyclosporin A were tested on porcine CAST promoter construct in order to study its effect on promoter expression (Cong et al., 1998b; Sensky et al., 2006). Raynaud et al. (2005) manage to perform a comprehensive study on bovine CAST promoter activity by isolating all four putative CAST promoters and trying to identify sequence elements responsible for their activity by 5' deletion analysis.

A common feature of these previous studies, and perhaps a reason for some of the diversity of results that they have generated, are oversights in the utilisation of appropriate controls. For example previous promoter studies did not include a transfection marker which was co-transfected in the host cell line where the CAST promoter construct was transfected. Furthermore, in

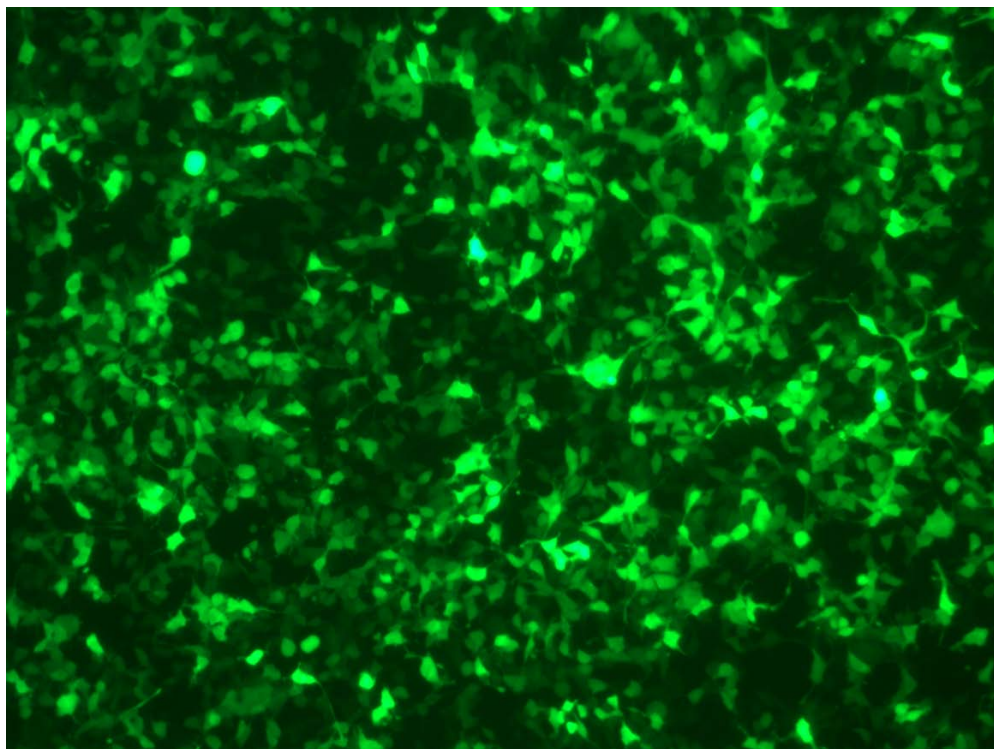
most of these studies there was no relevant positive control for treatment included in the study involving the use of anabolic agent. Therefore, the main objective of the work described in this section was to conduct a proper characterization of bovine CAST promoter activity with the inclusion of appropriate control in the experiment in order to extend the previous findings.

4.5 ZsGreen (Clontech) based promoter expression vector as a more suitable candidate for study of CAST promoter expression vector compared to EGFP based vector

Experimental study of bovine CAST promoter expression was started with EGFP as based vector. Promoter expression vector construct based on EGFP was generated from bovine CAST promoter region (Raynaud et al., 2005) and EGFP expression from this construct was quantified using flow cytometry. A slight different approach in fluorescence quantification was taken in this study where quantification of EGFP fluorescence from promoter construct was performed using FluoStar Optima Plate Reader (BMG Labtech). Initial experiment to optimise the experiment using the available EGFP based promoter expression vector revealed that EGFP was producing a low signal for detection purpose compared to an analogous promoter sequence in the ZsGreen promoter construct (data not included). Furthermore, fluorescence emission, observed using inverted fluorescence microscope, was low from cells transfected with EGFP construct compared to the ZsGreen (Figure 4.7) as had been previously reported by Ilagan et al. (2010). Therefore, it was decided during experimentation, to replace the EGFP based vector with ZsGreen reporter vector.



(a) GFP fluorescence



(b) ZsGreen fluorescence

Figure 4.7 Comparisons of fluorescence signal emitted from pZsGreen 1-N1 vector and pL3-TRE-LucGFP-2L reporter vector. Images of fluorescence signal from HEK293 cells transfected with 200ng per well of pZsGreen 1-N1 and pL3-TRE-LucGFP-2L vector both containing CMV promoter were captured 48 hour after transfection using inverted fluorescence microscope at 20× magnification.

4.5.1 Development of ZsGreen based promoter expression vector for CAST Type I, Type II and Type III promoter constructs

In the previous section, ZsGreen had been reported to provide higher intensity of fluorescence signal compared to the GFP based vector. Therefore in this section, the original clones of calpastatin promoter construct from Type I, Type II and Type III with their associated deletion construct derived from pEGFP-1 fluorescence based reporter vector were inserted into pZsGreen 1-1 reporter vector as indicated in Figure 4.8.

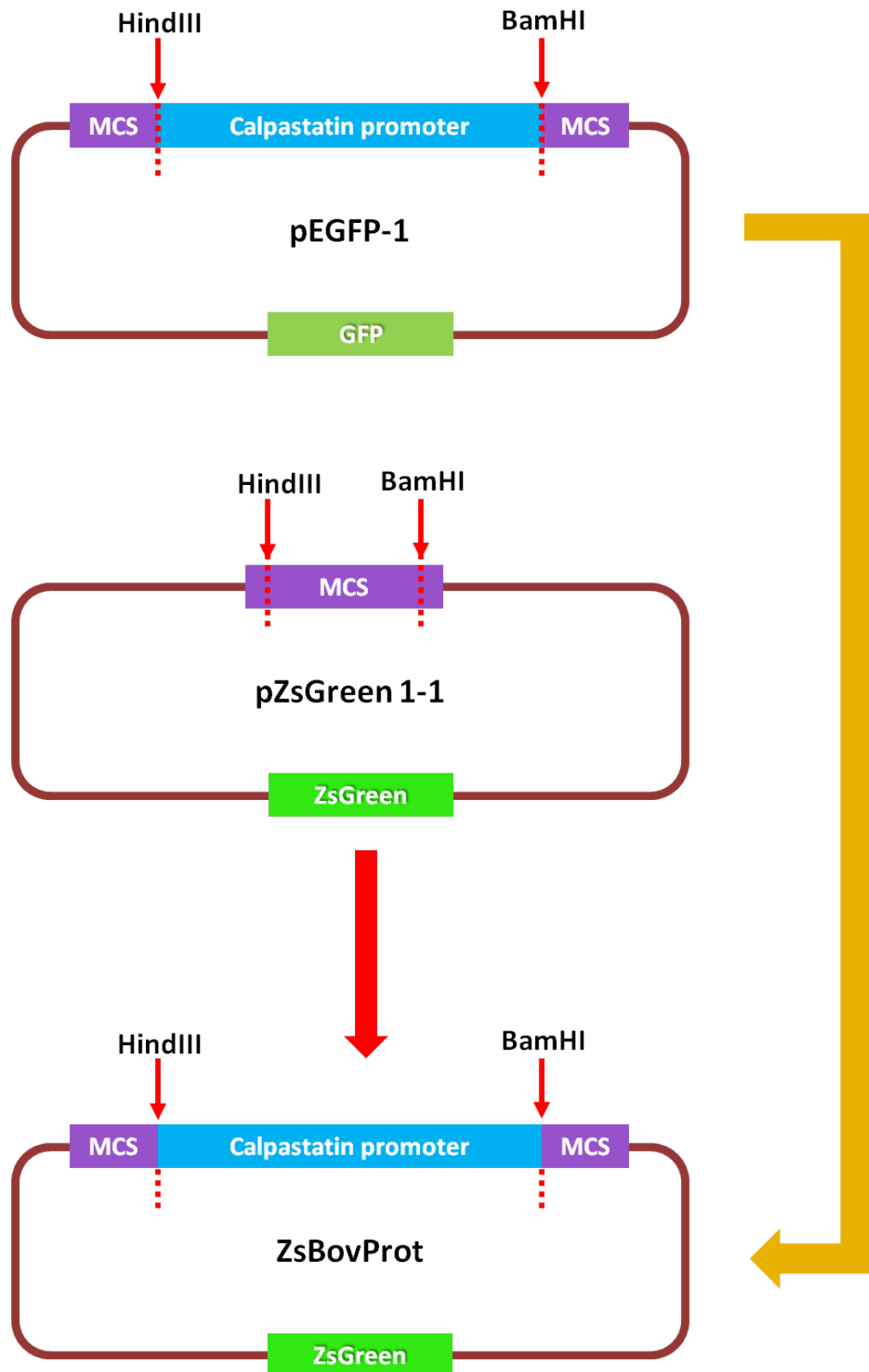


Figure 4.8 Replacement of GFP with pZsGreen based vector for calpastatin promoter study. Calpastatin promoter deletion sequences were released from its original promoter expression vector pEGFP-1 using HindIII and BamHI restriction enzymes and re-cloned into the multiple cloning site of pZsGreen 1-1 vector.

4.5.2 Co-transfected pDsRed-Express -N1 as a transfection marker

A suitable co-transfection marker was considered necessary to normalized fluorescence measurement from experiment involving study of promoter activity and gene expression. Reporter activity from the co-transfected marker can be used to indicate the efficiency of transient transfection (Schagat, 1996). Therefore in this study, pDsRed Express-N1 (Clontech) reporter vector was used as a transfection marker. The unmodified pDsRed Express N-1 plasmid contains CMV promoter that continuously expresses DsRed Express protein in the host cells. The pDsRed Express N-1 transfection marker needs to be co-transfected with bovine CAST promoter expression vector in order to normalize the expression value, therefore reducing variability in the data by factoring transfection efficiency in the transfected cells.

4.5.3 Effect of co-transfection (pDsRed Express N-1 Interaction) with ZsGreen based bovine CAST promoter vector (Typhoon Trio)

Before the study of bovine CAST promoter expression could be performed comprehensively, an initial experiment was set up to study the effect of co-transfecting pDsRed Express N-1 vector into a host cells with another ZsGreen based promoter expression construct. The main objective was to optimize the appropriate quantity of co-transfected vector with the EGFP based bovine CAST promoter expression vector, therefore reducing the interaction between both vectors in the host cells. In the previous section, initial experiment had been performed to compare ZsGreen fluorescence and GFP expression where ZsGreen had been reported to have greater fluorescence intensity. Therefore, the change had been made to use ZsGreen. In addition a more reliable and sensitive method was utilised for fluorescence based quantification and analysis of promoter activity. In previous experiments quantification using the fluorescence plate reader involved detaching of cells and transferring them into a new non-transparent white plate. This procedure introduced variation in replicates reading thus increasing the standard error means value for statistical analysis. For this reason a switch was made to future experiment involving fluorescence quantification was carried out using Typhoon Trio Fluorescence Imager (GE Healthcare) as fluorescence quantification could be performed directly on the same plates where the cells had been transfected. As a result there was a reduction in the variation from replicates thereby increasing the value of recorded data for statistical approach in analysing promoter activity.

The aim in this experiment was to find the right ratio of the two transfected vectors in order to reduce the interaction effect thus providing better and reliable results to quantify promoter expression. The interaction of co-transfected pDsRed Express N-1 vector on ZsGreen based vector expression was carried out using Typhoon Trio (GE Healthcare). The main objective of this experiment was to analyse interaction between the transfection marker

(pDsRed Express N-1) and the test ZsGreen based vector (pZsGreen 1-1 and pZsGreen 1-N1) and at the same time to establish a reliable method for analysis of bovine CAST expression in cell line through the use of a co-transfected control vector for normalisation of transfection efficiency

The interaction effect of pDsRed Express N-1 transfection marker was tested on the expression of pZsGreen 1-1 (negative control) and pZsGreen 1-N1 (ZsGreen positive). In order to investigate whether co-transfection of the pDsRed Express N-1 interfered, various quantities of this vector, ranging from 200ng to 2ng per well, were used for co-transfection with 200ng per well of negative control vector (pZsGreen 1-1) and ZsGreen positive vector (pZsGreen 1-N1) per well (Figure 4.9). The tested pZsGreen 1-N1 vector and pDsRed Express N-1 contained a CMV promoter to express both ZsGreen and DsRed fluorescence whilst the negative control vector (pZsGreen 1-1) did not contain any promoter sequences to express its fluorescence. In general, there was no effect of transfection marker on the expression of the negative control vector as promoter activity remains similar when differing quantities of pDsRed Express N-1 vector was co-transfected with 200ng per well of the negative control ZsGreen vector ($P>0.05$). This was the case in all transfected cell line C2C12, COS7 and HEK 293 cells.

However when the above experiment was repeated by replacing the negative control ZsGreen vector for one which contained a CMV promoter 5' to the ZsGreen there was an apparent interaction between the level of fluorescence generated from positive control ZsGreen and the co-transfected DsRed vector. For all the cells tested there was a significant difference ($P<0.05$) in the level of ZsGreen fluorescence signal dependent on the level of co-transfected pDsRed express N-1 vector. The effect of interference on the ZsGreen signal appeared to be an increase in the fluorescence recorded with the highest signal being at 100ng, 20ng and 50ng per well of pDsRed express N-1 co-transfection in C2C12, COS7 and HEK293 cells respectively.

ZsGreen positive vector reached its peak in its fluorescence value when the amount of co-transfected pDsRed Express N-1 vector was reduced from 200ng per well (well plate). In C2C12 cells, ZsGreen positive vector reach its highest expression in its fluorescence value when the quantity of co-transfected (pDsRed Express N-1) was reduced to 100ng per well while in COS7, ZsGreen vector reached its highest expression value at 20ng per well of co-transfected vector (Figure 4.9a). Lastly for HEK293 cell line, ZsGreen positive vector expression was at its maximum when the quantity of co-transfected vector was reduced to 50ng per well (Figure 4.9c). At this point where ZsGreen expression reached its peak, it was assume that the interaction between both vectors (ZsGreen positive and pDsRed Express N-1) were at its highest. At the lower levels of co-transfected pDsRed Express N-1 the value of ZsGreen expression was maintained at constant level in the interaction effect on ZsGreen positive was at the lowest when below 10-5ng per well co-transfected pDsRed Express N-1 vector was used.

It was concluded that there was an interaction between the transfection marker and the ZsGreen positive promoter expression vector ($P < 0.05$). Therefore, using the data from the interaction experiment, future transfection experiment involving bovine CAST gene promoter expression would be carried out using 5ng per well of transfection marker (pDsRed Express N-1) combined with 200ng per well of test vector (ZsGreen Bovine CAST promoter expression vector) in all cell line as at the concentration interaction between both vector was found to be at its lowest for C2C12, COS7 and HEK293 cell line.

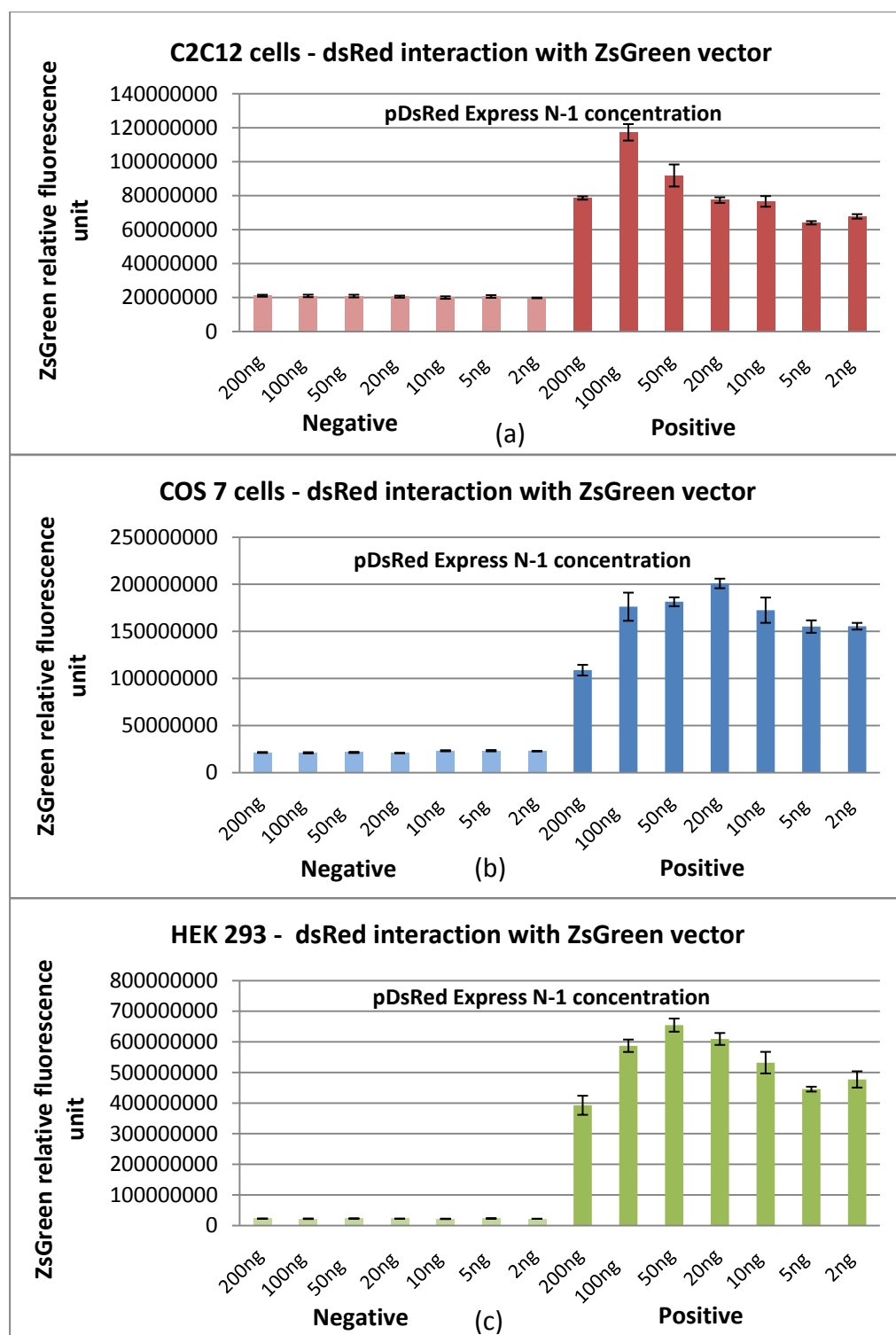
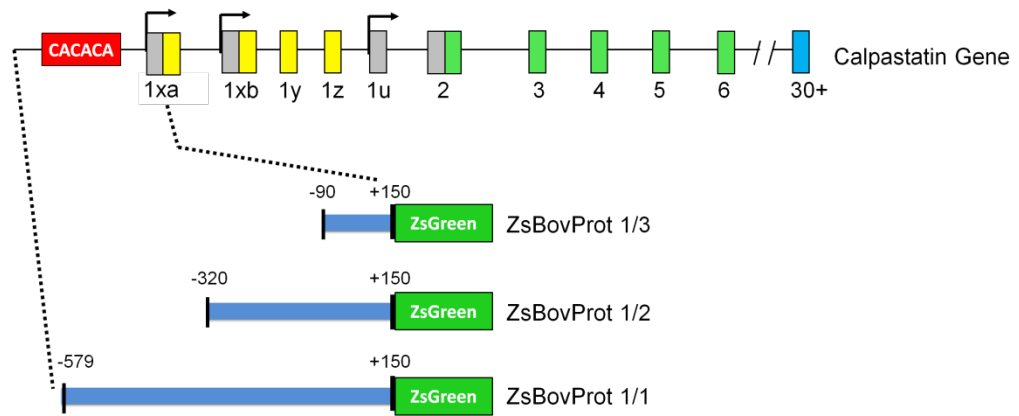


Figure 4.9 The effects of co-transfection with pDsRed Express N-1 on the fluorescence signal generated by vectors expressing 200ng ZsGreen per well. The interacting effects of ZsGreen vectors and incremental levels of pDsRed Express N-1 in A) C2C12 cells B) COS7 cells, C) HEK293 cells. Cells were seeded and transfected at 70% confluence with 200ng of pZsGreen 1-N1 (Positive) and pZsGreen 1-1(Negative) vector with variable amount of pDsRed Express N-1 (200ng, 100ng, 50ng, 20ng and 10ng). They were then incubated for 48 hours before fluorescence readings were taken for ZsGreen using Typhoon Trio examining fluorescence omitted at excitation wavelength of 493nm and emission wavelength of 505nm. Error bars are SEM and n=3. ANOVA was used to analyse the data.

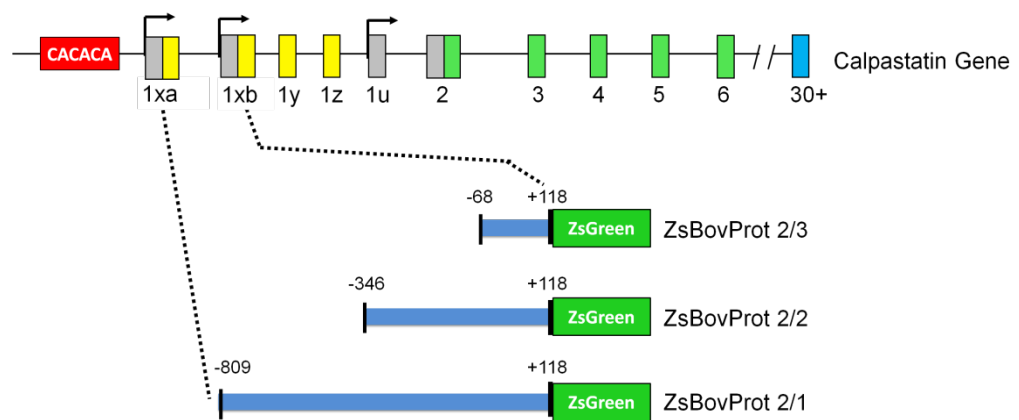
4.6 Activity of the bovine CAST promoters

In the previous experiment, bovine CAST promoter constructs were cloned into ZsGreen based promoter expression vector to increase sensitivity of fluorescence measurement (Section 4.5.3). Therefore, the CAST promoter construct of ZsGreen based reporter vector was used in this transfection experiment in order to quantify promoter activity from bovine CAST gene. In this experiment, activity of the bovine CAST promoters 5' to exons 1xa, 1xb and 1u were quantified using a new established method developed using Typhoon Trio (section 2.17.2). The main objective of this experiment was to characterize activity of the three main CAST promoters in mouse myoblast cells day 3 post-transfection (C2C12) and monkey kidney fibroblast cells (COS7) using the appropriate internal reporter vector co-transfection control (pDsRed Express N-1).

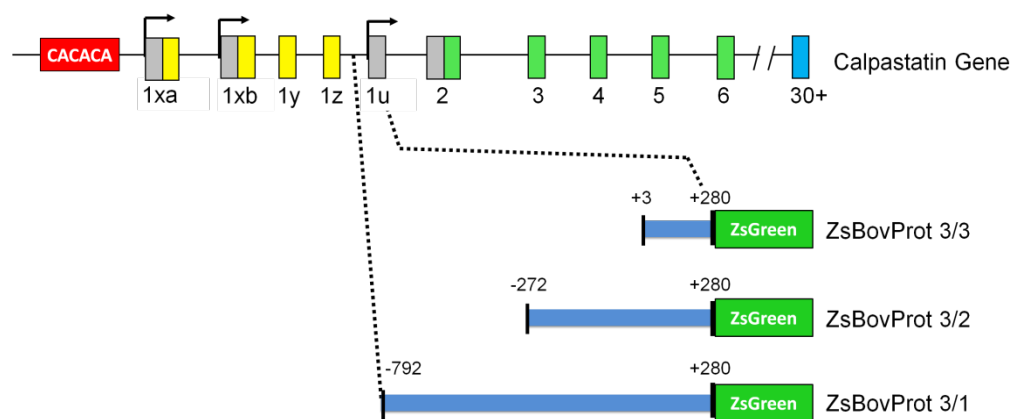
The ZsGreen based bovine CAST promoter vectors were developed to contain 5' deletions from the promoter regions adjacent to exon 1xa (ZsBovProt 1), exon 1xb (ZsBovProt 2) and exon 1u (ZsBovProt 3) (Figure 4.10). These constructs were transfected into C2C12 and COS7 cells along with pDsRed Express N-1 as internal reporter vector to indicate transfection efficiency. The quantification of fluorescence signal from expressed promoter vector was made 48 hours after transfection. In order to present the data, the ZsGreen expression value was normalized for its transfection efficiency using the expression of co-transfected pDsRed Express N-1 vector with the normalised values expressed relative to the pZsGreen 1-N1 (positive control) which was taken as being expressed at 100%. Overall, expression from CAST promoter deletions constructs was found to be significantly higher in C2C12 cells than COS7 cells ($P < 0.05$). This was contrary to previous report (Raynaud et al., 2005) where expression of CAST reported to be more prominent in COS7 cell lines than C2C12 cells.



(a)



(b)



(c)

Figure 4.10 ZsGreen based bovine calpastatin promoter construct made from 5' deletions sequence adjacent to the promoter region of exon 1xa, 1xb and 1u. (a) 5' deletions promoter construct made from promoter 1xa (b) 5' deletions promoter construct made from promoter 1xb (c) 5' deletions promoter construct made from promoter 1u. Numbering indicates nucleotide position relative to the start site of transcription.

4.6.1 Analysis of CAST promoter activity from 5' deletions promoter constructs in C2C12 cells

In C2C12 cell lines at 2 days post-transfection, all ZsGreen based 5' deletions constructs from the promoter regions associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were confirmed to be functional and was found to be significantly higher than negative control (pZsGreen 1-1) analysis ($P<0.05$). As shown in Figure 4.11, the longest construct, ZsBovProt 1/1 from exon 1xa promoter region produce the highest promoter activity of the three deletions construct ($P<0.05$). Deletion of 260bp sequence from the 5' end of the promoter region in ZsBovProt 1/1 to give ZsBovProt1/2 significantly ($P<0.05$) reduced the promoter activity. Within the deleted sequence, several trans-acting factors binding motifs sequences were identified such as four SRY, USF, Pbx-1, CdxA, C/EBPb, C/EBPa, C/EBP two GATA-1, two GATA-2, GATA-3, AP-1 and CP2 (appendix 16). Within this the deleted sequence was the short CA tandem repeat which had been previous identified in the bovine CAST promoter region (Cong et al., 1998) and had also been shown to be of variable length in different breeds of cattle (Nonneman et al., 1999). The shortest deletion construct for the promoter region of exon 1xa, ZsBovProt1/3 still retained its core promoter activity in C2C12 cell line ($P<0.05$). The sequence deleted from ZsBovProt1/2 contained multiple motifs including two GATA-1, GATA-3, two SP-1 and NFkB site (appendix 16). However, this deletion did not significantly affect the expression of CAST promoter ($P>0.05$) as expression remaining relatively similar to ZsBovProt1/2. The similar characteristics of ZsBovProt1/3 promoter expression with the second construct (ZsBovProt1/2) could be explained due to several transcription factor binding motifs sites remaining in the CAST promoter sequence such as two Sp1, three GATA-1, GATA-2 and GATA-3 site.

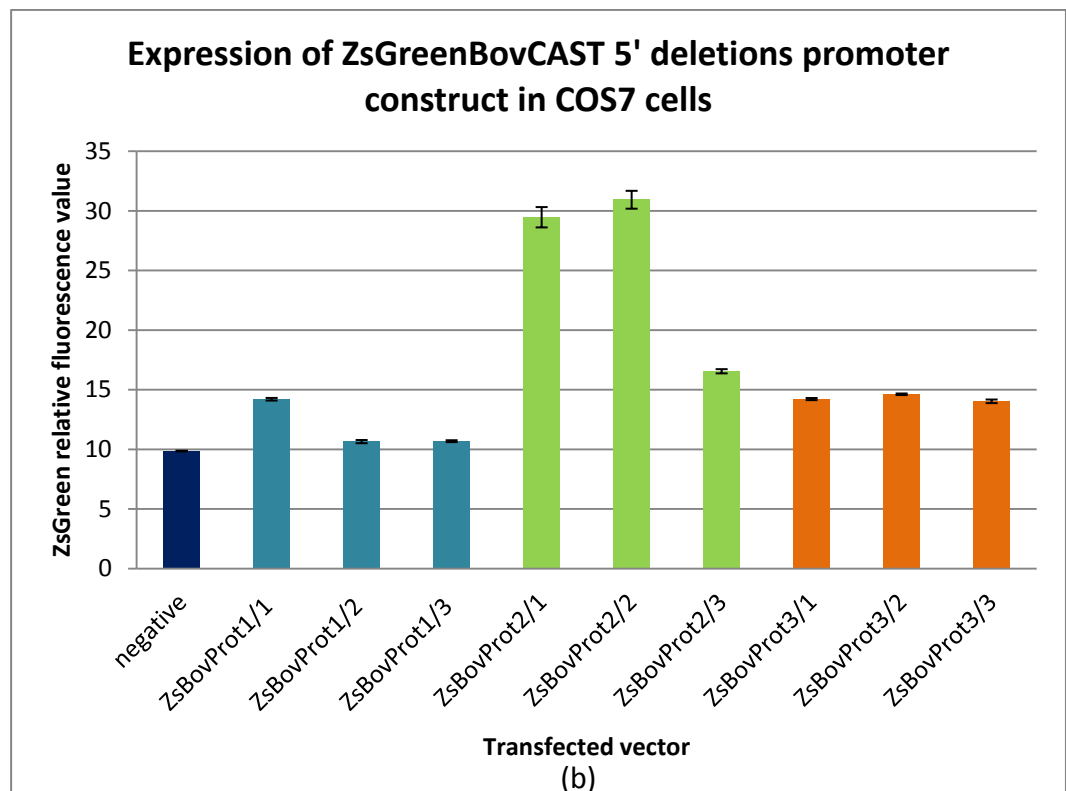
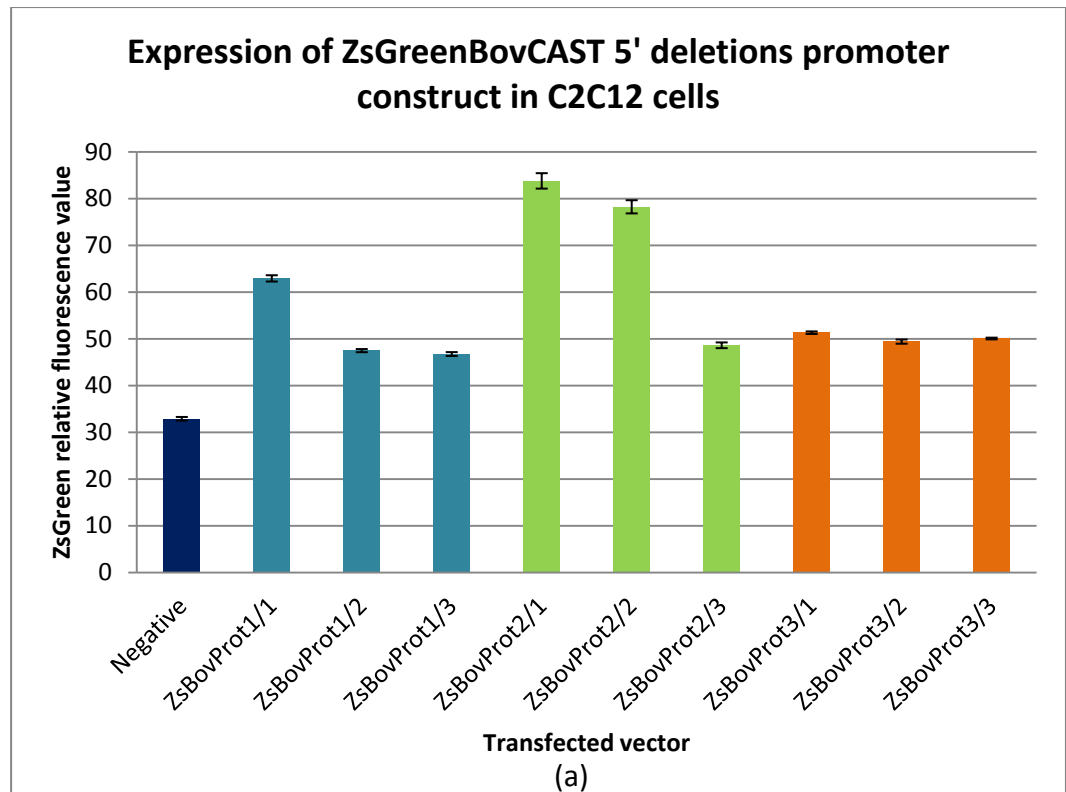


Figure 4.11 5' Deletion analysis of bovine CAST promoter activity. 5' deletion construct of CAST promoter region associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) and associated 5' deleted sequences were independently transfected into C2C12 (a) and COS7 (b) cell line. Transfection was carried out in cells at 70% of confluence and assessment of fluorescence was performed at 48 hour post-transfection. Error bars are SEM and n=3. ANOVA with post-hoc (bonferroni test) was used to analysed the data

The highest CAST promoter activity in C2C12 cells was recorded from the longest exon 1xb promoter construct, ZsBovProt2/1 as well as the second sequential 5' deletion ZsBovProt2/2 ($P<0.05$). The expression from ZsBovProt2/2 promoter was significantly decreased, ($P<0.05$) although slightly when compared to ZsBovProt2/1 activity, as 463bp 5' promoter sequences were deleted from the construct. The promoter activity of ZsGreenBovProt2/2 was still significantly high compared to other CAST promoter construct in C2C12 cell lines as the promoter region contained transcriptional factor binding protein sites such as the stacked binding site of GATA-1, GATA2 and GATA-3 site followed by multiple SP1 motifs located 5' of ZsBovProt2/2 sequence (appendix 17). Promoter activity in third deletion construct ZsBovProt 2/3, declined significantly ($P<0.05$) with activity reduced to almost half from the longest 1xb promoter construct. Within the region deleted were six Sp1 sites. Similar results of significant loss of promoter activity had also been reported in the previous study (Raynaud et al., 2005). The construct containing the longest region from the promoter associated with exon 1u, ZsBovProt 3/1 construct possessed the weakest promoter activity in C2C12 cells when compared with other similar promoter constructs derived from exon 1xa and 1xb. The promoter construct contains several GATA-1, SRY, and CdxA sites as well as one for NFkB (appendix 18). Deletion analysis of 1u promoter region did not produce any significant changes on quantified promoter activity with deletion constructs made from promoter 1u displaying promoter activity significantly higher than negative control ($P<0.05$). This finding was again in agreement with the study carried out previously using deletion construct made from promoter 1u (Raynaud et al., 2005) albeit these observations were from studies carried out in COS7 cell line. These findings suggest that the presence of transcription factor binding sites on sequences 5' to exon 1u may not be responsible or functionally active in regulating transcription of Type III CAST as expression of exon 1u deletion construct indicated no significant difference.

4.6.2 Analysis of CAST promoter activity from 5' deletions promoter construct in COS7 cells lines

Expression of ZsGreen based bovine CAST promoter construct in COS7 cell line was found to be significantly lower than in C2C12 cell line. This result was in contrary with the findings from previous reports where transcriptional activity of CAST promoter was higher in COS7 cells than in C2C12 cells (Raynaud et al., 2005). There are a few possible explanations from these results. At the time of the experiment, both cell types C2C12 and COS7 cells were set up so that transfection can be performed when the seeded cells reached 70% to 80% confluency. C2C12 cells were known to have a faster proliferating rate compared to the COS7 cells. The doubling time for C2C12 cells was reported as 12 hours (Bardouille et al., 2001) whereas COS7 cell lines was observed to have longer doubling time of 24 hours. The proliferation rate of C2C12 cell lines which was higher may contribute to a higher expression level of CAST promoter in the cell line compared to COS7. There was also possibility that bovine CAST gene was exhibiting preferential expression of the transcript which had been indicated by a tissue specific expression of the gene where its promoter activity was recorded higher in mouse myoblast cells (C2C12) than in monkey kidney fibroblast cells (COS7).

The highest activity of CAST promoter in COS7 cells was recorded from the construct of putative 1xb exon promoter (Figure 4.11b). This characteristic was similar to the results of CAST promoter expression in C2C12 cells as putative promoter from exon 1xb recorded the highest activity compared to other putative promoter construct (Figure 4.11a)

Longest construct from exon 1xa promoter region, ZsBovProt1/1 was reported to be functional in COS7, as promoter activity was observed as significantly higher than negative control vector ($P < 0.05$). However, further deletions of 5' sequences from 1xa promoter region rendered a very low expression from ZsBovProt1/2 and ZsBovProt1/3 deletion construct which was not significantly different to the negative control ($P < 0.05$). This finding however was contrary to the results from the expression of this promoter in

C2C12 cell line as construct ZsBovProt1/2 and 1/3 still retained its core promoter activity when compared to the negative control.

In general, the expressions of deletion constructs from exon 1xb were significantly higher than negative control ($P < 0.05$). The longest promoter construct ZsBovProt2/1 reported the highest value of promoter activity in COS7 cell line. Further deletions of 463bp sequences in ZsBovProt 2/2 promoter construct did not significantly affect its activity as the core promoter activity remained strong. There was no differential in the expression between ZsBovProt2/1 and ZsBovProt2/2 construct ($P > 0.05$). Promoter activity from the shortest deletion constructs ZsBovProt2/3 was reduced significantly ($P < 0.05$) compared to ZsBovProt2/1 and ZsBovProt 2/2 deletion construct. The pattern of promoter activity from 1xb deletion construct was the same as the result found in C2C12 cells as promoter activity from the two longest constructs were closely similar in expression value and reduction in promoter activity was only seen in the shortest construct as it lost motifs such as GATA-1, GATA-2 and SP1 sites from the promoter sequence (Appendix 17).

CAST promoter deletion constructs from exon 1u in C2C12 cell recorded expression values which were significantly higher than the negative control vector ($P < 0.05$). However, there was no differential in transcriptional activity between deletion construct of ZsBovProt3/1, ZsBovProt 3/2 and ZsBovProt3/3. Promoter activity from the longest construct (ZsBovProt3/1) which contains 1072bp upstream 5' sequences was not significantly different compared to the expression from the shortest construct ZsBovPro3/3 which had 277bp 5' 1u promoter sequences. This was again consistent with expression of this deletion construct in C2C12 cells.

4.6.3 Summary of bovine CAST promoter expression in C2C12 and COS7 cell lines

In conclusion, bovine CAST promoter constructs developed using ZsGreen based fluorescence reporter vector was proven to be functional in mouse myoblast (C2C12) and monkey kidney fibroblast (COS7) cells (Figure 4.11) with all the full length putative calpastatin promoter constructs from exon 1xa, 1xb and 1u displayed promoter activity which was significantly higher compared to the negative control ($P \leq 0.05$). The characteristic of promoter expression exhibited was similar to the results of Raynaud et al. (2005). On the other hand, bovine CAST promoter expression was found to be more prominent in C2C12 cells than in COS7 cells. Analogous to Raynaud et al. (2005) the ZsBovProt2/1 construct of 1xb promoter showed the highest activity in both cell types. There was no differential in promoter expression between ZsBovProt1/1 and ZsBovProt 3/1 in COS7 cells although there was significant difference in C2C12 cells with ZsBovProt1/1 recorded 16.18% higher activity than ZsBovProt1/1 ($P < 0.05$).

4.7 Responsiveness of bovine CAST promoter towards treatments of secondary messenger for anabolic agents

An increase in muscle mass by skeletal muscle hypertrophy can be induced in mammals by administration of β -adrenergic agonist and growth promoters. Previous studies had shown that administration of β -adrenergic agonist in animal could affect the overall expression of CAST gene, which consequentially led to a negative effect on meat quality due to inhibition of calpain by calpastatin protein (Killefer and Koohmaraie, 1994; Koohmaraie et al., 1991a). The increased level of CAST specific mRNA in bovine was demonstrated in cattle administered with cimaterol (Parr et al., 1992). The effect of β -adrenergic stimulation was also shown in porcine, as the level of CAST mRNA and protein translation was significantly increased in skeletal and cardiac muscle of pigs administered with clenbuterol treatment (Parr et al., 2001). It was proposed that β -adrenergic stimulation may act via cyclic adenosine monophosphate (cAMP) responsive elements (CRE) in CAST promoter region as administration of dibutyryl cAMP, a constitutive activator for protein kinase activity, may increase transcriptional activity from CAST promoter by phosphorylation of CRE binding protein and its binding to CRE site in CAST promoter region (Cong et al., 1998b) (Figure 4.13). Their study managed to characterise bovine CAST promoter expressions using transient transfection of promoter construct and identified the region which contains the CRE motif. The promoter sequence initially designated as promoter region of exon 1xa in the study was later confirmed as exon 1xb CAST promoter region by Parr et al. (2001). The exact location of CRE binding site identified by Cong et al. (1998b) was in the intronic region between exon 1xa and exon 1xb of bovine CAST gene sequence (Figure 4.12). This intronic region had been shown to contain other TF binding motifs such as GATA-1, GATA-2 and Sp1 (appendix 17), which could potentially affect transcriptional activity of the calpastatin gene.

Hypertrophy in skeletal muscle could also be mediated through calcineurin signalling pathway that is Ca^{2+} dependent (Semsarian et al., 1999) and potentially mediated through the transcription factors with GATA-2 and NF-ATc1 (Musaro et al., 1999). Therefore, it was concluded the importance of testing the effect of cAMP and calcium (Ca^{2+}) related stimuli on regulation and expression of calpastatin as the gene was found to be elevated in hypertrophic growth and associated with meat toughness. In the following experiments, agonists which activate protein kinase A (PKA) activity and subsequently potentially mediate their effects on gene transcription through CRE motifs were used to further assess the responsiveness of CAST promoters. In addition, calcium ionophore was also used to investigate its effect on the activity of bovine CAST promoter, potentially being mediated through Ca^{2+} signalling pathway such as that associated with calcineurin or through the presence of GATA-2 motifs in the promoter region.

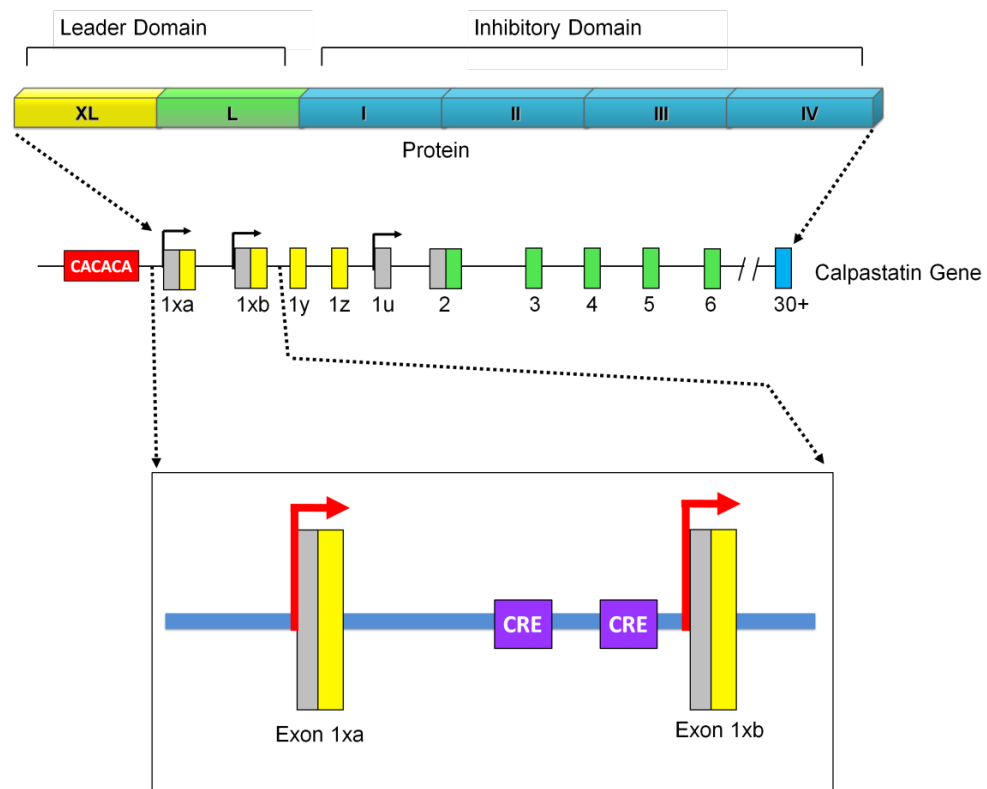


Figure 4.12 Location of potential CRE motifs identified in the intronic region of bovine calpastatin 1xb promoter region. The exact location of CRE motifs identified by Cong et al. (1998b) in the promoter region of exon 1xa was later confirmed by Parr et al. (2001) as the promoter region for exon 1xb for bovine calpastatin gene.

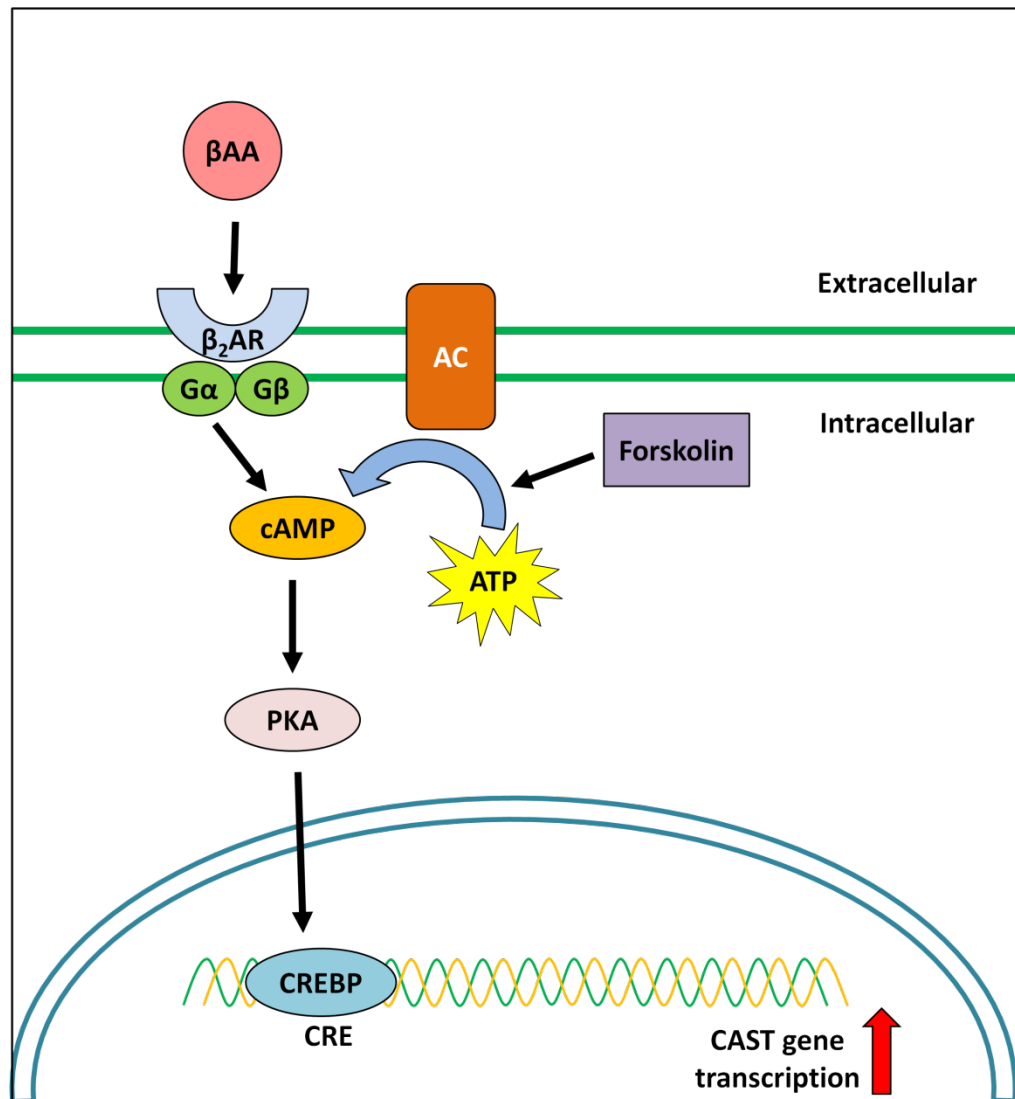


Figure 4.13 Proposed beta adrenergic agonist stimulation of calpastatin gene transcription via cAMP response element binding protein (CREBP) activated by phosphorylation of protein kinase A (PKA). Signalling pathway started with administration of β -adrenergic agonist (β AA) which will stimulate β_2 -adrenergic agonist receptor (β_2 AR) and subsequently activate $G\alpha$ protein followed by cAMP formation by adenylyl cyclase (AC), triggering phosphorylation of CREBP by PKA. Binding of CREBP to the CRE sites located in the promoter region will increase transcriptional activity of the gene.

4.7.1 Effect of dibutyryl cAMP treatment on bovine CAST promoter expression

Previous results had shown administration of dibutyryl cAMP (db cAMP), a activator in protein kinase signalling pathway, managed to increase transcriptional activity of bovine CAST promoter (Cong et al., 1998b). However in this experiment there was neither a proper positive control for the treatment or co-transfection control. The aim in this experiment was to characterise the response from the three main promoters 1xa, 1xb and 1u with the use of a treatment control towards treatment of db cAMP. Initial results from transfection experiment with three of cell lines HEK293, C2C12 and COS7 revealed that CAST promoter expression was more prominent in C2C12 and COS7 cells (data not shown). Therefore, these cell line of mouse myoblast (C2C12) and monkey kidney fibroblast (COS7) were used to compare response from CAST promoter towards treatment of anabolic agent in different tissue type and organisms of origin.

C2C12 and COS7 cells line were transfected with ZsGreen based bovine CAST promoter construct and then treated with 2mM dibutyryl cAMP 24 hours after transfection and responsiveness of CAST promoter 5' deletion constructs was measured using Typhoon Trio fluorescence imager after 24 hours of treatment. In order to validate findings of treatment with dibutyryl cAMP and to confirm its effectiveness, a positive control vector for treatment which was a GFP based CRE reporter vector (SABiosciences), was included in the experiment. The objective of this experiment was to determine whether bovine CAST promoter expression could be regulated through cAMP-activated process which are mediated through cAMP-dependent PKA as it have been proposed previously that the CAST gene is a target for PKA (Parr et al., 1992).

In general, expression of bovine CAST promoter in both C2C12 and COS7 cells was affected by a 24 hour treatment with 2mM dibutyryl cAMP (Figure 4.14 & 4.15). In both cell types the activity of all the bovine CAST promoter constructs displayed decreases in promoter activity relative to cells which had not been treated with dibutyryl cAMP. The transfected CRE reporter construct displayed significant increase in its promoter activity with administration of dibutyryl CAMP in both cell types ($P<0.05$) indicating the effectiveness of the treatment.

In C2C12 cells, there was a significant decrease in activity for the promoter region associated with exon 1xa in ZsBovProt1/2 and the shortest construct, ZsBovProt 1/3 ($P<0.05$) (Figure 4.14). However, there was no significant effect on transcriptional activity of the longest construct in exon 1xa when treated with dibutyryl cAMP relative to the non treated transfected cells($P>0.05$). Deletion of transcriptional factor binding motifs from the second longest construct of exon 1xa (ZsBovProt1/2) such as SRY, GATA-1, GATA-2, GATA-3 and AP-1 site including the short CA tandem repeat sequences presumably triggers the significant reduction in promoter activity when treated with dibutyryl cAMP as this reduction was not observable in the longest exon 1xa construct (ZsBovProt 1/1). Transcriptional activity was also reduced with dibutyryl cAMP treatment for construct associated with exon 1xb, as ZsBovProt2/1 and ZsBovPort 2/2 displayed significant reduction in its promoter activity ($P<0.05$) relative to the nontreated transfected cells. The shortest construct from the promoter region associated with exon 1xb, ZsBovProt2/3 decreased its promoter activity compared to the non-treated transfected cells, however this was not statistically significant.

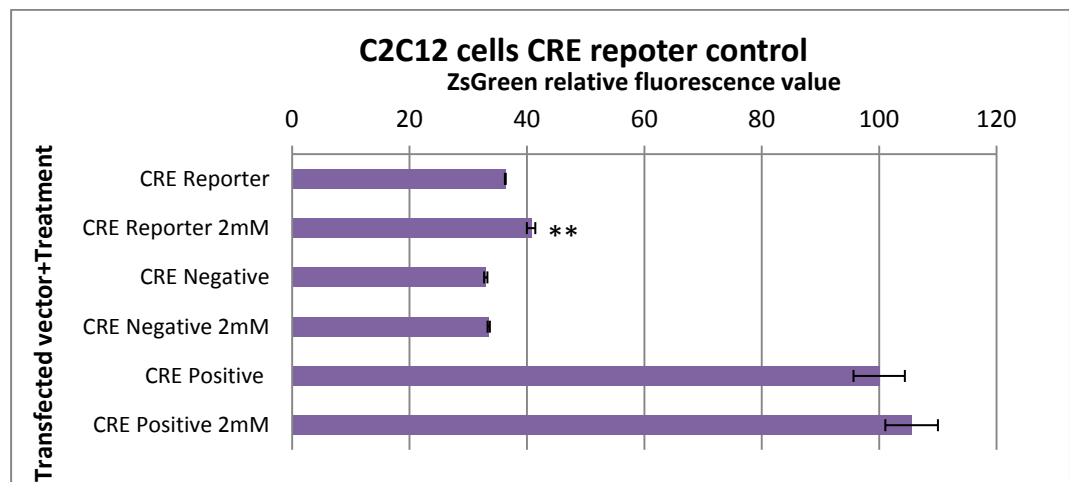
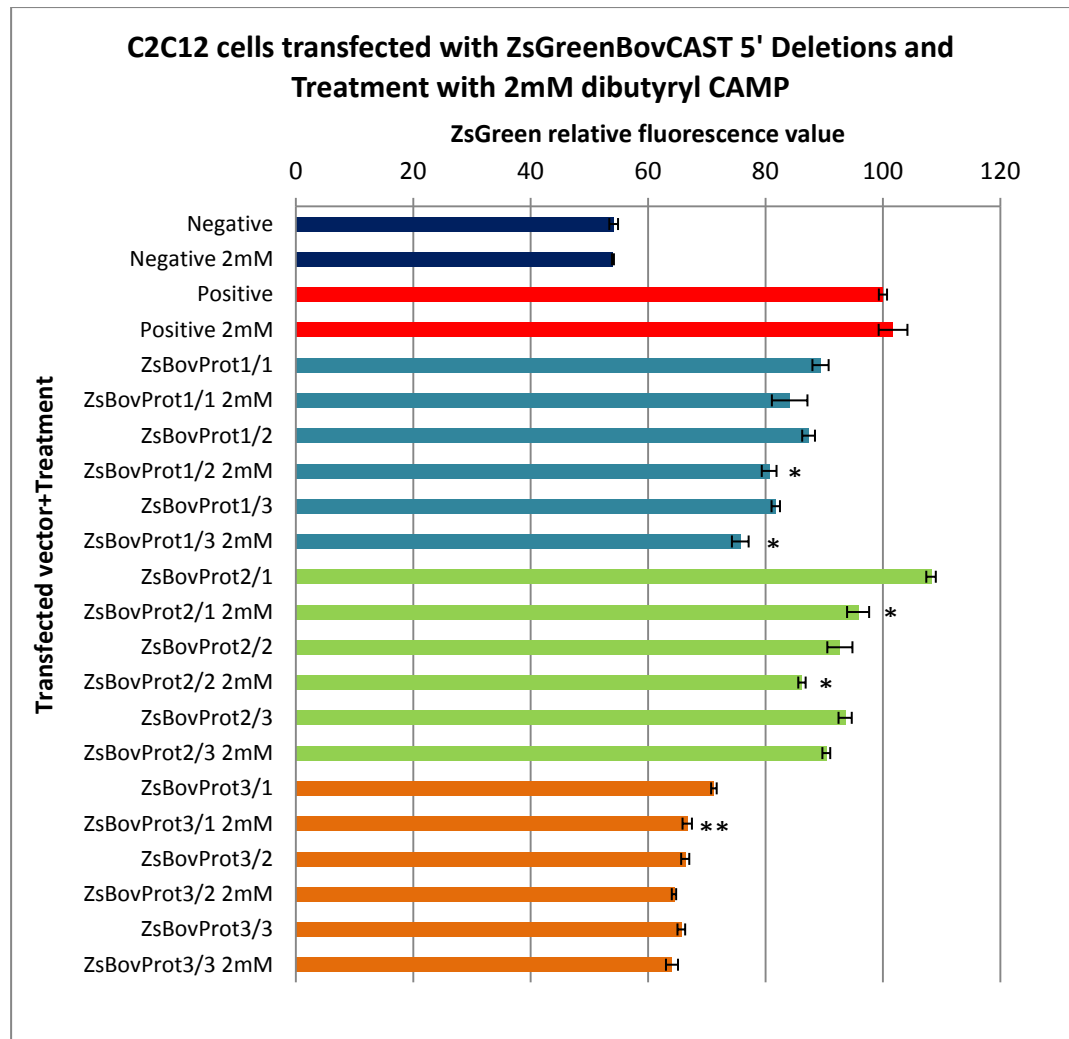


Figure 4.14 The effect of 2mM dibutylrlyl cAMP treatment on bovine CAST promoter activity in mouse muscle C2C12 cells. 5' deletion construct of CAST promoter region associated with exon 1a (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into C2C12 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 2mM dbcAMP for 24 hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * ($P < 0.05$), ** ($P < 0.01$). Error bars are SEM and $n = 3$. T-Test was used to analyse the data.

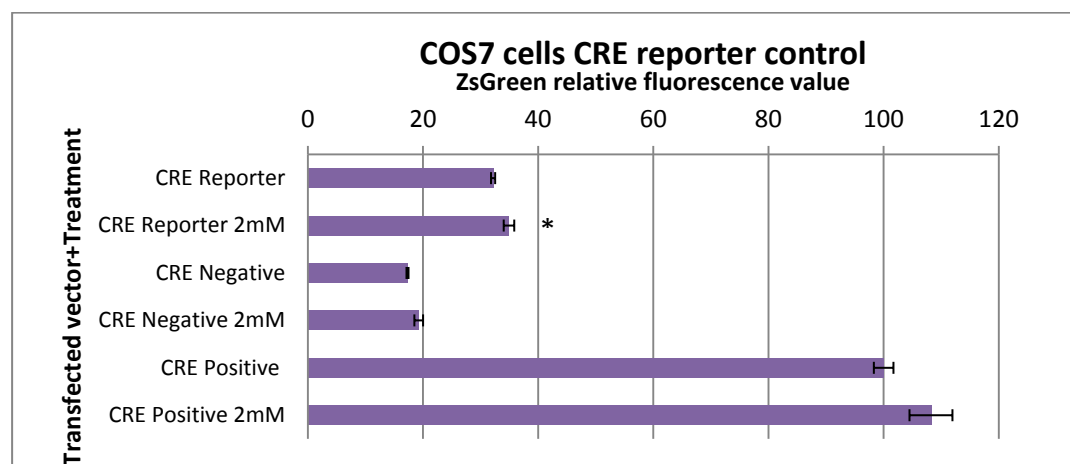
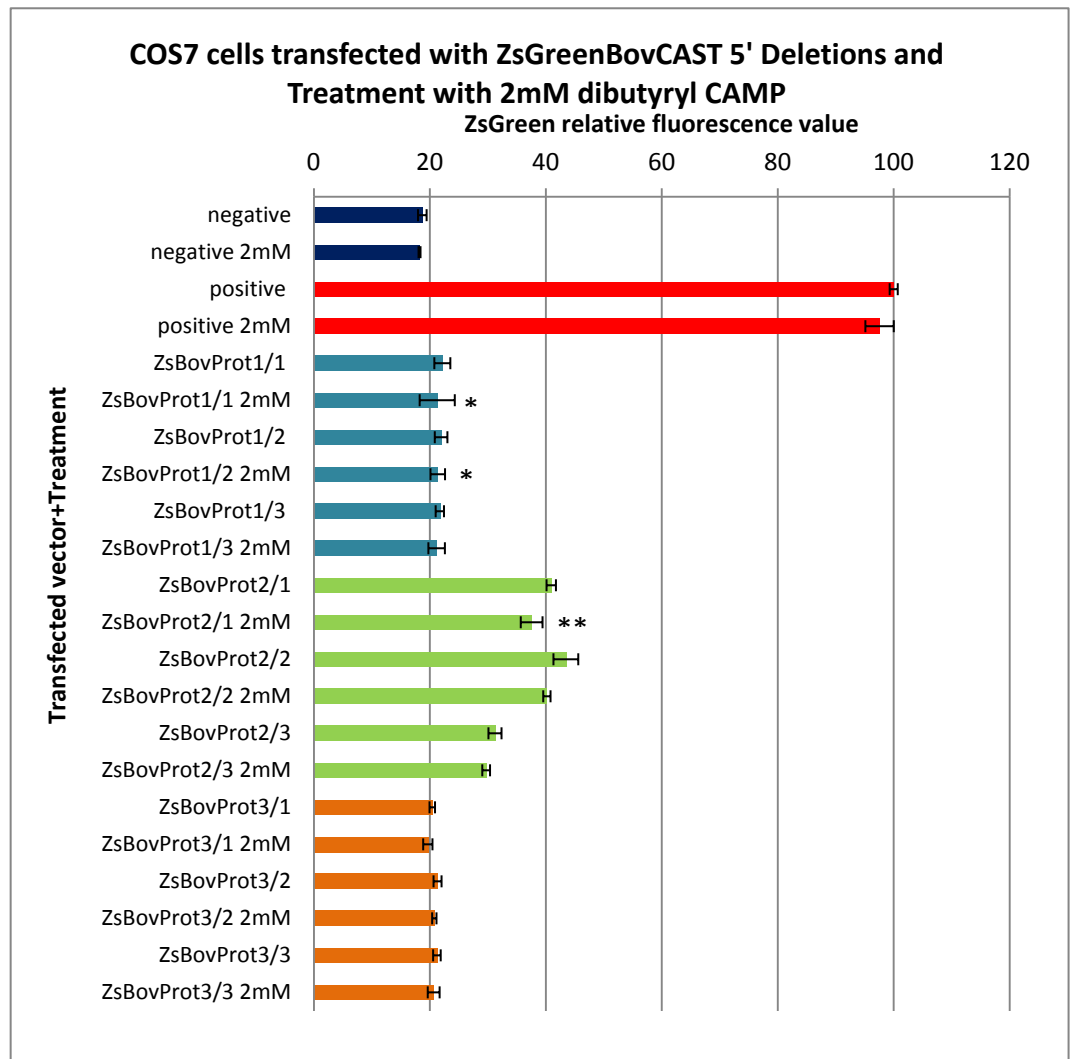


Figure 4.15 The effect of 2mM dibutyryl cAMP treatment on bovine CAST promoter activity in monkey kidney COS7 cells. 5' deletion construct of CAST promoter region associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into COS7 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 2mM dbcAMP for 24hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * ($P < 0.05$), ** ($P < 0.01$). Error bars are SEM and $n = 3$. T-test was used to analyse the data.

Furthermore, promoter construct ZsBovProt3/1 which contains longest promoter region associated with exon 1u recorded significant decrease ($P<0.01$) in its promoter activity when treated with dibutyryl cAMP. The shorter promoter constructs from exon 1u, ZsBovProt 3/2 and ZsBovProt 3/3, however displayed no significant differences compared to the non-treated promoter construct.

Reduction in promoter activity was also demonstrated in bovine promoter construct transfected in COS 7 cells line albeit there was lower promoter activity observed from CAST promoter transfection in the cell line (Figure 4.15). Reduction in activity was observable from the two longest construct in exon 1xa, with ZsBovProt 1/1 and ZsBovProt 1/2 recorded significant reduction in their promoter activity ($P<0.05$). For the promoter region associated with exon 1xb, only the longest construct displayed a significant decrease in its promoter activity ($P<0.05$) with treatment with dbcAMP. In contrast to observation in C2C12 cells, there was no significant difference in promoter activity from 1u promoter construct when administered with dibutyryl CAMP. This was shown on all deletions construct from promoter 1u as deletions of 5' sequences did not appear to affect responsiveness of promoter activity in response to dibutyryl CAMP treatment.

When compared between cell lines, transfection of CAST promoter constructs into C2C12 cells exhibit more sensitivity towards dibutyryl cAMP treatment as more 5'deletion CAST promoter constructs displayed reduction in their promoter activity with higher significance level from statistical test ($P<0.01$) although this did not define magnitude of the response from the treatment.

4.7.1.1 Summary of bovine CAST promoter responsiveness towards administration of dibutyrylCAMP

To conclude, administration of dibutyryl cAMP on bovine CAST promoter was capable to suppress transcriptional activity of the tested promoters. The suppression of transcriptional activity were observed generally in all promoter construct including from promoter responsible for transcription of Type I, II and III calpastatin and the effect was more noticeable in mouse myoblast cell lines (C2C12) than in monkey kidney fibroblast cells (COS7). Furthermore, the effect of suppression observed in bovine CAST transcriptional/translational of promoter activity differs depending on the types of promoter and cells used for transfection. Interestingly, the bovine promoter deletion construct from exon 1xb which is reported to contain two cAMP responsive elements (Cong et al., 1998) did not demonstrate elevation in its promoter activity when subjected to dibutyryl CAMP although the promoter deletion construct derived from this experiment (ZsBovProt2/1, 2/2 and 2/3) contains the same CRE region as proposed in the previous study. In order to verify the effectiveness of dibutyryl CAMP treatment, (CRE reporter vector) was included as a positive control for treatment in the experiment. Finally, the Inclusion of CRE reporter vector in C2C12 and COS7 cells transient transfection experiment managed to provide an indication of the effectiveness of dibutyryl cAMP treatment on promoter construct.

4.7.2 Effect of forskolin treatment on bovine CAST promoter expression

In the previous section, responsiveness of bovine CAST promoter had been tested with the administration of cAMP homolog, dibutyryl cAMP an activator for PKA in C2C12 and COS7 cells. The findings in the experiment indicate suppression CAST promoter transcriptional activity when it was subjected to the treatment. Therefore, to further confirm these results, cells transfected with bovine CAST promoter construct will be treated with forskolin, an agent which stimulates formation of cAMP through activation of adenylate cyclase.

The responsiveness of bovine CAST promoter towards forskolin treatment were measured using C2C12, mouse myoblast cells and COS7, monkey kidney fibroblast cell lines as host for transient transfection of calpastatin ZsGreen based promoter construct. Transfected promoter constructs were treated with 10 μ M forskolin an agent which causes the increased formation of endogenous cytosolic cAMP. Treatment was performed on day 1 post-transfection for 24 hours before fluorescence was quantified. The objective of this experiment was to determine whether bovine CAST promoter expression was influenced by changes in endogenous cAMP levels mediated by the administration of forskolin. As had been shown in the previous section it was apparent that administration of cAMP analogue dbcAMP was able to influence the level of activity of the promoters associated with exons 1xa, 1xb and 1u.

In general, forskolin treatment on C2C12 and COS7 cell lines transfected with bovine CAST promoter deletion construct did not significantly affect transcriptional activity of most CAST promoter activity (Figure 4.16 & 4.17). This was shown in C2C12 cells as only the longest construct from promoter 1xb (ZsBovProt 2/1) displayed significant reduction in its promoter activity ($P < 0.01$). The CAST promoter constructs derived from the promoter regions 5' to exon 1xa and 1u did not have any significant response to administration

of forskolin, neither did any of the 5' deletion constructs from these promoters.

Furthermore, in COS 7 cell lines, the effect of forskolin treatment on CAST promoter construct was only visible in exon 1u promoter deletion construct where a significant decrease in promoter activity was observed in ZsBovProt 3/2. This reduction was not observable in C2C12 cell lines. Decrease in promoter activity was also recorded from expression of ZsBovProt 3/1 and ZsBovProt 3/3 in COS7 cell lines although it was later determined as not significant when compared to the untreated transfected cells.

Interestingly, CRE reporter which acted as a positive control for treatment in both cell types was reported to be highly elevated with the administration of forskolin ($P < 0.0001$) and the elevation of CRE reporter activity was apparent in C2C12 cells.

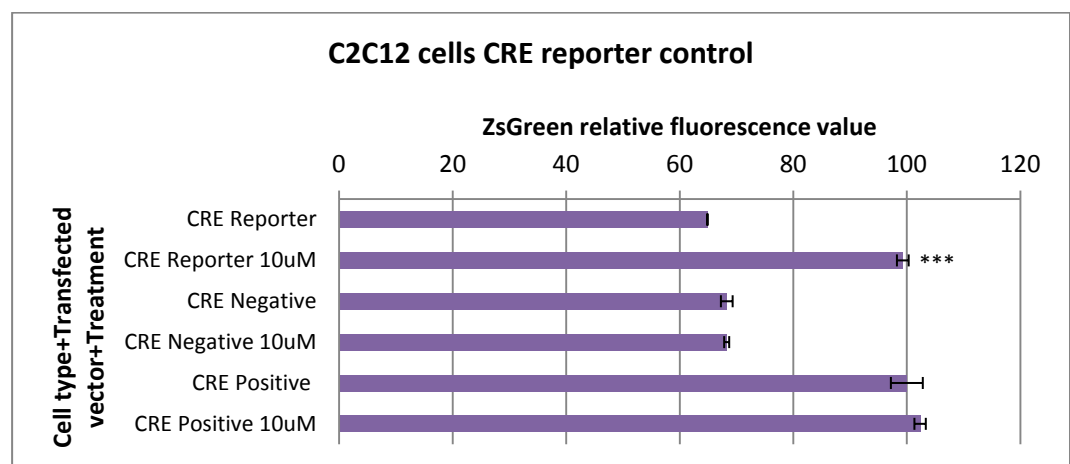
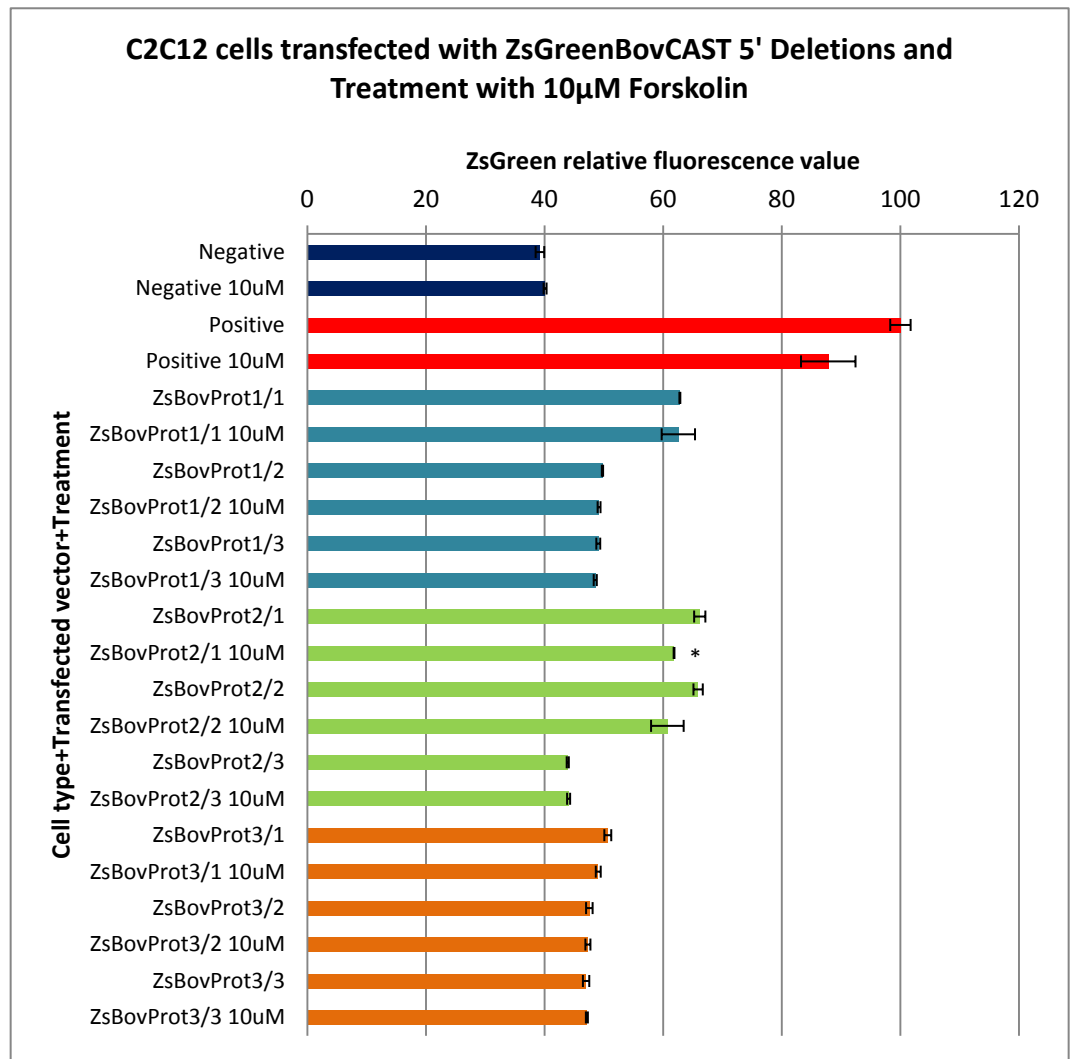


Figure 4.16 The effect of 10 μ M forskolin treatment on bovine CAST promoter activity in mouse muscle C2C12 cells. 5' deletion construct of CAST promoter region associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into C2C12 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 10 μ M forskolin for 24hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * (P<0.05), ** (P<0.01), *** (P<0.001). Error bars are SEM and n=3. T-test was used to analyse the data.

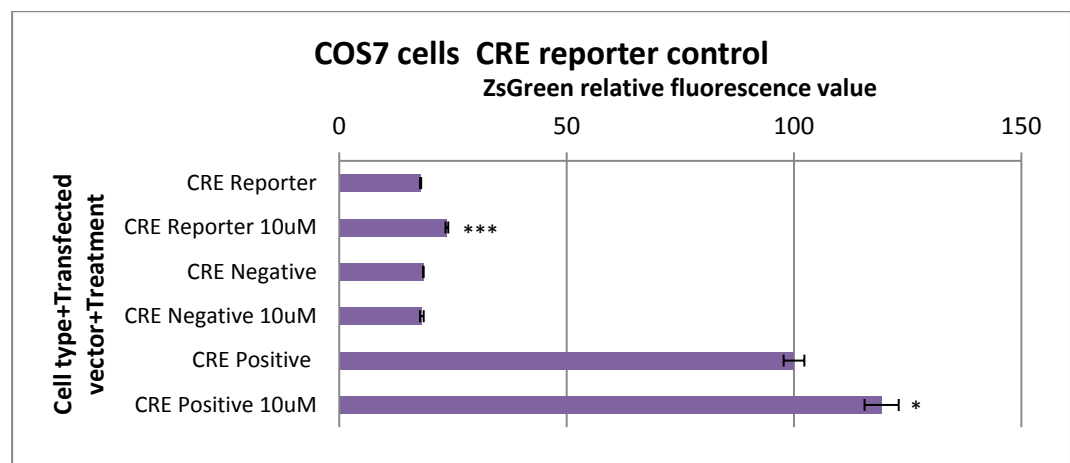
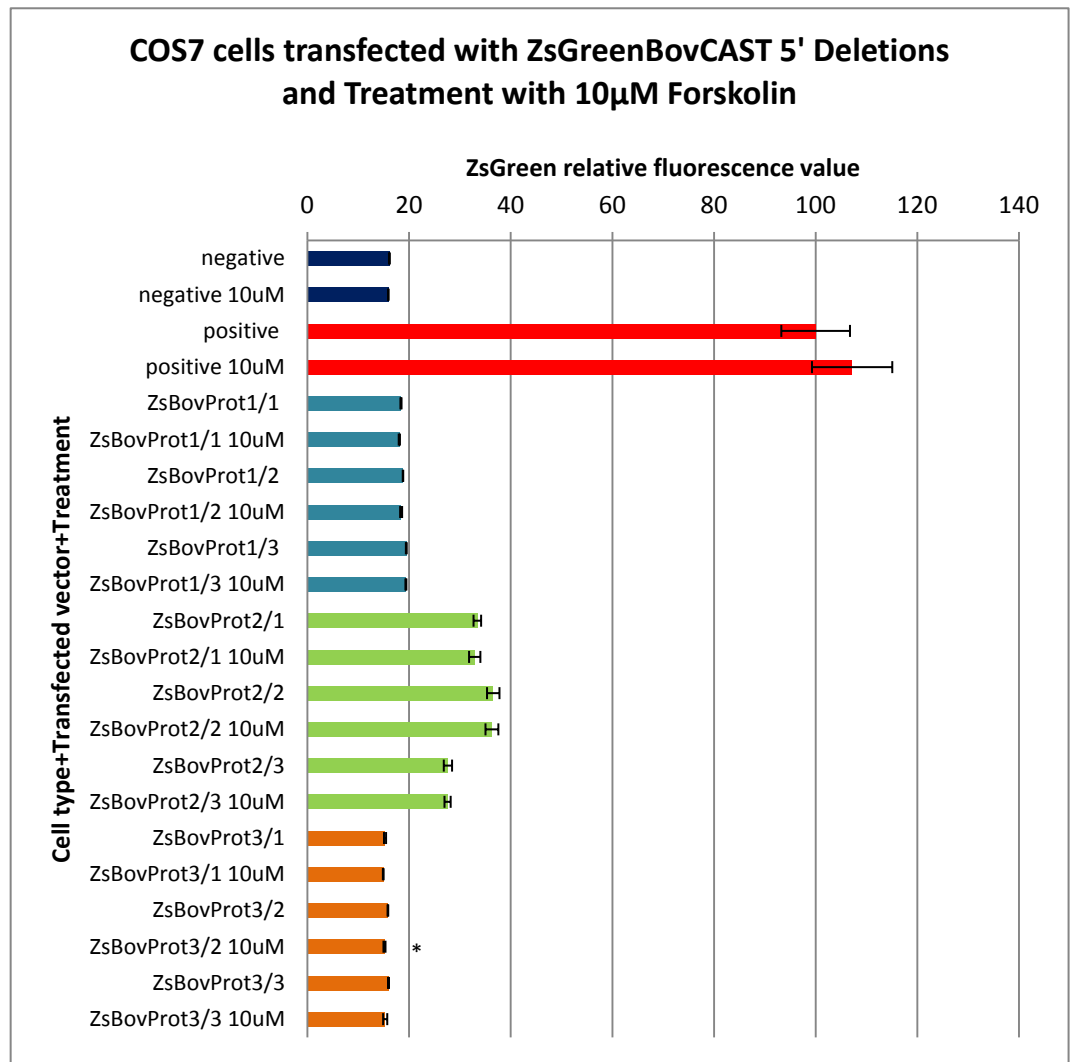


Figure 4.17 The effect of 10 μ M forskolin treatment on bovine CAST promoter activity in monkey kidney COS7 cells. 5' deletion construct of CAST promoter region associated with exon 1 α (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into COS7 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 10 μ M forskolin for 24hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * (P<0.05), ** (P<0.01). Error bars are SEM and n=3. T-test was used to analyse the data.

4.7.2.1 Summary of bovine CAST promoter responsiveness towards administration of forskolin

In summary, the treatment of bovine CAST promoter deletion construct in C2C12 and COS7 cells does not induce any significant response on the transcriptional/translational activity of the CAST transfected promoters as only construct ZsBovProt 2/1 and ZsBovProt 3/2 exhibited changes in their promoter activity. The changes displayed by these promoter constructs was specific to the cell lines where it was transfected with ZsBovProt2/1 displayed decrease in activity when transfected in C2C12, mouse myoblast cell line while ZsBovProt3/2 displayed decrease in activity in COS7, monkey kidney fibroblast cell lines. These changes were assumed as not associated with the response of CAST promoter expression administered with dibutyryl cAMP as no similar patterns of promoter expression were seen from both treatment with either forskolin or dibutyryl cAMP.

4.7.3 Responsiveness of bovine CAST 1xb promoter toward administration of forskolin with variable concentration

Promoter activity from the sequence 5' to exon 1xb was found to be the highest relative to other CAST promoter sequences when transfected in COS7 and C2C12 cell lines (Figure 4.11). These results were in agreement with previous reports where exon 1xb promoter constructs responsible for transcription of Type II bovine calpastatin was reported to have the highest expression value compared to the promoters believed to be responsible for Type I and Type III calpastatins (associated with exons 1xa and 1u respectively) (Raynaud et al., 2005). Cong et al (1998b) had also described how the activity of exon 1xb promoter region was sensitive to cAMP signalling pathway, as putative CRE element proposed in the promoter was shown to be responsive to dibutyryl cAMP treatment. However in these studies, this region was shown to have a positive effect on the activity of the promoter associated with 1xb. These studies combined with those described above indicated that the strongest promoter region in the bovine CAST gene was that associated with exon 1xb.

In order to further investigate the responsiveness from this promoter region, a dose response of forskolin administration experiment was setup. Promoter activity from bovine promoter associated with exon 1xb, ZsBovProt2/1 which contains the longest 5' sequences was tested against different concentrations of forskolin treatment in a range from 10 μ M to 0.5 μ M (Figure 4.18). The dose response of forskolin treatment was tested in two different cell lines of mouse myoblast, (C2C12) and monkey kidney fibroblast cells (COS7). Generally, results from the administration of forskolin exhibited a significant decrease of promoter 1xb activity both in C2C12 and COS7 cells. There was a significant effect from the treatment of forskolin to the promoter activity of 1xb ($P < 0.05$) in both C2C12 and COS7 cells. In C2C12 myoblast cells, a significant decrease of transfected ZsBovProt 2/1 promoter activity was observed selectively only with 10 μ M and 1 μ M forskolin treatment compared to the control vector of non-treated ZsBovProt2/1 plasmid ($P < 0.05$). However in COS7 cells, there was a significant decrease of 1xb CAST promoter activity which was exhibited with all the doses of forskolin.

In conclusion, the administration of forskolin on 1xb bovine CAST promoter construct transfected in both C2C12 myoblast and COS7 fibroblast cells exhibited a significant decrease in the promoter activity and this was similar to the previous results (Section 4.7.2) where a decrease in promoter activity was observed from 1xb CAST promoter construct. The response from the treatment also appears to be differential across cell lines. The reduction shown in both cell lines was however not subjected to any pattern of dose response curve from varying concentration of administered forskolin.

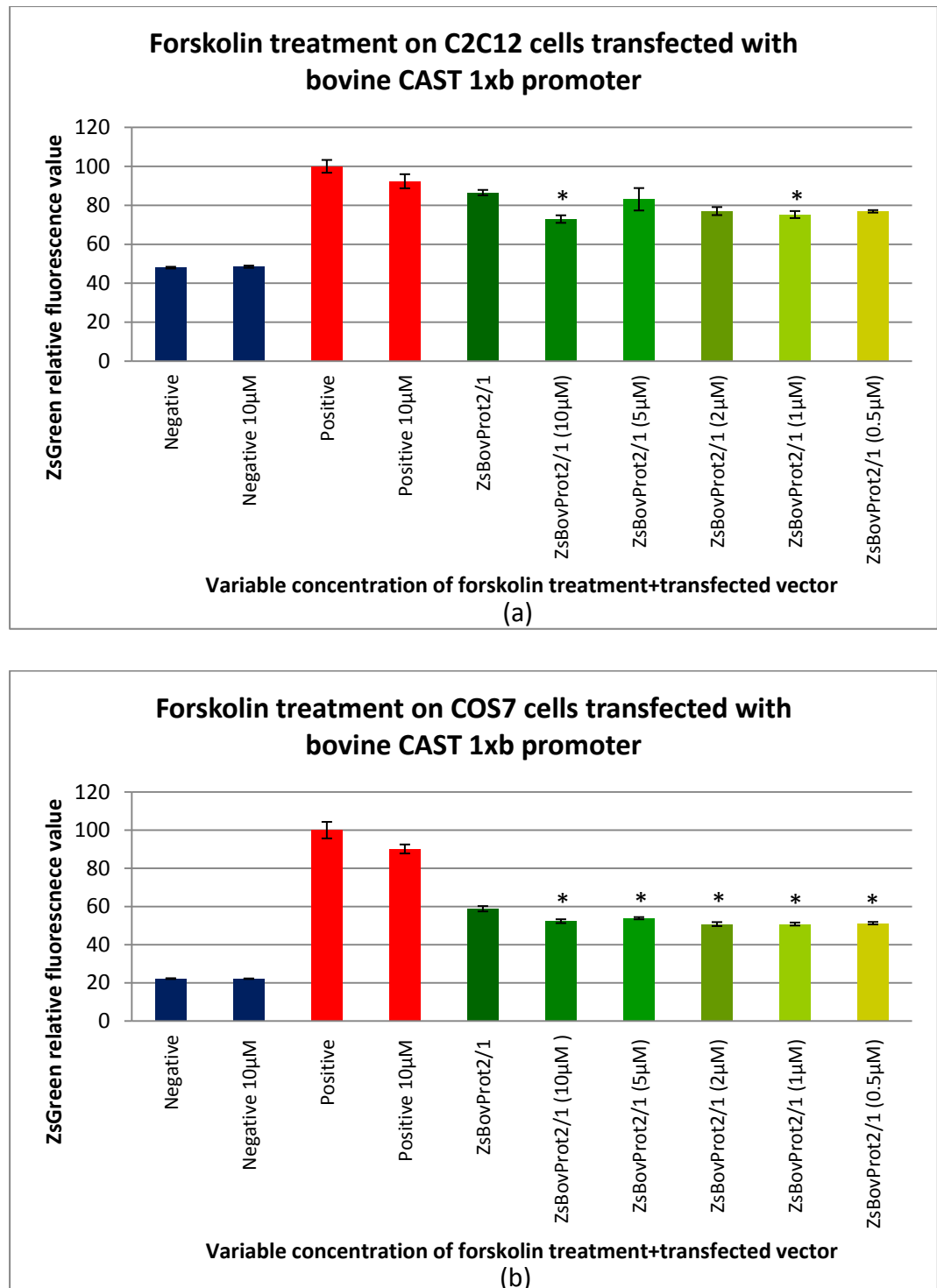


Figure 4.18 The effect of variable concentration of forskolin treatment on bovine CAST 1xb promoter activity in monkey kidney COS7 cells and mouse muscle C2C12 cells. Longest 5' construct of CAST promoter region associated with 1xb (ZsBovProt2/1) were transfected into (a) COS7 and (b) C2C12 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post-transfection cells were exposed to (10 μ M, 5 μ M, 2 μ M, 1 μ M and 0.5 μ M) forskolin for 24hours before assessment of fluorescence was made. There was significant effect of forskolin treatment on the activity of bovine 1xb CAST promoter construct in C2C12 and COS7 cells (ANOVA: $P < 0.05$). Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * ($P < 0.05$), ** ($P < 0.01$). Error bars are SEM and $n = 3$. ANOVA with post-hoc (dunnett test) was used to analysed the data.

4.7.4 Endogenous calpastatin expression in forskolin treated C2C12 cells transfected with bovine CAST promoter construct

Administration of forskolin treatment on bovine calpastatin promoter deletion construct transfected in C2C12 mouse myoblast cells had a selective effect only to promoter deletion construct from promoter 1xb as significant reduce in promoter activity was recorded with the treatment. Other promoter deletion construct associated with exon 1xa and 1u, however did not appear to be responsive with the treatment relative to their non-treated control. It was not known whether administration of forskolin affect expression from the endogenous calpastatin in the transfected C2C12 cells. Therefore in this experiment, quantitative RT-PCR will be carried out to investigate the effect of forskolin treatment on the expression of the endogenous calpastatin from the C2C12 cells transfected with bovine promoter deletion construct. The aim was to measure generic calpastatin expression from mouse C2C12 muscle cells transfected with bovine CAST promoter construct.

C2C12 cells were transfected with bovine calpastatin putative promoter construct associated with exon 1xa, 1xb and 1u, 24 hour after cells were seeded and treatment was carried out the next day with 10 μ M forskolin. C2C12 cells were harvested 24 hours after the treatment for total RNA extraction. For quantitative RT-PCR, the same primers and method in section 4.3 was used to measure generic mouse calpastatin expression from the extracted total RNA of transfected C2C12 cells.

Figure 4.19 shows the endogenous mouse CAST expression from C2C12 cells transfected with bovine CAST promoter construct. Generally, there was significant effect of forskolin treatment on the expression of the endogenous mouse calpastatin ($P < 0.01$). There was also significant effect of types of transfected bovine cast promoter construct on the overall expression of endogenous mouse calpastatin through statistical analysis ($P < 0.05$). However, there was no interaction between factors of forskolin treatment and the types of transfected bovine cast promoter construct on the

expression of endogenous mouse calpastatin ($P>0.05$). From the quantitative RT-PCR data, it clearly shows that expression of generic endogenous mouse CAST was affected with forskolin treatment and the type of response reported was in the form of increase in generic endogenous calpastatin expression. This finding was in contrast with the response from transfected bovine CAST promoter as administration of forskolin generally reduces the promoter activity of transfected construct.

To summarise, quantification of generic calpastatin expression from transfected C2C12 cells revealed that it was significantly affected with forskolin treatment and the types of transfected vectors on the expression of generic mouse calpastatin.

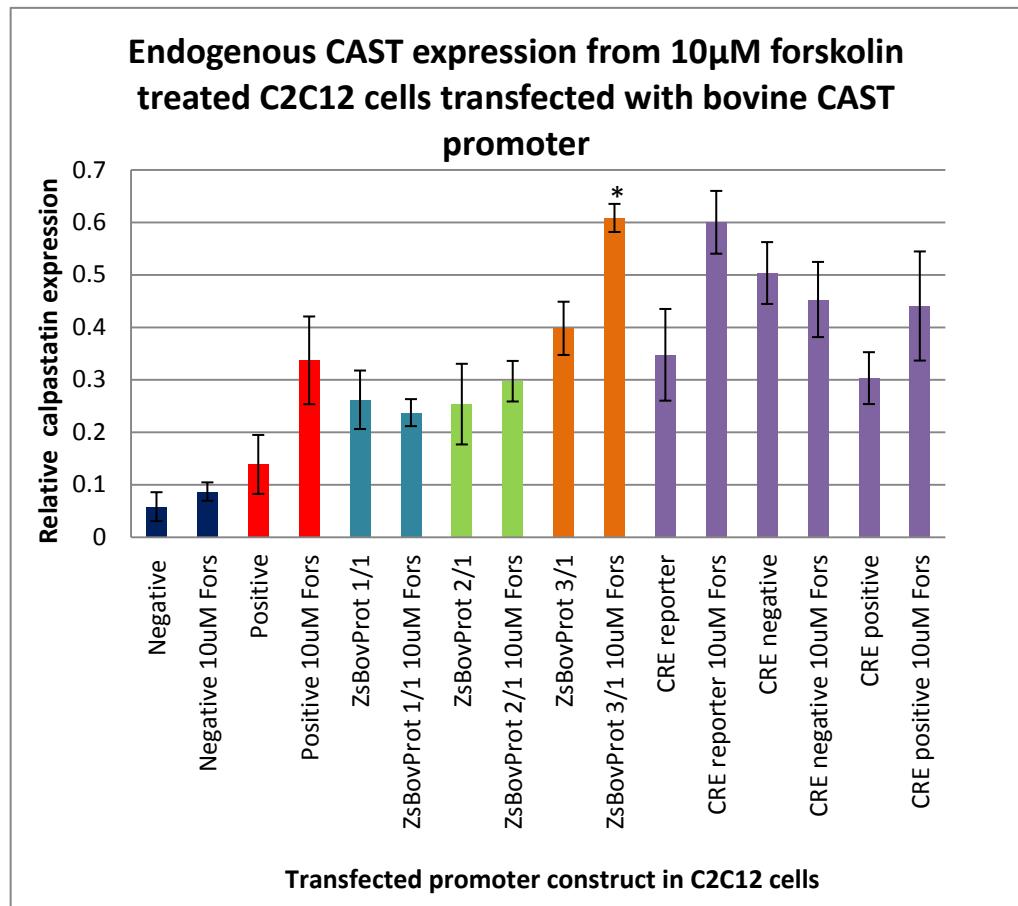


Figure 4.19 Quantitative RT-PCR of CAST gene expression over a mouse C2C12 muscle cells transfected with bovine CAST promoter construct and treated with 10 μ M forskolin. Total RNA from C2C12 cells was prepared post 2 day after transfection. Equal quantities of total RNA persample were utilise to synthsis first strand cDNA which was then subjected to PCR using primers for mouse CAST. CAST expression relative to GAPDH was plotted on the graph, Error bars are SEM and n=3. Significant effect of forskolin treatment on the generic endogenous mouse calpastatin activity was observed (ANOVA; $P < 0.01$). Significant effect of transfected vectors on the generic endogenous mouse calpastatin activity was observed (ANOVA: $P > 0.05$). There was no interaction between factors of forskolin treatment and types of transfected vectors on generic endogenous mouse calpastatin activity (ANOVA: $P > 0.05$). ANOVA and T-test was used to analyse the data.

4.7.5 Effect of calcimycin treatment on bovine CAST promoter expression

Skeletal muscle hypertrophy has been reported to be mediated by a Ca^{2+} dependent calcineurin signalling pathway induced by IGF-I (Semsarian et al., 1999). It was important to investigate whether calpastatin transcriptional activity was involved in the calcium signalling pathway using diagnostic agent related to calcineurin pathway as the gene contains other potential transcriptional motifs that could be responsive to activation or deactivation of transcription factors. Therefore, the main objective of this experiment was to further investigate whether expression from the CAST promoters was sensitive to calcium related stimuli thereby indicating potential links of CAST gene expression regulation by Ca^{2+} signalling pathway such as those mediated by calcineurin or which are linked skeletal muscle hypertrophy (Semsarian et al., 1999).

The responsiveness of bovine CAST promoter construct towards administration of calcimycin (A23187), a calcium ionophore which increase cytosolic calcium was tested on C2C12 mouse myoblast cells and COS7 monkey kidney fibroblast cells transfected with CAST promoter construct. Transfected cells were treated with $1\mu\text{M}$ of calcimycin as had been previously used in a study examining the responsiveness of the porcine CAST promoters (Sensky et al., 2006).

Overall, the effect of treating C2C12 and COS7 cells that had been transfected with CAST promoter constructs with $1\mu\text{M}$ calcimycin on bovine CAST was a significant reduction in the promoter activity (Figure 4.20 & 4.21). In C2C12 cells, promoter deletion construct made from exon 1xa (ZsBovProt 1/1), exon 1xb (ZsBovProt 2/1) and exon 1u (ZsBovProt 3/1) showed a highly significant decrease in their promoter activity ($P < 0.001$). The significant decrease in promoter activity was not only seen in the longest CAST promoter construct but also in 5' deletion constructs of each promoter regions of exon 1xa, 1xb and 1u.

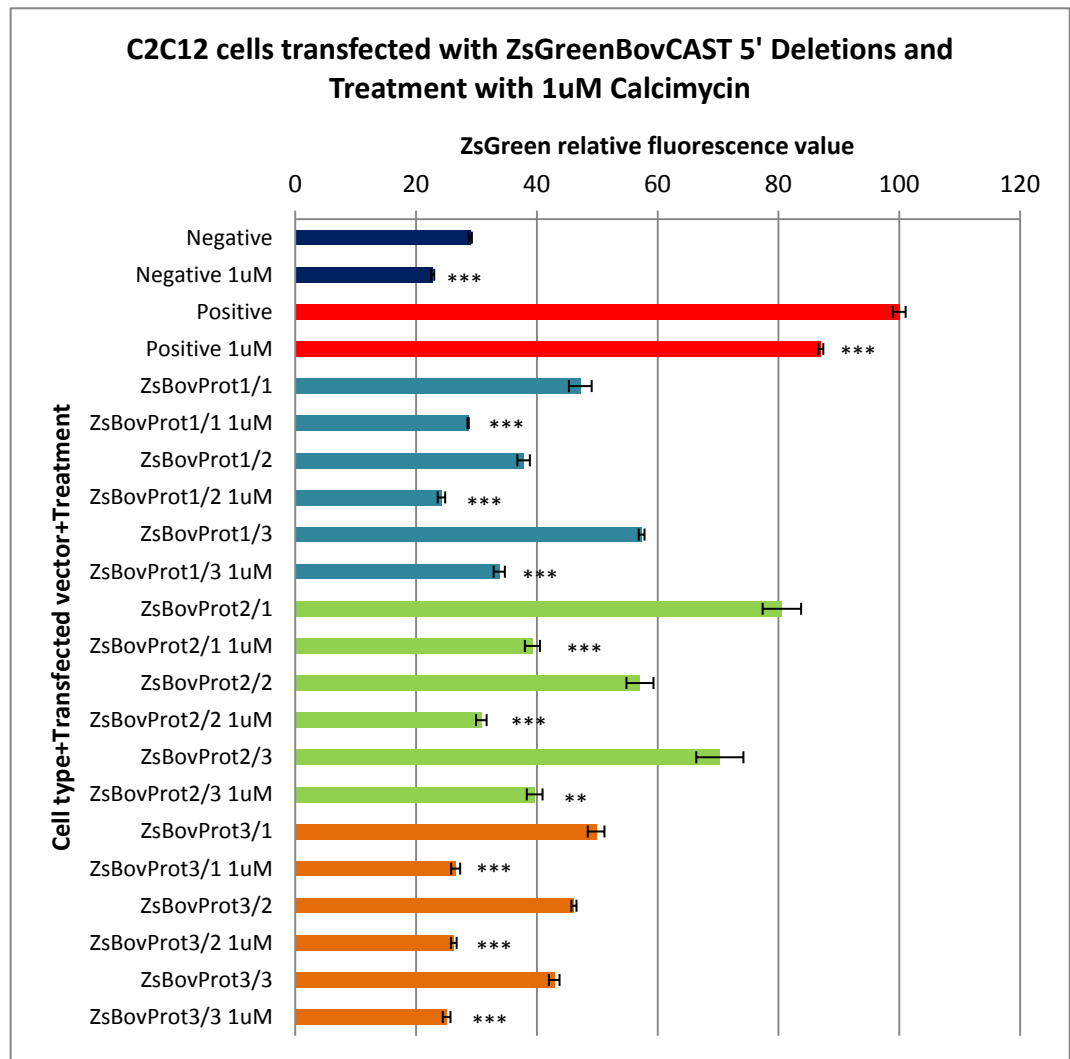


Figure 4.20 The effect of 1 μ M calcimycin treatment on bovine CAST promoter activity in mouse muscle C2C12 cells. 5' deletion construct of CAST promoter region associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into C2C12 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 1 μ M calcimycin for 24hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * (P<0.05), ** (P<0.01), *** (P<0.001). Error bars are SEM and n=3. T-test was used to analyse the data.

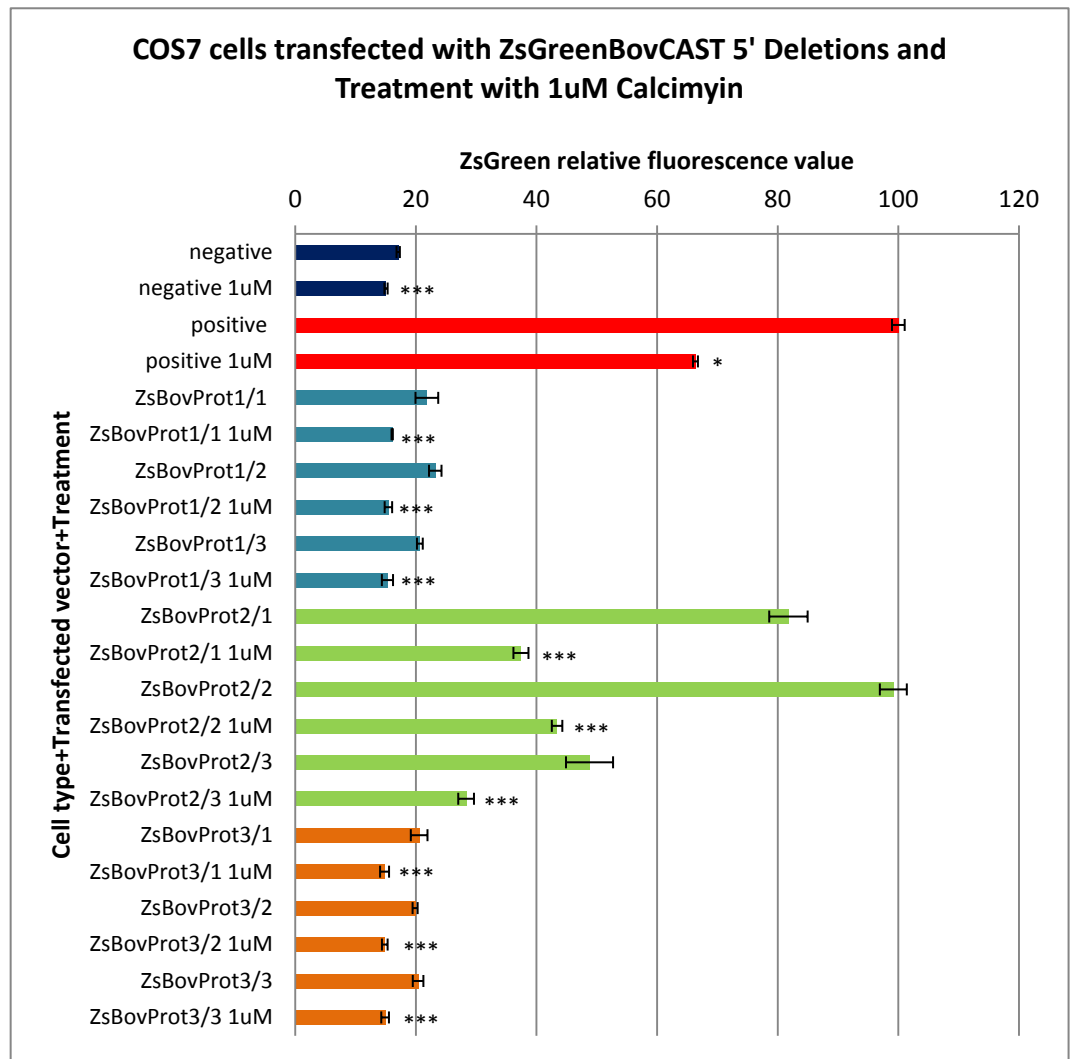


Figure 4.21 The effect of 1 μ M calcimycin treatment on bovine CAST promoter activity in monkey fibroblast COS7 cells. 5' deletion construct of CAST promoter region associated with exon 1xa (ZsBovProt1), 1xb (ZsBovProt2) and 1u (ZsBovProt3) were transfected into COS7 cell line. Transfection was carried out in cells at 70% of confluence. At 24 hour post transfection cells were exposed to 1 μ M calcimycin for 24hours before assessment of fluorescence was made. Significant decreased in CAST promoter activity was observed as a result from the treatment relative to the control: * (P<0.05), ** (P<0.01), *** (P<0.001). Error bars are SEM and n=3. T-test was used to analyse the data.

As for the COS7 cells the same trend of reduce in promoter activity applies for transfected ZsGreen based bovine CAST promoter constructs treated with the calcimycin. CAST promoters associated with exon 1xa, 1xb and 1u along with the 5' deletions recorded highly significant decrease in their promoter activity relative to the non-treated control ($P < 0.001$).

4.7.5.1 Summary of bovine CAST promoter responsiveness towards administration of calcimycin

The administration of calcium ionophore in transfected cell line of myoblast and fibroblast cell types indicated a major decrease in activity of bovine CAST promoter activity. In conclusion, although a few studies have suggested a relationship between elevated concentrations of calcium and its importance in calcium dependent signalling pathways which are related to muscle hypertrophy, there was no indication or direct evidence that can be related to CAST promoter activity. However, the data from our experiment suggest that excess of cytosolic Ca^{2+} significantly reduced the activity of bovine CAST gene promoter.

4.7.6 Summary of bovine CAST promoter responsiveness towards administration of anabolic agent.

Bovine calpastatin promoter had been tested for its responsiveness against agents or components involved in hypertrophic growth associated with pathway related to cAMP and calcium signalling pathway. Overall, studies in this aspect revealed that the response shown by bovine calpastatin promoter with testing of these agents were displayed as reduction in its transcriptional activity relative to the control value. Bovine CAST promoter deletion constructs were showing decrease in its promoter activity when administered with dibutyl cAMP and the effect varies between cell lines. Further studies on this effect, using forskolin which induces cAMP formation, only managed induced significant response from promoter deletion construct associated with 1xb and 1u in C2C12 and COS7 cells as the effect varies between cell lines. The reported data from these experiments raised a question whether administration of these treatment were affecting expression of the endogenous calpastatin as the transfected cells also contains calpastatin gene. For this reason, further investigation was made using quantitative RT-PCR which reported that forskolin treatment significantly affects the expression of endogenous calpastatin in the transfected cells in the form of increase in the endogenous mouse CAST gene expression.

Furthermore, administration of calcimycin on transfected bovine calpastatin promoter deletion construct significantly reduced promoter activity compared to the non-treated control. The inclusion of positive control for treatment, however manage to validate findings from these experiment as CRE reporter displayed significant increase in its transcriptional activity when measured against the non-treated control. To conclude, there was no direct evidence or prove that could associate calpastatin transcriptional regulation towards calcineurin and cAMP signalling pathway that were involved in hypertrophic growth as the investigation were only performed on the basis of measuring response from transfected bovine calpastatin promoter.

Chapter 5: Relationship Between Calpastatin Short CA Tandem Repeat and Meat Tenderness

5.1 Introduction

Characterisation of bovine calpastatin promoter region immediately 5' to exon 1xa revealed polymorphic short CA tandem repeat region which had variable length in different cattle breeds suggesting a use as an informative polymorphisms site and the possibility of its use as a marker associated with meat tenderness (Nonneman et al., 1999). The short CA tandem repeat region was also reported to be located in a similar in a region 5' to exon 1xa CAST promoter in porcine thereby suggesting an apparent significance of the polymorphic site in the most distal 5' promoter region of the calpastatin gene (Figure 5.1) (Parr et al., 2001).

As described in section 3.6, variation in the length the CA repeat was shown between cattle from two different sources. It was suggested that the short CA tandem repeat region located 5' to the transcriptional start site (TSS) in exon 1xa in CAST gene may play a role in regulating the transcriptional activity of the gene. This will indirectly influence meat quality, as calpastatin is known to be inhibitory to calpain activity, which is responsible for protein degradation in meat and thereby tenderness. Therefore, the hypothesis was that there is an association between polymorphisms of the short CA tandem repeat region in CAST promoter region and tenderness of meat.

The main objective of this study was to establish analysis of variability in calpastatin 1xa promoter region and at the same time to characterize the promoter activity of gene sequences had polymorphism of differing CA tandem repeat length in order to define whether this was associated with variable meat tenderness.

[illegible]

5.2 Development of vector for cloning short CA tandem repeat amplicons

As described in section 3.6 variation short CA tandem repeat in the promoter associated with 1xa had been demonstrated through the analysis of PCR amplicons generated across this region from genomic DNA templates. To characterise the effect that varying CA tandem repeat length had on the activity of the CAST 1xa promoter this region in targeted CAST exon 1xa promoter sequences from different genomic DNA sources were amplified and cloned into a fluorescence-based promoter mammalian cell reporter expression vector and then transfected into a variety of cell lines.

5.2.1 Site directed mutagenesis of ZsBovProt1/1 promoter construct

As described in section 3.6 a variety of PCR amplicons had been generated across CA tandem repeat region contained in the 1xa promoter. By selecting different positions for the PCR primers these amplicons varied in length from the shortest 123bp to the longest 555bp theoretical size. By sequencing these amplicons it was clear that variation in length was due to differences in the number of nucleotides within the CA repeat indicating that polymorphisms existed within this region of the promoter. Therefore a cloning strategy was devised to clone the short CA tandem repeat amplicons from the promoter region.

Promoter construct from exon 1xa (BovProt1/1) had been made available by Raynaud et al. (2005) appendix 15. This promoter construct contains 729bp sequences of exon 1xa bovine CAST promoter located 150bp 3' to the TSS inserted into the GFP based promoter expression vector pEGFP-1. In the previous chapter, 1xa calpastatin promoter sequences from BovProt1/1 promoter construct had been recloned into pZsGreen 1-1 expression vector.

In order to clone variable short CA tandem repeat region into ZsBovProt1/1 vector, a minor base modification was made to the vector sequences. This introduced a novel HindIII restriction site in ZsBovProt1/1 vector sequences

and this was performed in the 1xa promoter sequence inserted in the promoter reporter vector using site directed mutagenesis (Figure 5.2) section 2.13.2. This allowed insertion of 295bp amplicons which spanned the CA tandem repeat region. These amplicons were generated from cattle genomic DNA using primers which contained XhoI and HindIII restriction sites at their 5' and 3'ends. However, changing of three nucleotides in the promoter construct in order to engineer a Hind III site inevitably altered transcriptional factor binding site in the promoter region. This was demonstrated using the bioinformatics programme TF search (Akiyama, 1995) which identified putative transcription factor binding sites on the original sequence and the engineered mutated sequence. The output of this showed a potential conversion of CEBP to a HSF2 binding site (Figure 5.3). It was however unknown whether this conversion would significantly affect promoter activity.

The generation of amplicons of the CA tandem repeat from genomic DNA extracted from cattle of with meat of differing quality was performed using primers added with restriction site XhoI and HindIII (appendix 7) as to accommodate the complimentary site for ligation of the amplicons located in mutated ZsBovProtIHindIII vector.

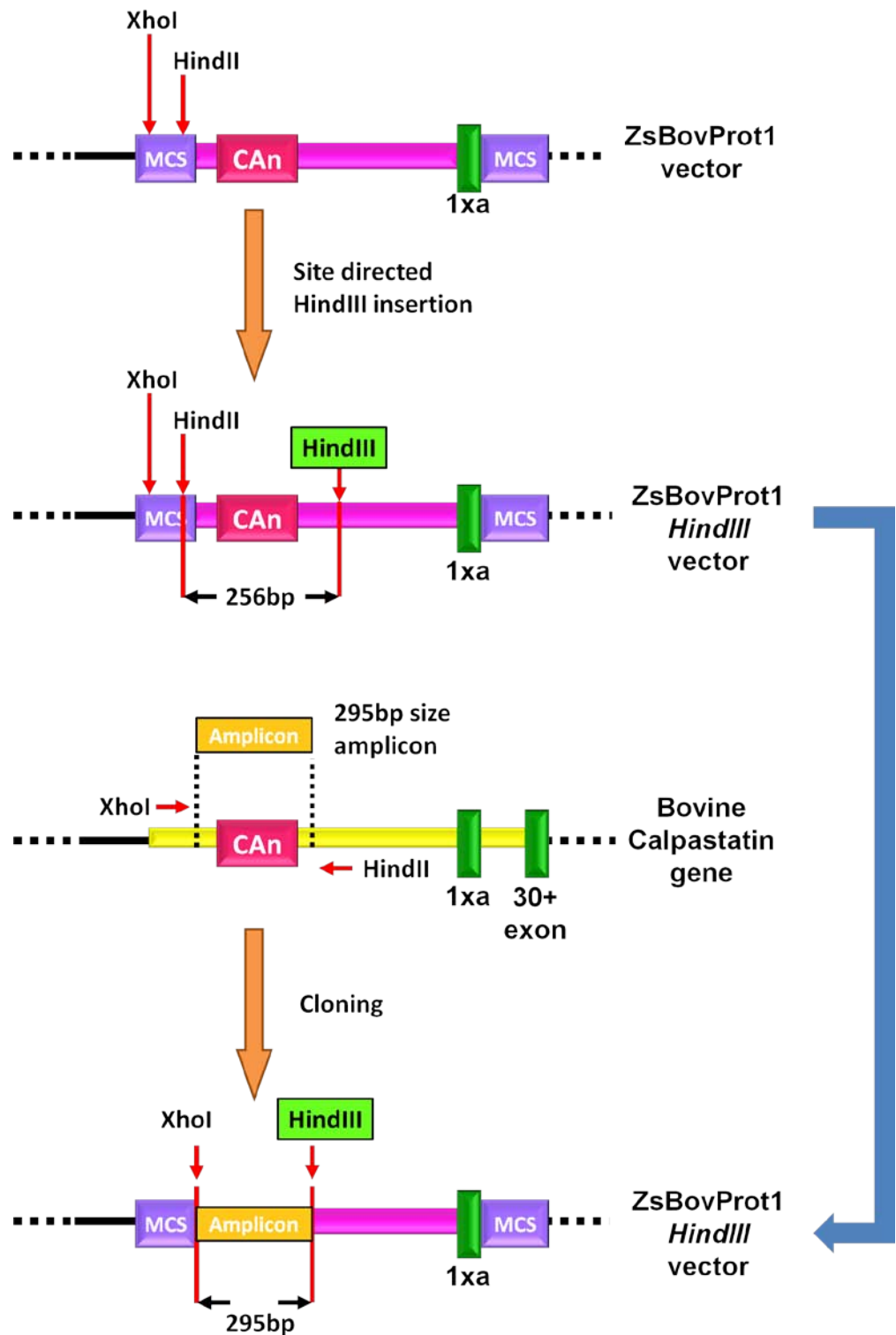


Figure 5.2 Site directed mutagenesis on BovProt1/I vector introduced a HindIII restriction site within the 5' sequence of bovine calpastatin exon 1xa promoter region. The introduction of novel HindIII restriction site will allow cloning of variable length of short CA tandem repeat region from different cattle source.

Comparison of TF search before and after mutagenesis (85point threshold score)							
a) ZsBovProtI/I (original Sequence)							
301	GTTGG	TAA	TCTCCTCCTA	GTTA	ACTCAG	AGCAGATTGC	AGAAATGCTG
	----->						
						entry	score
						C/EBP	86.2
b) ZsBovProtIHindIII (mutant sequence)							
301	GTTGG	<u>AAGCT</u>	TCTCCTCCTA	GTTA	ACTCAG	AGCAGATTGC	AGAAATGCTG
	----->						
	<-----						
	<-----						
						entry	score
						HSF2	94.9
						HSF2	92.3
						HSF1	85.9

Figure 5.3 Potential conversion of transcriptional factor binding site highlighted using TF search ver1.3 on mutated BovProtIHindIII promoter sequences. TF searched at the threshold score of 85 on the original and mutated BovProtI/I sequence revealed a potential conversion of transcriptional factor binding site on the mutated promoter sequences. Bases bolded in red indicate those altered to generate a HindIII site (underlined).

5.3 Variation in the CA repeat from meat of differing quality

Samples of bovine meat of varying quality were kindly provided by Dr Ronald Bardsley (University of Nottingham) which originated from the Tendercheck Shear Force Research Project Program. This samples contains meat sample from three different breeds which were Charolais, Limousin and Simmental. The tenderness quality from each bovine meat sample had been previously assessed using Warner Bratzler shear force value (Table 5.1). Of 17 samples the range of shear force values varied from the most tender 3.998kg to the most tough 7.319kg.

Genomic DNA was extracted from all the meat samples indicated in Table 5.1. The polymorphic site containing short CA tandem repeat were amplified by PCR using 1xCASTand 3 primers covering 203bp of DNA sequences in the 5' promoter region of CAST exon 1xa as described in section 3.5. In order to compare size differences of the amplified PCR product from bovine genomic DNA samples from carcasses of varying shear force value was assessed by non-denaturing acrylamide gel electrophoresis. Figure 5.4 shows size differences in the amplicons. The variation in the size of DNA bands was similar as been reported in section 3.4.2. There was a large variation in terms of tandem CA repeat length. As described in section 3.6.1, the assumption was made that the lowest band was the actual product from the amplification. This assumption was made based on the results in chapter 3.6.1 where size of the furtherst migrating band in the amplicons reflected the size of theoretical product.

Table 5.1 Samples of bovine meat of varying quality and breed from the Tendercheck Shear Force Program. The samples had been sorted according to Warner-Bratzler shear force value. Amplicons of 295bp length containing short CA tandem repeat region from calpastatin 1xa promoter had been cloned into ZsGreenBovProt1HindIII vector from samples which have highlighted in yellow. Shear force value was determined by third party in Tendercheck shear force program and data was used with permission as described in Section 2.3.

Sample number	Warner-Bratzler shear force value	Cattle breed
10	3.998	Charolais
14	4.011	Charolais
12	4.298	Charolais
13	4.301	Limousin
6	4.582	Limousin
3	4.923	Charolais
15	5.271	Simmental
17	5.334	Charolais
2	5.336	Charolais
9	5.389	Charolais
16	5.703	Limousin
5	5.95	Charolais
1	6.194	Charolais
7	6.21	Charolais
8	6.456	Charolais
11	6.618	Charolais
4	7.319	Charolais

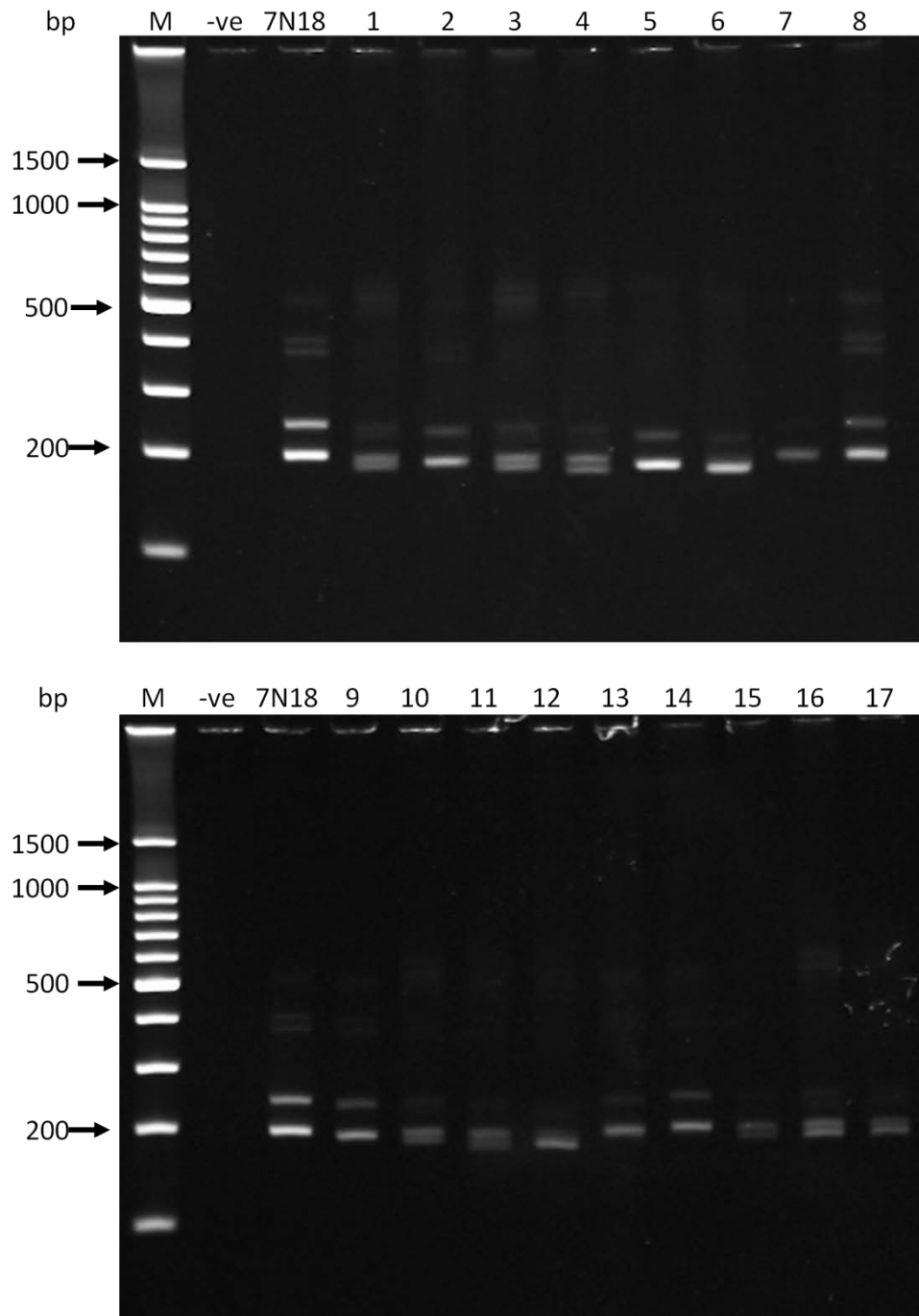


Figure 5.4 Variation of short CA tandem repeat length in 203bp size PCR product amplified from cattle gDNA with differing meat tenderness. Primer 1xaCASTand3F/R was used to amplify the CA tandem repeat region and the variation in length was observed on non-denaturing acrylamide gel. (M) indicates 100bp DNA marker, (-ve) negative control and (7N18) is bovine BAC clone.

5.4 Cloning and sequencing of CAST short CA tandem repeat region in ZsGreen fluorescence based promoter expression vector

To assess CAST Type I promoter activity, amplicons from the short CA tandem repeat region were cloned into pZsBovProtIHindIII plasmid construct which contained the engineered HindIII site utilising the engineered cloning site as described above. A predicted 295bp size amplicon was generated from the 5' of 1xa promoter region which included the short CA tandem repeat region. Six DNA meat samples of cattle with varying tenderness ranging from the lowest to the highest shear force value were chosen for cloning of the CA tandem repeat sequence Figure 5.4. Successfully cloned insert of CA repeat amplicons were sequenced to determine the precise length of the tandem repeat Figure 5.6. Sequencing of CA tandem repeat ZsBovProtIHindIII constructs revealed large variation in terms of tandem repeat length across shear force value (Figure 5.5). ZsBovProtIHindIII(14) promoter construct originated from the most tender meat had a 58bp CA repeat sequence whilst ZsBovProtIHindIII(4) promoter construct originated from the most tough meat was 40bp long. Furthermore, ZsBovProtIHindIII(15) and ZsBovProtIHindIII(16) promoter constructs which both originated from cattle which had of shear force between the most tender and toughest meat range had CA repeat sequences with 58bp and 60bp long respectively. DNA sequencing also showed that CA repeat with the length of 60bp, 58bp and 40bp long appeared to be in more than one construct. Only one promoter construct had a CA repeat with 54bp length, ZsBovProtIHindIII(8).

Table 5.2 Length of tandem repeat determined from sequencing of ZsBovProt1HindIII plasmid construct. Sequencing of CA tandem repeat construct ZsBovProt1HindIII from meat samples revealed differing length of CA repeat.

Sample number	Warner-Bratzler shear force value (kg)	CA repeat length (bp)
14	4.011	60
12	4.298	40
15	5.271	58
16	5.703	60
8	6.456	54
4	7.319	40

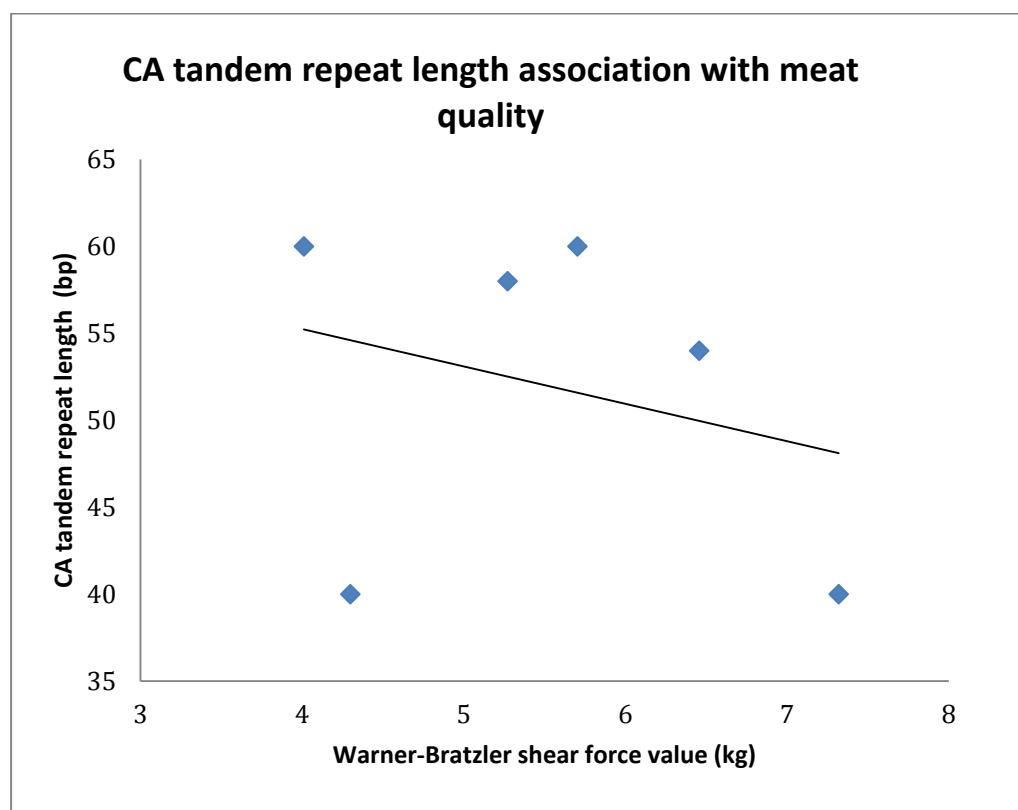


Figure 5.6 Relationship between CA tandem repeat length with meat tenderness. Length of CA tandem repeat from DNA sequencing was plotted against the shear force value assessed from the original meat sample to indicate the relationship with meat tenderness. $n=6$ and $r=0.28$ ($P>0.05$). Pearson coefficient correlation was used to analyse the data.

Figure 5.6 indicates association of CA tandem repeat length with the meat tenderness. In general, the relationship observed from the graph was higher shear force value in meat was associated with a shorter length of CA tandem repeat. Statistically there was no correlation between both length of CA tandem repeat in 1xa calpastatin promoter region and the shear force value of the meat sample ($P>0.05$).

Cloning of the short CA tandem repeat was made from the most furtherst migrated band in the gel; sequencing of this band showed only a single length of CA tandem repeat. Interestingly, from the individual animals examined several had CA tandem repeat which was the same length suggesting there are there are defined lengths of the CA repeat which results distinct alleles appearing at the same locus for the CAST short CA tandem repeat thereby generating polymorphisms. These alleles were presented in more than one loci as CA repeat were 60bp, 58bp and 40bp long. The tandem CA repeat of 54bp length was found in only one construct.

To conclude, there was no trend that can be associated with meat tenderness as the CA short tandem repeat size differs and scattered across the shear force value within the tested meat samples. The main finding was that more than one construct contains the same size of CA tandem repeat suggesting that there are specific lengths of the CA repeat within this loci. Therefore, from this observation, it was concluded that at least four types of alleles represented as $(CA)_n$ existed at the same locus in calpastatin exon 1xa promoter region.

5.5 Characterisation of promoter 1xa activity based on varying length of CA repeat

The final experiment in this thesis involved characterizing the effect of varying CA tandem repeat length on the activity of the 1xa promoter. This was determined using a variety of ZsBovProtIHindIII promoter expression constructs, which differed in the length of short CA tandem repeat region, transfected into C2C12 mouse myoblasts or COS7 monkey kidney fibroblast cell lines. The activity of the promoters was measured using Typhoon Trio fluorescence detector, 48 hours after transfection (Figure 5.7). The objective was to determine whether the polymorphisms of short CA tandem repeat length influenced the activity of the 1xa promoter. The hypothesis being that variation in the CA tandem repeat in the 1xa promoter would influence transcriptional activity of gene encoding calpastatin which would thereby influence calpain mediated meat tenderness. This experiment was designed to determine the effect of length of CA repeat on calpastatin 1xa promoter activity. It was known from previous experiment that within the animal there are two alleles and it was hypothesized that the combination of both alleles may possibly affect the promoter expression.

In general, promoter expression from the transfected ZsBovProtIHindIII promoter constructs with varying CA tandem repeat length was recorded as significantly higher compared to the ZsGreen based negative control vector ($P < 0.05$). Overall in C2C12 cells, promoter activity from 1xa promoter construct with differing CA tandem repeat length was found to be significantly higher compared to the control than the same constructs transfected in COS7 cell lines, as in the C2C12 cells the increase in promoter activity was at least 75% higher than the negative control vector. There was however the exception with ZsBovProtIHindIII(12) construct containing 40bp length of CA repeat sequence as its promoter activity was significantly lower compared to the negative control vector ($P > 0.05$).

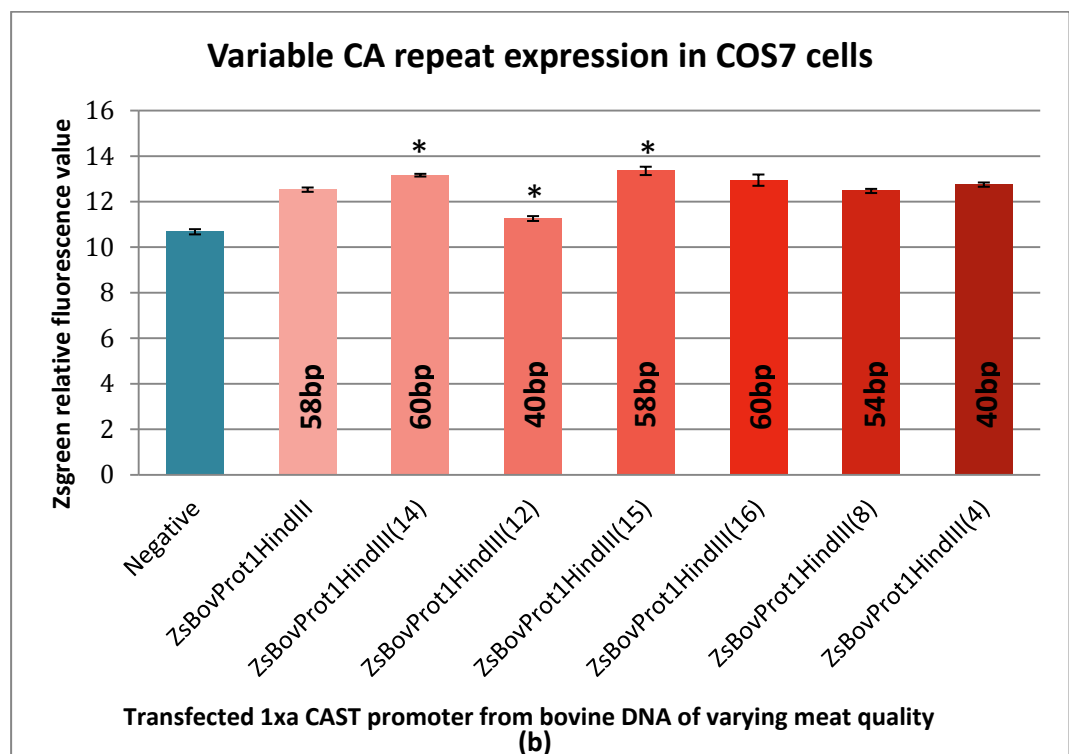
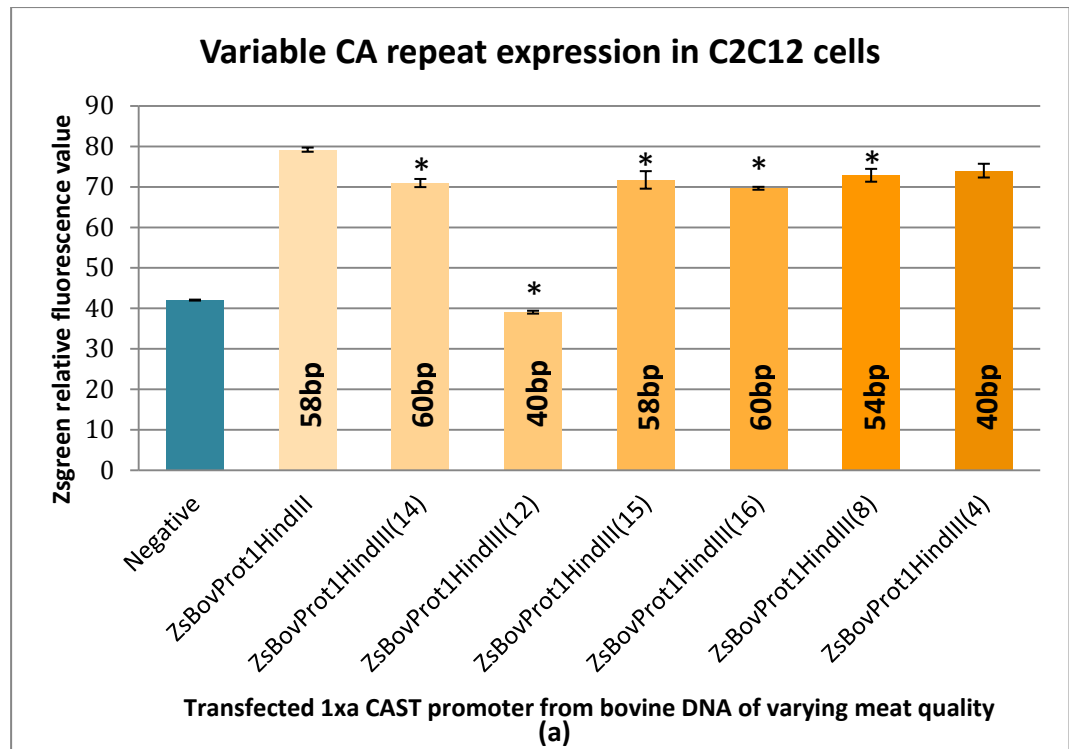


Figure 5.7 Promoter expression from ZsBovProt1HindII promoter construct containing CAST 1xa promoter with differing CA tandem repeat length in (a) C2C12 and (b) COS7 cells. The length of CA repeat have been labelled for each promoter construct and the graph have been sorted from the lowest to the highest shear force values based on the origin of the CA repeat. ANOVA analysis reported significant means difference between tested promoter construct ($P < 0.001$). Dunnett's pairwise comparison test with unaltered ZsBovProt1HindIII indicate significant difference* ($P < 0.05$). Error bars are SEM and $n = 3$. ANOVA with post-hoc (dunnett test) was used to analysed the data.

As been reported previously, the variation in the CA repeat length from the provided sequencing data of ZsBovProtIHindIII promoter construct did not present any particular trend associated with the shear force value of the meat samples. Statistical analysis using one way ANOVA on the tested ZsBovProtIHindIII promoter constructs (sample 14, 12, 15, 16, 8 and 4) showed that there was significant difference in terms of expression from each tested promoter construct ($P < 0.001$) of varying CA repeat length in the C2C12 cell line. Additional *post-hoc* statistical test using Dunnett's pairwise comparison found difference in relative fluorescence value from construct from samples 14, 12, 15, 16 and sample 8 which contains 60, 40, 58, 60 and 54bp length of CA tandem repeat when it was compared to the control ZsBovProtIHindIII construct. However, there was no significant difference in the promoter activity of construct from sample 4 containing 40bp length of CA repeat compared to the control ZsBovProtIHindIII promoter construct using Dunnett's comparison test ($P > 0.05$).

Transfection of 1xa promoter constructs in COS7 cell lines in general produced promoter activity which were relatively significantly higher than the negative control ($P < 0.05$). Using one way ANOVA, promoter expression from 1xa promoter construct containing CA tandem repeat region of varying meat tenderness was shown to be significantly different within the tested samples ($P < 0.001$). Additional *post-hoc* test using Dunnett's comparison test shows that the significant different in promoter expression was observed from construct derived from CA tandem repeat from sample 14, 12 and 15 compared to the control ZsBovProtIHindIII plasmid.

In conclusion, there was no direct association between transcriptional activity from CAST 1xa promoter and the meat tenderness of CA tandem repeat derived promoter construct. Furthermore, there was also indication or trend in CAST 1xa promoter expression and the length of CA tandem repeat sequence for each of the tested promoter construct.

Chapter 6: Discussion & Future Work

The main aim of the experiments performed in this thesis was to investigate the effect of CA tandem repeat length polymorphism and its effects on the transcriptional activity of calpastatin Type I promoter. Furthermore, this was done to assess whether there was any indication of a correlation between the CA length polymorphisms and the meat quality in terms of its shear force value. The main hypothesis in this study was built on the argument that meat tenderness is influenced by calpain mediated proteolytic activity which is regulated by its inhibitor calpastatin. The activity of the inhibitor, calpastatin is known, in part, to be regulated by the transcriptional activity of the promoters which are responsible for producing different types of calpastatin transcripts and therefore calpastatin isoforms. To further character potential factors regulating expression, we also examined the potential regulatory role of elements such as CA tandem repeat polymorphisms as well as other agents associated with increase in transcriptional activity of the gene (dibutyryl cAMP and calcimycin), thereby the level of mRNA and subsequently its translation to protein. It was hypothesized; this could affect the activity of calpain activity and in the end affect tenderness of the meat. The main hypothesis of this study was the CA length polymorphisms affects the transcriptional activity of Type I promoter. Furthermore, it was hypothesise the length of CA repeat polymorphism could be correlated to the tenderness of the meat. If this hypothesis was true, it could potentially help to improve tenderness in meat by selection of animal prior to slaughter using a genetic base test, thus reducing the additional cost in meat tenderisation and thereby total production costs.

The novel findings in this thesis are:

The assessment made on CA tandem repeat polymorphisms:

- Amplicons made spanning the CA tandem repeat region from cattle of different source using designated primers yield varying size of PCR product.

Assesment of calpastatin promoter expression:

- Development of new method based on Typhoon fluorescence imager to quantify calpastatin promoter activity and establishing inclusion of transfection control marker in the assessment of bovine calpastatin promoter activity
- Comparative evaluation on transcriptional activity of calpastatin promoters in C2C12 cell and COS7 cells lines shows that these have higher activity in C2C12 cells.

Assessment of calpastatin promoter response towards treatments of anabolic agent:

- Calpastatin promoter activity for Type I, II and III activity was reduced 5-8% when treated with dibutyryl cAMP ($P<0.05$)
- There was no significant effect of forskolin on calpastatin promoter Type I, II and III activity ($P>0.05$)
- The calcium ionophore (calcimycin) treatment significantly reduced 40-50% of activity from Type I, II and III calpastatin promoter ($P<0.05$)

The assessment on CA tandem repeats length polymorphism and promoter activity in association to meat tenderness:

- No relationship found between CA repeat length and meat tenderness ($P>0.05$)
- Changing the length of CA tandem repeat for Type I CAST promoter appears to affect its transcriptional activity ($P<0.05$)
- Within the samples analysed by sequencing, there are defined lengths of the CA repeat

6.1 Characterisation of CA repeat in bovine calpastatin promoter

In summary the work presented in this thesis identified the variation of CA tandem repeat in the region 5' to the transcription start site of bovine Type I calpastatin using PCR-based approach which was developed by this research. The main aim was to develop a suitable PCR technique to amplify the CA tandem repeat region therefore differentiate the length of the amplified region in cattle of different breed and meat tenderness.

Based on the developed PCR based techniques used to identify the CA tandem repeat polymorphism, it was suggested that the length of CA tandem repeat in the calpastatin 1xa promoter region varies between animals. This was clearly shown as when amplicon products were generated using primers which annealed to fixed regions either side of the CA repeat, they varied in length dependent on the cattle gDNA source. Furthermore, the amplicons produced were indistinct their size being difficult to assess on the native agarose gel.

To further help our investigation and improve the band clarity in order to differentiate size of amplicons generated spanning the CA repeat region, variety of gel systems with varying concentrations of the matrix constituents were used. Comparisons were made between 2% (w/v) agarose, 3% (w/v) metaphor agarose and 7.5% (w/v) non-denaturing polyacrylamide gel system, and the result have proven that the non-denaturing polyacrylamide gel provide greater results and clarity of migrated bands with higher separation of individual bands. Furthermore the advantage of using high percentage acrylamide gel system has been recognised in other research as an effective medium to assess polymorphisms in DNA sequences (Savov et al., 1992; Southern, 2001). However, inclusion of denaturing systems in our examination did not improve resolution. Therefore, the non-denaturing system was chosen as it was the best viewing medium to observe the variation of length from the amplicons. With the non-denaturing polyacrylamide gel system at 7.5% (w/v), it was made apparent that the

amplicons generated from the CA repeat region produced several individual bands resulted from the amplification of a single homogenous DNA source originating from cloned bovine gDNA (BAC 7N18). From this observation, it was hypothesised that the formation of multiple bands was a function of the length of the PCR, as short amplicons would allow heteroduplex formation. To clarify this issue, steps were taken to enhance the PCR specificity. Longer amplicons were generated, although the variation in amplicon size was maintained even after the predicted distance between primers was increased for the amplicons suggesting that the presence of multiple bands were not due to the length of amplicons, rather it is more related to the functional aspect of the sequence polymorphisms. This phenomenon was also observed in different gene in other species. It was reported that amplification of dinucleotides CA repeats in human dystrophin gene generates non-specific additional bands caused by slippage of polymerases during chain elongation in PCR reaction (Murray et al., 1993). This could results in error during amplification of the polymorphic region similar to what have been observed in this thesis.

The second approach to clarify this issue involved nested PCR strategy, as it was hypothesised that the multiple bands were a result of nonspecific PCR and that the nested approach would allow a second round of amplification of specific products (Kirkpatrick and Monson, 1993). However this approach was also unsuccessful suggesting that the multiple bands produced were forms of the expected template. To fully investigate the hypothesis that the multiple bands were generated as a result of nonspecific amplification, third approach was utilised to improve PCR specificity by increasing temperature of the annealing step of the PCR process. PCR amplifications were performed using a series of annealing temperature from the 46°C to 66.5°C in order to improve the primer binding to the DNA template. However, amplicons produced from this PCR reaction remain similar with multiple non-specific bands across the increasing annealing temperature range with no increase in PCR specificity again further suggesting that the multiple bands were from a

single source. The region which was being amplified in this process consisted of a high proportion of repetitive sequence (CA repeat) and previous studies as well as examination of this region of the calpastatin gene across species had shown that this region is high in GC content (~60%) and is conserved across species (Ishida et al., 1991; Killefer and Koohmaraie, 1994). Regions with high GC content also was associated with rise to stable secondary structures which could reduce the efficiency of amplification by serving as pause or termination sites (McDowell et al., 1998).

It should be noted that these experiments predominantly utilised a template source from bovine BAC clone (7N18) which was homogenous in its DNA sequence and was isogenetic. Utilising this source which contained a single template target site produced multiple bands using the same primers which are similar with the amplification product made from purified genomic cattle DNA further suggesting that the heterogeneity of the bands produced by PCR was due to the nature of the sequence being amplified. Indeed when the apparent heterogenous amplicons generated from the CA repeat region were cloned into a vector, the inserts were identified as being of homogeneous sequence, although showing variation in the length of the CA repeat further suggesting that heterogeneity was a function factors associated with the specific sequence rather than non-specific amplification of the sequence. Furthermore when these clones, were amplified again using primers spanning the CA repeats, similar results of multiple bands were observed on the gel. It was hypothesised that this could be due to the nature of the plasmids in the supercoiled formed as this could affect amplification of the CA repeat region (Chen et al., 2007). To clarify this issues, plasmid were linearised using restriction enzyme. However, there was no improvement observed on the amplification of tandem repeat region from the linearised plasmid as amplicons still produce multiple non-specific bands. This observation emphasized, that the nature of the plasmid was not the cause of the non-specific bands in amplicons and this were confirmed by comparing

bands sizes through calculation method derived based on bands migration on gel.

Our original intention and aim was to examine length polymorphisms from the CA repeat region and to establish its association with meat tenderness. Through our findings, characterisation of the polymorphic region using the PCR based technique was proven to be difficult as amplification from single homogenous source of DNA yield non-specific products. However, it was concluded that the multiple bands observed from the amplification of CA repeat region was caused by the nature and characteristic of the nucleotides repeats as observed by other. Furthermore, characterisation of CA repeat length differences was possible based on the lowest bands observed from the amplicons. Therefore, using this method and gene sequencing, we reported variation in terms of CA tandem repeat region from cattle of different breed based on the findings in the PCR experiment and gene sequencing.

6.2 Characteristic of calpastatin promoter expression

The study on the transcriptional activity of bovine calpastatin promoter had been carried out in two types of cell lines C2C12 and COS7 cells. Transfection experiment was carried out in various cell lines to analyse response of calpastatin promoter as these cells lines might contains differing transcriptional factors that regulate transcriptional activity of the gene and the response might be different as the gene might be switch on or off depending on the presence of transcription factors that are specific to the gene. Through transfection experiment of calpastatin promoter expression construct, it was concluded that the transcriptional activity of the promoter corresponds for Type I, II and Type III transcripts were most active in C2C12 mouse muscle cell line. Furthermore, through RT-PCR experiment, it was found that the expression of endogenous mouse calpastatin gene was not affected by differentiation of C2C12 cells. These findings were in contrast to earlier reports from Raynaud et al. (2005) who reported that calpastatin

promoter expression was higher in COS7 cell than C2C12 cells. It was proposed that the shorter doubling time in C2C12 than COS7 contributed to these findings (Bardouille et al., 2001).

It should be noted that the difference in the characteristic of transcriptional activity of these promoters in C2C12 and COS7 cell lines was probably caused by difference in the types of reporter gene, transfection marker and fluorescence measuring based equipment used in this thesis compared to the previous study. The original reporter gene (GFP) for calpastatin promoter expression vector was replaced with (ZsGreen) in our study as it produced brighter fluorescence as well as increasing the efficiency in its detection. Furthermore, a transfection marker was also included during transfection of calpastatin promoter construct in order to normalized variation caused by varying transfection efficiency in cells (Schagat, 1996). The inclusion of transfection marker was not done by previous research of calpastatin promoter expression particularly in bovine (Cong et al., 1998b; Raynaud et al., 2005) thereby contributing to the variation in the results generated. The results reported in this study were generated by the use of new fluorescence based measuring equipment (Typhoon Trio Fluorescence Imager) which had a greater sensitivity of fluorescence detection than conventional method using a plate reader. This new method of quantifying fluorescence managed to reduce variation in replicates which were often observed in the previous study of calpastatin promoter in either bovine or porcine gene from 12-25% to 1-3% in our experiment (Cong et al., 1998a; Parr et al., 2004; Sensky et al., 2006).

The study firstly optimised the amount of co-transfected transfection marker as from our assessment, there was evidence of significant interaction ($P < 0.05$) between ZsGreen positive vector and the level of transfected marker, pDsRed Express N-1. The interaction between both promoters could be explained as certain promoters were stronger than others (Qin et al., 2010). The stronger promoter could sequester basal factors, consequently repressing activity of the weak promoter. In our experiment, both vectors of

ZsGreen positive and pDsRed Express N-1 contains the same CMV promoter. It was proposed that the interaction observed in our experiment could be the results where either one of these promoters were competing for the availability of transcriptional factors in the cell lines which in turn could affect the constitutively expressed fluorescence gene (Shifera and Hardin, 2010).

Interaction between both vectors was apparent when higher amount of transfection marker was used, therefore only 5ng was used in later experiment as it provides the lowest interaction between ZsGreen based promoter vector and transfection marker. Data of calpastatin promoter activity was generated through transfection studies and was normalised against transfection marker expression to reduce variability caused by transfection efficiency (Schagat, 1996). There were no issues with our experiment as the activity of co-transfection control (pDsRed Express N-1) was not affected by transcriptional activity of transfected ZsGreen based vector or any of the experimental treatment.

Through observation, all three calpastatin promoters for Type I, II and Type III transcripts were functional in C2C12 and COS7 cell lines using the ZsGreen based reporter vector with higher transcriptional activity than the control vector ($P < 0.05$). The longest promoter construct for Type I, II and Type III displayed similar pattern of promoter activity observed previously in bovine with Type II promoter exhibit the highest promoter activity in both cell lines (Raynaud et al., 2005). This was however different in pigs as transcriptional activity of Type I and Type II promoter was reported to be approximately 50% less efficient compared to Type III promoter (Parr et al., 2004). This was explained by Parr et al. (2004) as there is a possibility of presence of negative enhancer sequence 5' upstream of Type I and II porcine CAST promoter which could down regulate their activity. Furthermore, in agreement with Raynaud et al. (2005) deletion analysis of promoter for Type III transcripts did not produced significant changes to the promoter activity. This suggested that potential transcription factor binding sites such as GATA-1 and HSF1 potential site found in the in the promoter region of deletion construct

ZsBovProt3/1 may not be responsible in regulating transcriptional activity for Type III transcript although transcriptional activity of the promoter was still significantly higher with the shortest deletion construct compared to the control vector. The presence of five potential SP-1 motifs in all Type III constructs could explain the maintained of transcriptional activity of the promoter as the presence of this potential site was a characteristic known in mammalian gene promoter which lack TATA box (Kolell and Crawford, 2002). Expression of these TATAless promoters were usually driven by other promoter element in this case is SP1 in order to activate transcriptional activity of the promoter (Huber et al., 1998). Furthermore, SP1 binding site is normally involved in the determination of transcription start sites (Kollmar et al., 1994). A TATAless mammalian promoter containing SP1 binding site and also high in GC content is also associated with constitutive expression of the gene (Ye et al., 1993).

Apart from looking at the potential link of polymorphic region in calpastatin type I promoter and meat tenderness, it was also an interest in this study to assess transcriptional activity of bovine calpastatin promoter. The work showed promoter expression similar to that observed in the previous research. Furthermore, the study managed to improve the processes used to assess promoter activity. Finally, the inclusion of transfection marker validated our finding on transcriptional activity of calpastatin promoter in both C2C12 and COS7 cell lines.

6.3 Calpastatin promoter response towards treatment of anabolic agent

The nature of calpastatin gene which contains multiple promoter activity were similar to findings in other gene such as the growth hormone receptor (Jiang, 1998) where multiple variance of the gene are expressed as the results of transcriptional activity from different promoters in the gene. As with other promoter, the calpastatin promoter contains motifs or elements which could possibly responsive to the activation or deactivation transcriptional factor binding protein consequently affecting the transcriptional activity of the gene particularly in an association with hypertrophic growth as calpastatin mRNA and protein were found to be highly elevated in animals in those condition (Duckett et al., 2000; Parr et al., 1992). Therefore, the main hypothesis was built to assess whether activity of calpastatin promoter could be affected with administration of these components. The inclusion of a proper positive control have always been an oversight in the previous experiment involving response of calpastatin promoter towards treatment of chemical agents involved in the hypertrophic signaling pathway (Cong et al., 1998b; Sensky et al., 2006). For this reason, experiment involving calpastatin promoter response in this thesis was carried out using a proper positive control for treatment (CRE reporter) (SABiosciences) in order to generate a more reliable data.

In general, the treatment of dibutyryl cAMP on cells transfected with calpastatin promoter construct demonstrates that most of the promoters including their deletion construct recorded significant decrease in their promoter activity. The observed findings were in contrast with findings reported by Cong et al. (1998b) as promoter for Type II displayed significant increase in transcriptional activity when it was treated with dibutyryl cAMP. It was hypothesised that Type II promoter transcriptional activity could be elevated with dibutyryl cAMP treatment as CRE motifs are located at -148 and -333 upstream from TSS. However significant decrease in transcriptional activity was observed in this thesis. The inclusions of a positive control for

dibutyryl cAMP treatment in our experiment confirmed that the treatment was effective and was able to elevate transcriptional activity of the positive control vector. This control was not included in the previous research done by other groups. It was also noted that the reduction in activity of Type I promoter were similar to the response recorded in porcine calpastatin when it was administered with dibutyryl cAMP as significant decrease in promoter activity was recorded (Sensky et al., 2006). In porcine the CRE sites were identified in the 5' region of Type III promoter but in bovine CREs are located in the 5' region of Type II promoter (Cong et al., 1998b; Parr et al., 2001). This high density of CREs in Type III reported in porcine probably explains the significant the increase in this promoter's activity (Parr et al., 2001), although in our study a significant decrease of promoter activity was observed in bovine Type III promoter when it was treated with dibutyryl cAMP; there was no CRE site located in the 5' region of Type III bovine calpastatin promoter.

Furthermore, administration of forskolin, which in theory will increase formation of cAMP in the cytosol, does not show any significant changes to the calpastatin promoter activity although the positive control for treatment was significantly induced ($P<0.001$) with the treatment. In porcine, promoter for Type III transcripts recorded increase in transcriptional activity while Type I promoter activity were reduced from the treatment similar to the response recorded from dibutyryl cAMP treatment (Sensky et al., 2006). Dose response of forskolin administration on the longest construct for Type II calpastatin promoter shows significant decrease of promoter activity ($P<0.05$) compared to the control promoter construct (non-treated) although the negative response was not subjected to any pattern of dose response curve from the variable concentration of forskolin use for the treatment.

In order to investigate, whether endogenous CAST was affected by treatment of forskolin quantitative RT-PCR were carried out on the C2C12 cells transfected with promoter construct associated with Type I, II and Type III calpastatin. There was significant effect ($P<0.05$) of forskolin treatment on the transcriptional activity of the endogenous calpastatin of transfected

C2C12. Furthermore, it was also shown from this experiment where the type of transfected calpastatin promoter construct significantly affect expression of endogenous CAST ($P<0.05$). These results suggest that the presence of CAST promoter construct in the cells could produce transcriptional interference which in turns affects expression from the endogenous gene. However, there was no direct evidence to explain our findings.

Apart from investigating response of calpastatin promoter to cAMP mediated activation of the gene, calcium related stimuli was also investigated, as elevated concentrations of calcium had been linked to hypertrophy in cardiac muscle through activation of calcium-calceineurin related pathway (Wilkins and Molkenin, 2004). The activation of this pathway would lead to upregulation in the transcriptional activity of gene related to hypertrophic growth. In our findings, the administration of calcimycin managed to significantly reduce ($P<0.001$) transcriptional activity from all tested calpastatin promoter construct with its deletion. Compared to previous study in porcine, only promoter from Type III shows significant decrease in the promoter activity with calcimycin treatment (Sensky et al., 2006). Therefore, it may be possible that results generated from this experiment may occur as the consequence of the treatment itself where cells started to die as they were treated with calcimycin, subsequently contributing to the sudden drop of its transcriptional activity. This observation was supported by literatures which observed calcimycin as an agent which could induce apoptosis or cell death in mouse lymphoma cell lines as well as lens epithelial cells and the effect was enhanced with the increase in the concentration of calcimycin (Caron-Leslie et al., 1994; Li et al., 1995). In our case, only partial of this effect was observed as the concentration used for the treatment was not high as used by others.

To summarise, it could be said that treatment of cAMP was able to show regulation in the forms of suppression in CAST promoter activity although it was not known what could cause this effect. Furthermore, assessment on administration of forskolin indicates that there was no direct effect of the

agent on the promoter as transcriptional activity remains unchanged with the treatment. Interestingly, the treatment of these agents was capable to induce differential in response from CAST promoters depending on the cell lines where it was transfected. This could be the results as different cells where the promoters were transfected behave differently with the provided stimulus although both cells are of mammalian origins. It could also be said that CAST promoter is more responsive in cells which is related to muscle than kidney as different cells probably contains regulatory elements or transcription factors that are specific to the cell or tissue type as mammalian gene promoters harbours reputational control of this effect (Smith et al., 2007).

6.4 CA repeats length polymorphisms and its relation to promoter activity in association with meat tenderness.

In the literature it has been proposed that the intronic CA repeat region has significantly been associated as a possible regulator of mammalian alternative splicing (Hui et al., 2005). This evidence suggests the possibility of this region to play a role in the event of alternative splicing. However, in our case the CA tandem repeat region is located 5' upstream the TSS. Furthermore, previous publication which managed to identify the polymorphisms in the promoter region for Type I calpastatin suggest that the tandem CA repeat could be potential marker for linking the genetic traits and meat quality (Nonneman et al., 1999). Therefore in this study we examine the hypothesis of possible links between the length polymorphisms of CA tandem repeat and meat tenderness of the samples which have determined by its shear force value.

The length of CA tandem repeat in calpastatin promoter region had been shown to be different in size in two different individual cattle. This was shown on sequencing data and amplicon size determination from DNA sourced from BAC 7N18 vector and DNA source from BovProt1/1 plasmid. In order to further analyse variability of this region and its association to meat tenderness, DNA samples of cattle with variation in tenderness as

determined by shear force were purified and the CA tandem repeat region were amplified.

To fulfil our aims and objective, a cloning strategy were devised and the CA tandem repeat region of cattle with differing meat tenderness and breeds were cloned into the developed ZsGreen promoter expression vector (ZsBovProtIHindIII). Sequencing of these plasmids revealed large variation in the CA repeat length with the longest containing 60bp size of CA repeat length and the shortest containing 40bp length of CA repeat sequences. This proved our first hypothesis that amplification of this region from different cattle yield different length of CA repeat.

To test our second hypothesis, the CA repeat sequences were plotted against the shear force value evaluated from their original sample. However, there was no correlation ($R=0.28$) found between the length of the CA repeat sequences and the tenderness of the meat ($P>0.05$). This may be the case of only a small sample were tested for this correlation ($n=6$). The sequencing data also revealed that more than one plasmid contains the same length of CA tandem repeat. Therefore it was theorized that these may indicates the presence of multiple potential alleles appearing at the same locus which represent the polymorphism of the CA tandem repeat in the promoter region of calpastatin gene, as others have reported in the intron 1 region of IGF-I gene (Estany et al., 2008). The existence of these polymorphisms may have direct influence in the expression of calpastatin gene as the CA tandem repeat is located at the promoter region for Type I calpastatin ~483bp before the TSS, a region which is often associated with variance observed in calpastatin transcripts either in pigs, cattle or rat (De Tullio et al., 2007; Parr et al., 2001; Raynaud et al., 2005). To test our third hypothesis, Zsgreen based 1xa promoter expression construct with varying length of CA repeat sequence were transfected into C2C12 cell lines and assessment of promoter activity was made. It was revealed that there was significant effect of CA tandem repeat length on the calpastatin promoter expression ($P<0.05$) when it was compared to the control vector (ZsBovProtIHindIII).

The variation observed in the transcriptional activity of CAST Type I promoter was more apparent in C2C12 cell lines as only three constructs displayed significant difference in their expression in COS7 cell lines when compared to the control promoter expression vector ($P < 0.05$). The importance of the CA tandem repeat sequence in the transcriptional activity of Type I Calpastatin promoter was displayed in bovine as deletion of the tandem repeat from the promoter region of Type I promoter expression construct significantly diminished its activity (Raynaud et al., 2005). Therefore the importance of this numbers of CA repeat was further repeated with the experiment carried out in this thesis which proven our original hypothesis that the polymorphism of CA tandem repeat length in the promoter region for Type I calpastatin does significantly affect transcriptional activity of the promoter. It was however not known how this length variation largely affects the transcriptional efficiency of the promoter as this experiment only defines variation in the transcriptional activity of the promoter construct with varying length of CA repeat compared to the control promoter construct.

Although there was no direct evidence which defines the correlation between the length of CA tandem repeat polymorphisms and meat tenderness, it was clear that the transcriptional activity of Type I promoter was significantly affected by the changing the length of the sequence. Previous study managed to correlate the tenderness traits and genetic markers which are presents in more than one gene related to meat tenderness (Barendse et al., 2007; Casas et al., 2006). SNP sites were located in calpain and capastatin gene of large pool of samples in order to associate it with tenderness traits in animals and other characteristic of meat quality. Compared to our study, there are vast differences in terms of the number of polymorphic sites, samples population and the number of meat quality traits seeks in their experiment. This may explained the results we reported as the data generated in this study were restricted because of the limitation produced from the experiment.

6.5 Conclusion

This study managed to define the CA tandem repeat length polymorphisms located in the promoter region of Type I bovine calpastatin by developing a reliable method of cloning the tandem repeat region. However, characterisation of CA tandem repeat length on gel using PCR amplification product were proven to be difficult as multiple bands were observed in the gel. Gathered observation indicates that non-specific product could be generated from slippage during PCR as well as the nature of the nucleotide repeat which are prone to error during amplification. Through sequencing of successfully cloned CA repeat amplicons, it was found that this region contains variable length of CA tandem repeat and the length differs in individual cattle of different breed. In our investigation to associate the CA repeat length polymorphisms with meat tenderness, we found that there was no correlation ($P>0.05$) between both although it might be caused by the relatively small sample used in this study. Interestingly we found evidence or possibility that the CA repeat in the promoter regions for Type I calpastatin were actually consist of alleles with different length of CA repeats which appears in the same locus in the gene. Furthermore, we found that the activity of calpastatin Type I promoter was significantly affected as length of the CA tandem repeat were changed in the promoter region although it was unknown how this occurs.

In the study calpastatin promoter, we found that the promoters for Type I, II and Type III were functional in C2C12 and COS7 cells lines although the activity was more efficient in C2C12 cell lines. Furthermore, investigation of promoter response towards agent such as dibutyryl cAMP, forskolin and calcium ionophore generates data which was contradicting to the research done previously as treatment of dibutyryl cAMP and forskolin did not increase CAST promoter activity. However, our results were validated with the inclusion a positive control for these treatment which was not been done previously. The contradicting results may be generated by the different methodology and materials use in the previous experiment such as reporter

vector, transfection reagent, fluorescence measurement as this could also affect transcriptional activity of the gene itself.

6.6 Future work

The meat tenderness aspect was always the main criteria and objective that were considered during the course of this project as we examined transcriptional activity of calpastatin, an endogenous inhibitor of calpain activity. This is crucial in meat quality aspect as the enzymatic activity of calpain were largely responsible for degradation of myofibrillar protein during post mortem period in animals. The project carried out in this thesis has been focusing on method development which in turn helps us to define length variation of the CA repeat located at 5' upstream promoter region of Type I calpastatin and subsequently determined its association with meat tenderness. Although, in our report there was no correlation between the CA tandem repeat polymorphisms and meat quality, there was evidence that changing the length of polymorphisms in this promoter region significantly affect transcriptional activity of the gene. In order to associate the polymorphisms with meat tenderness, we hope that the new develop method could be use in the experiment which incorporates a larger sample size in order to further validates our finding. Furthermore, we hope that we can include other polymorphisms located in the gene such as a single nucleotide polymorphisms located at 3' untranslated region of the gene in this correlation therefore a genetic based method could be developed in order to select animal prior to slaughter. Finally it would be interesting if these polymorphisms could be linked directly to the activity or level of the protein.

Appendix 1

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

LOCUS NW_001495281 3121 bp DNA linear CON 29-JUL-2008
 DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.
 ACCESSION NW_001495281 REGION: 770041..773161
 VERSION NW_001495281.2 GI:194668375
 KEYWORDS WGS.
 SOURCE Bos taurus (cattle)

```

1021 tgccatggca ggggtaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tcactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct ttaagatga accaaagaca
1201 aaagcaatct ggcctcctcg caaacgaaat tcaacagcct cctgaaaggc aatgggggtt
1261 aaatttagga ctgttcttcg gttttgattg gaacccaaga cttgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgca c aaaacacacc caggcccg tg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag a ccaattttgt ttctgcaatc cgc ttctctca tccagagggt ccaggccccg
1501 ggccatctga gttggtaaata tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgagg cttgagatgt agacggggcc acgccccggg
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccgcca
1681 tctccagccc ctctctctcg cgaccactg gggcaccagg gcgacctcgg gtgggggtgg
1741 gtgtccctgg gagaagatac gggaccagg gtgtgagttg caaacaggca gccccgggct
1801 gctgccgccc gggcgctgcc aactgcaggc aggaagggga gggccctgcc cggcgaaggg
1861 gagctctcgc gggtcggggc tgggtcggaa aagctgcctc acaggcgcg cccgccagccc
1921 ctccgcgccc tcgctccctc ccagcgctcc ccggtctccag cctcccttcc aggctccgcc
1981 gcgcccgcgc ggaggcagcg ctgcgaccgg cctcgccatg tcccagccgg gcccggaagc
2041 cgccgcctcc ccgcggcccc ggcgcgctgc ccgccacacc caggaggtga gtggcgctcc
2101 taccgcgggg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccacccctcc caggcccgga
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 ggcccgcgcg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg
  
```

Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	1xaCASTtand1 F	CAAAACACACCCAGGCCCG	61	123bp
Reverse	1xaCASTtand1 R	GCGGATTGCAGAAACAAATTGG	58.4	
Reverse (antisense)		CCAATTTGTTTCTGCAATCCGC		

Appendix 2

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

LOCUS NW_001495281 3121 bp DNA linear CON 29-JUL-2008
 DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.
 ACCESSION NW_001495281 REGION: 770041..773161
 VERSION NW_001495281.2 GI:194668375
 KEYWORDS WGS.
 SOURCE Bos taurus (cattle)

```

1021 tgccatggca gggttaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tcaactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct tttaatgatga accaaagaca
1201 aaagcaatct ggctctctcg caaacgaaat tcaacagcct cctgaaaggc aatgggggtt
1261 aaatttagga ctgtttctcg gttttgattg gaacccaaga cttgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaaacacac caggcccggtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgtttctctc tccagagggt ccaggccccg
1501 ggccatctga gttggtaaata tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccgcga
1681 tctccagccc ctctctctcg cgacccactg gggcaccaag gcgacctcgg gtgggggtggg
1741 gtgtccctgg gagaagatac gggaccagag gtgtgagttg caaacaggca gcccccgggt
1801 gctgccgccc gggcgctgcc aactgcaggc aggaagggga gggccctgcc cggcggaagg
1861 gagctctcgc gggtcggggc tgggtcgga aagctgcctc acaggcgcg cgcgcagccc
1921 ctccgcgccc tcgctcctc ccagcgctcc ccggctccag cctcccttcc aggcctcgcc
1981 gcgcccgcgc ggaggcagcg ctccgcagcg cctcgccatg tcccagccg gcccggaagg
2041 cgcgcgctcc ccgcggcccc ggcgcgctgc ccgccacacc caggaggtga gtggcgctcc
2101 taccgcggg gttgagcgcg gggaggatct cggggcccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccaccctcc caggcccgga
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcg atggccctg gtcccggcca agttcactgg gcgcggcgca agccacattg
  
```

Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	1xaCASTtand2 F	AAAACAAGTAAAGCCGCACAAAA	55.3	153bp
Reverse	1xaCASTtand2 R	TGGATGAGGAAGCGGATTG	56.7	
Reverse (antisense)		CAATCCGCTTCCTCATCCA		

Appendix 3

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

LOCUS NW_001495281 3121 bp DNA linear CON 29-JUL-2008
 DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.
 ACCESSION NW_001495281 REGION: 770041..773161
 VERSION NW_001495281.2 GI:194668375
 KEYWORDS WGS.
 SOURCE Bos taurus (cattle)

```

1021 tgccatggca gggttaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tactgacaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct tttaatgatga accaaagaca
1201 aaagcaatct gccctcctcg caaacgaaat tcaacagcct cctgaaaggc aatgggggtt
1261 aaatttagga ctgtttctcg gttttgattg gaacccaaga cttgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaacacacc caggcccgtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgcttctctca tccagagggg ccaggccccg
1501 ggccatctga gttgggtaaat tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccgcga
1681 tctccagccc ctctctctcg cgacccactg gggcaccaag gcgacctcgg gtgggggtgg
1741 gtgtccctgg gagaagatac gggaccagag gtgtgagttg caaacaggca gcccccgggt
1801 gctgccgccc gggcgctgcc aactgcaggc aggaagggga gggccctgcc cggcggaagg
1861 gagctctcgc gggtcggggc tgggtcggaa aagetgcctc acaggcgcgc ccgccagccc
1921 ctccgcgccc tcgctccctc ccagcgctcc ccggctccag cctcccttcc aggcctccgc
1981 gcgcccgcgc ggaggcagcg ctgcgaccgg cctcgccatg tcccagccgg gcccgaaagg
2041 cgcgcgctcc ccgcggcccc ggcgcgctgc ccgccacacc caggaggtga gtggcgctcc
2101 taccgcccgg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccaccctcc caggcccgga
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcgg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg
  
```

Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	1xaCASTtand3 F	TCATTGCACTTTCAAAACAAGT	54.7	203bp
Reverse	1xaCASTtand3 R	AATTTACCAACTCAGATGGCCC	58.4	
Reverse (antisense)		GGGCCATCTGAGTTGGTAAATT		

Appendix 4

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

LOCUS NW_001495281 3121 bp DNA linear CON 29-JUL-2008
 DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.
 ACCESSION NW_001495281 REGION: 770041..773161
 VERSION NW_001495281.2 GI:194668375
 KEYWORDS WGS.
 SOURCE Bos taurus (cattle)

```

1021 tgccatggca gggttaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tcaactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct tttaatgatga accaaagaca
1201 aaagcaatct ggcctcctcg caaacgaaat tcaacagcct cctgaaaggc aatgggggtt
1261 aaatttagga ctgtttctcg gttttgattg gaacccaaga cttgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaacacacc caggcccggtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgcttctctca tccagaggggt ccaggccccg
1501 ggccatctga gttgggtaaat tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccgcga
1681 tctccagccc ctctctctcg cgacccactg gggcaccaag gcgacctcgg gtgggggtggg
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1921 ctccgcgccc tcgctccctc ccagcgctcc ccggctccag cctcccttcc aggcctcgcc
1981 gcgcccgcgc ggaggcagcg ctccgcacgg cctcgccatg tcccagccgg gcccgaaagg
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2101 taccgcccgg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccaccctcc caggcccggg
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcgg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg
  
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Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	RE1xaF	AAACTCGAGTTCAACAGCCTCCTGAAAGG *XhoI restriction site	66.7	295bp
Reverse	RE1xaR	GAGAATTTACCAACTCAGATGGCC	61	
Reverse (antisense)		GGCCATCTGAGTTGGTAAATTCTC		

Appendix 5

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

LOCUS NW_001495281 3121 bp DNA linear CON 29-JUL-2008
 DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based on Btau_4.0), whole genome shotgun sequence.
 ACCESSION NW_001495281 REGION: 770041..773161
 VERSION NW_001495281.2 GI:194668375
 KEYWORDS WGS.
 SOURCE Bos taurus (cattle)

```

961 aagaaactgt ctgaaacaca gcaccgcaga gagcagtgac cccgaggctc acattgtcaa
1021 tgccatggca gggttaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtctc ttaagatga accaaagaca
1201 aaagcaatct ggctcctcg caaacgaaat tcaacagcct cctgaaaggc aatggggttt
1261 aaatttagga ctgttcttcg gttttgattg gaacccaaga cttgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaacacacc caggcccggtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgcttctctc tccagagggt ccaggccccg
1501 ggccatctga gttggtaaatt tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
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1861 gagctctcgc gggtcggggc tgggtcgga aagctgcctc acaggcgcg cggccagccc
1921 ctccgcgccc tcgctccctc ccagcgctcc ccggctccag cctcccttcc aggctccgcc
1981 gcgcccgcgc ggaggcagcg ctgcgcacgg cctcgccatg tcccagccgg gcccgaagcc
2041 cgccgcctcc ccgcggcccc ggcgcgctgc ccgccacacc caggagggtga gtggcgctcc
2101 taccgcccgg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccaccctcc caggcccgga
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcgg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg
  
```

Tandem CA repeat : Highlighted Red

Exon 1xa: Highlighted Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	555bp 5'1xa F	TCTGAAACACAGCACC GCAG	59.4	555bp
Reverse	RE1xaR	GAGAATTTACCAACTCAGATGGCC	61	
Reverse (antisense)		GGCCATCTGAGTTGGTAAATTCTC		

Appendix 6

Real-Time PCR oligonucleotides designed in mouse Calpastatin

Sequence details from NCBI database:

LOCUS AB026997 2622 bp mRNA linear ROD 02-OCT-1999
 DEFINITION Mus musculus CAST mRNA for calpastatin, complete cds.
 ACCESSION AB026997
 VERSION AB026997.1 GI:6006274
 KEYWORDS calpastatin.
 SOURCE Mus musculus (house mouse)

```

1201 tctgatttca cctgtagttc tccaactgga aagcaaagtg agaaagagaa gtctactggg
1261 gaaattttca aagctcagtc tgcaggagtg accagaagtt ctgttccccc caaggagaag
1321 aaaaggaagg tggaagagga ggtcataaat gatcaagcac ttcagggtct gtcagattca
1381 ctggggcacc ggccagccaga tcctccgagc cacgttagcc aagctgaaca agtcaaagag
1441 gcaaaagcaa aagaagaaaag gcaggagaag tgtggtgaag atgaggacac agtcccagct
1501 gagtacaggt taaaaccagc aaaggataaa gatggaaaac cactattgcc agagcctgaa
1561 gaaacatcta agagcctgag tgagtcggag ctgattgggg agctttcagc agattttgac
1621 cgatctacat atcaagacaa accatctacg ccagctgaaa agaaatccaa tgacacatcc
1681 caaactcctc cgggggagac tgtgcctcgg gcctccatgt gcagtatacg gtcagcgcca
1741 cccaaactag catccttgaa gggcgtggta ccagaagatg ctggtgaaac cttggctgga
1801 agcctgggga caagggaagc agatccagaa catgaaaaaa ctgtggagga taaagtcaag
1861 gagaaagcta aagaagaaga gcatgaaaaa cttggtgaaa aagaagaaac agtgccctct
1921 gattatcgac tagaagaagt caaggataag gatggaaaac cactcctgcc caaagaatcc
1981 caaggaacaac ttgcaccctt aagcgatgac ttccttcttg atgccttgtc tcagggacttc
2041 tccagtcctgg caaacatatc gtctccttgaa tttgaagatg ccaaactttc tgctgccatt
2101 tctgaagtag tttctcagac acctgctcca agcaccatg cagcagctcc actgcctggc
2161 actgagcaga aagacaaaaga acttgatgat gccttggatg aactttctga cagtcttgga
2221 caaaggccgc ctgatccaga tgagaacaaa ccactggatg acaaagtga ggagaaaatc
2281 aaaccagagc atagcgagaa actgggagaa agagacgaca ccatccccc tgaatacagg
2341 catctcttgg ataatgatgg gaaggacaaa ccagagaagc caccgactaa gaaaacagag
2401 aaacctgatc aggaccggga cccattgat gccctctcag aagatttgga tagctgcccc
2461 tcaactacag agacctcaaa gaatacagca aaggggaaga gcaagaagac ttcaagttcc
2521 aaagcatcca aggacggaga gaaaacaaag gactcttcca agaagacaga ggaagtgtcc
2581 aagccaaagg ctaaagaaga tgcaagacac agttaatgaa tt
  
```

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	musCASTF	AAACCACTCCTGCCCAAAGA	57.3	100bp
Probe	musCAST Probe	CCAGGAACAACCTTGCACCCTTAAGCG	66.4	
Reverse	musCASTR	TGTTTGCCGGACTGGAGAAG	59.4	
Reverse (antisense)		CTTCTCCAGTCCGGCAAACA		

Appendix 7

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region

Sequence details from NCBI database:

```

LOCUS       NW_001495281                3121 bp    DNA        linear    CON 29-JUL-2008
DEFINITION  Bos taurus chromosome 7 genomic contig, reference assembly (based
            on Btau_4.0), whole genome shotgun sequence.
ACCESSION   NW_001495281 REGION: 770041..773161
VERSION     NW_001495281.2 GI:194668375
KEYWORDS     WGS.
SOURCE      Bos taurus (cattle)
1021 tgccatggca gggtaaacag gttttcttct gtcagatctt cacaacttgg cagcaaccac
1081 aggcagacaa tcaactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct ttaagatga accaaagaca
1201 aaagcaatct ggctctctcg caaacgaaa t tcaacagcct cctgaaagg aatggggttt
1261 aaatttagga ctgttcttcg gttttgattg gaaccaaga ctgtttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaacacacc caggcccgtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgcttctca tccagagggt ccaggccccg
1501 ggccatctga gttggtaaat tctc tctccta gtaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccacca
1681 tctccagccc ctctctctcg cgaccactg gggcaccaag gcgacctcgg gtgggggtggg
1741 gtgtccctgg gagaagatac gggaccagg gtgtgagttg caaacaggca gccccgggct
1801 gctgccgccc gggcgctgcc aa ctgcag aggaagggga gggccctgcc cggcgaaggg
1861 gagctctcgc gggtcggggc tgggtcggaa aagctgcctc acaggcgcgc ccgccagccc
1921 ctccgcgccc tcgctccctc ccagcgcctc ccggtctccag cctcccttcc aggctccgcc
1981 gcgcccgcgc ggaggcagcg ctgcgaccgg cctcgccatg tcccagccgg gcccggaagcc
2041 cgccgcctcc ccgcggcccc ggcgcgctgc ccgccacacc caggaggtga gtggcgctcc
2101 taccgcgggg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccacccctcc caggcccggg
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcgg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg

```

Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

PstI restriction site: Highlighted Pink

SacI restriction site: Highlighted Blue

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	RE1xaF(XhoI)	AAACTCGAGTTCAACAGCCTCCTGAAAGG *XhoI restriction site	66.7	295 bp
Reverse	RE1xaR(HindIII)	GAGAAGCTTCCAACCTCAGATGGCC	64.4	
Reverse (antisense)		GGCCATCTGAGTTGGAAGCTTCTC*		

*Changes were made to the forward and reverse primers in order to introduce a HindIII restriction site during site directed mutagenesis.

Appendix 8

PCR oligonucleotides designed in bovine calpastain exon 1xa promoter region for site directed mutagenesis

Sequence details from NCBI database:

```

LOCUS      NW_001495281                3121 bp    DNA        linear    CON 29-JUL-2008
DEFINITION Bos taurus chromosome 7 genomic contig, reference assembly (based
            on Btau_4.0), whole genome shotgun sequence.
ACCESSION  NW_001495281 REGION: 770041..773161
VERSION    NW_001495281.2 GI:194668375
KEYWORDS   WGS.
SOURCE     Bos taurus (cattle)
1021 tgccatggca gggttaacag gttttcttct gtcagatctt cacaaactgg cagcaaccac
1081 aggcagacaa tcaactgcaga ggaggagcct tttctagtct ggcttataac atttcacttc
1141 aagcttggtta taattcacag ccccaaacca ggaagtgtct ttaagatga accaaagaca
1201 aaagcaatct ggctctctcg caaacgaaat tcaacagcct cctgaaaggc aatggggttt
1261 aaatttagga ctgttcttcg gttttgattg gaacccaaga ctgttccac atgaaacatc
1321 attgcacttt caaaacaagt aaagccgcac aaaacacacc caggcccgtg tgtgtacaca
1381 cacacacaca cacacacaca cacacacaca cacacacaca cacaatcaca acacacggag
1441 tcacacacag accaatttgt ttctgcaatc cgcttctca tccagagggt ccaggcccgc
1501 ggccatctga gttggtaa at tctcctccta gtttaactcag agcagattgc agaaatgctg
1561 tctaattctt gagattctta gggagtgggg cttgagatgt agacggggcc acgccccggt
1621 tccgctctcc ttgcacaact gcaagccaag tctaggcaag tcggggggaa aaccccgcga
1681 tctccagccc ctctctctcg cgaccactg gggcaccaag gcgacctcgg gtgggggtgg
1741 gtgtccctgg gagaagatac gggaccagg gtgtgagttg caaacaggca gcccccgggt
1801 gctgccgccc gggcgctgcc aactgcaggc aggaagggga gggccctgcc cggcgaaggc
1861 gagctctcgc gggtcggggc tgggtcgga aagctgcctc acaggcgcgc ccgccagccc
1921 ctccgcgccc tcgctccctc ccagcgctcc ccggctccag cctcccttcc aggetccgcc
1981 gcgcccgcgc ggaggcagcg ctgcgaccgg cctcgccatg tcccagccgg gcccgagacc
2041 cgccgcctcc ccgcggcccc ggcgcgctgc ccgccacacc caggaggtga gtggcgctcc
2101 taccgccggg gttgagcgcg gggaggatct cggggccccc cgaagcctcg ggtccacctt
2161 cagaggaggg ttagccttcc tggccttctg tccctgggcg ccacccctcc caggcccgga
2221 gaaacatctg gagagagacg ggtgtggact cagaacctgc ctctgggcag gtgaccggag
2281 gggcccgcgg atggccctgg gtcccggcca agttcactgg gcgcggcgca agccacattg

```

Tandem CA repeat : Bolded Red

Exon 1xa: Bolded Blue

Site directed mutagenesis target: Highlighted Pink

Primer Type	Primer Name	Primer Sequence	Tm °C	Product length
Forward	SDM F CAST 1xa	CGGGCCATCTGAGTTGGAAGCTTCTCCTCCTAGT TAACTC *	74.6	123bp
Reverse	SDM R CAST	GAGTTAACTAGGAGGAGAAGCTTCCAACCTCAGA TGGCCCG	74.6	
Reverse (antisense)	1xa	CGGGCCATCTGAGTTGGAAGCTTCTCCTCCTAGT TAACTC *		

*Changes were made to the forward and reverse primers in order to introduce a HindIII restriction site during site directed mutagenesis.

Appendix 9

Ingredients formulated for 1 litre of NZY+ broth media.

Constituent	Quantity/Volume	Description
NZ amine (casein hydrolysate)	10g	pH adjusted to 7.5 using NaOH
Yeast extract	5g	
Sodium chloride (NaCl)	5g	
Deionized H ₂ O	1litre	
Magnesium Chloride (MgCl ₂)	12.5ml of (1M)	The following were added prior to use
Magnesium Sulfate (MgSO ₄)	12.5ml of (1M)	
Glucose (C ₆ H ₁₂ O ₆)	10ml of (2M)	

Appendix 10

Calculation of quantity estimation of DNA in each band from HindIII digested Lambda DNA size markers.

Band no	bp of band	Proportion of DNA in band	% of DNA in band	Quantity of DNA (ng) per band			
				Loading volume (ng)			
				500	1500	2500	3500
1	23130	0.4769	47.69	238	715	1192	1669
2	9416	0.1941	19.41	97	291	485	679
3	6557	0.1352	13.52	68	203	338	473
4	4361	0.0899	8.99	45	135	225	315
5	2322	0.0479	4.79	24	72	120	168
6	2027	0.0418	4.18	21	63	104	146
7	564	0.0116	1.16	6	17	29	41
8	125	0.0026	0.26	1	4	6	9
Total	48502						

Appendix 11

Single factor anova analysis for comparison of mouse calpastatin level expression in C2C12 cells between different days during cell differentiation.

Anova: Single Factor

SUMMARY						
Groups	Count	Sum	Average	Variance		
Day 1	3	71.59709	23.8657	0.226148		
Day 3	3	69.88847	23.29616	0.087028		
Day 5	3	69.82187	23.27396	0.216861		
Day 7	3	71.10994	23.70331	0.395928		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.788635	3	0.262878	1.135587	0.391375	4.066181
Within Groups	1.851929	8	0.231491			
Total	2.640564	11				

Appendix 12

Raw data from quantitative RT-PCR using 36B4 house keeping gene from experiment. Data which fell in the range of average plus or minus two standard deviation was considered acceptable to be pooled and used as standard curve for quantitative RT-PCR experiment.

Transfected vectors in C2C12 cells	Cp
Negative	23.16 19.83 19.65
Negative 10uM Fors	20.38 18 19.27
Positive 1-N1	19.64 19.26 19.17
Positive 1-N1 10uM Fors	19.01 19.15 18.47
ZsBovProt 1/1	19.24 19.44 18.98
ZsBovProt 1/1 10uM Fors	18.62 18.81 19.96
ZsBovProt 2/1	18.29 19.07 19.65
ZsBovProt 2/1 10uM Fors	19.24 18.81 19.47
ZsBovProt 3/1	19.97 18.2 19.01
ZsBovProt 3/1 10uM Fors	19.69 19.78 18.98
CRE reporter	19.47 19.96 18.13
CRE reporter 10uM Fors	19.25 19.63 19.2
CRE negative	19.19 20.35 20.14
CRE negative 10uM Fors	18.46

	19.15
	19.66
CRE positive	19.04
	19.07
	19.7
CRE positive 10uM	18.73
	18.96
	19.67
pZsGreenBovProt1HindIII	18.11
	19.27
	19.69
pZsGreenBovProt1HindIII 10uM	18.26
	19.44
	19.48

Average data value	19.30
Standard Deviation	0.78
Average value + 2 × (Standard deviation)	20.86
Average value – 2 × (Standard deviation)	17.74

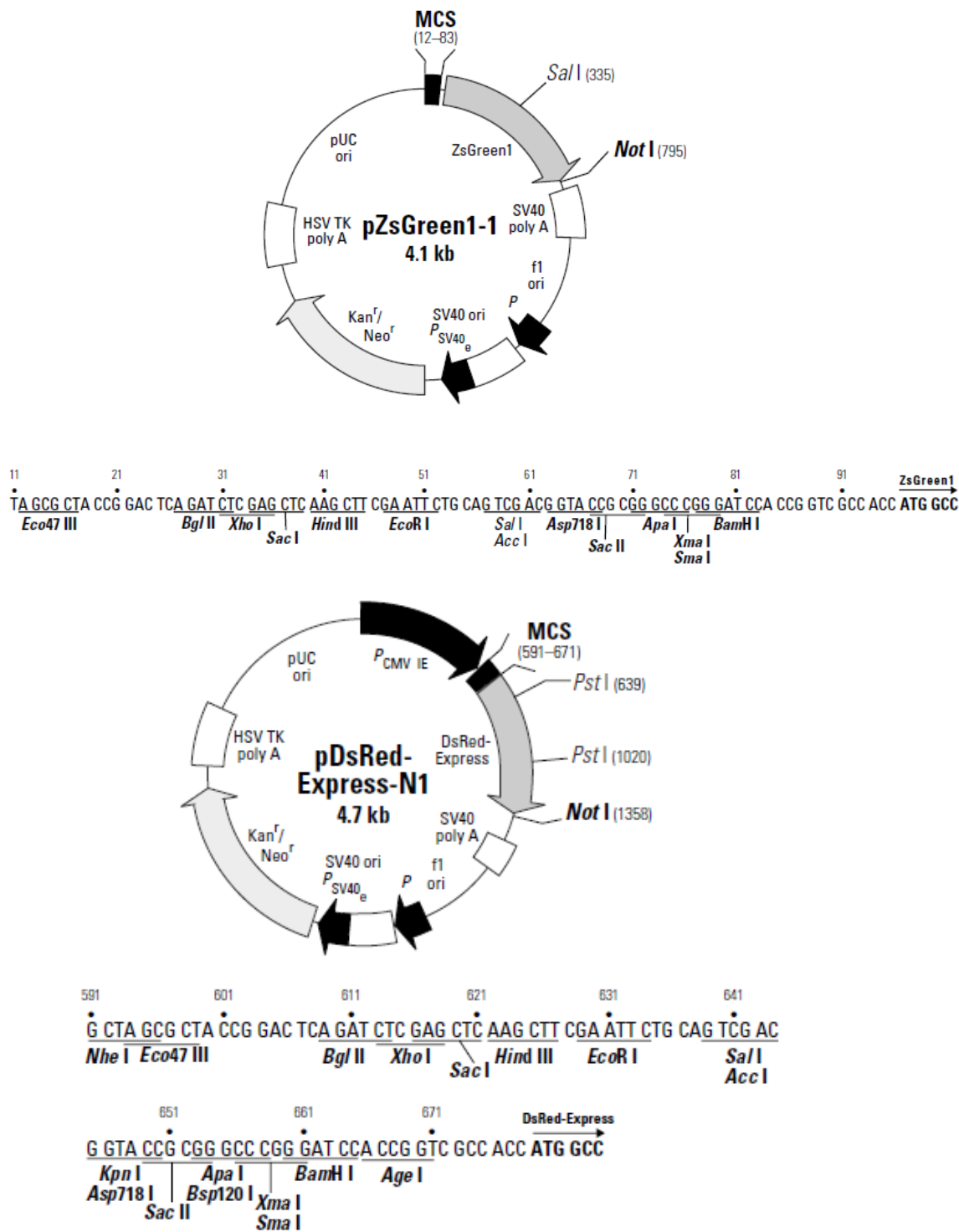
Appendix 13

Picture of a Typhoon trio fluorescence based imager used for scanning fluorescence emitted from transfected promoter construct



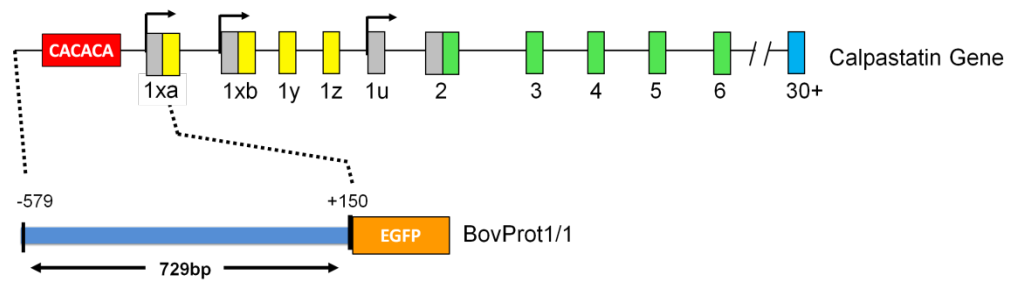
Appendix 14

Transfected promoter construct based from pZsGreen 1-1 promoter expression vector and the co-transfected pDsRed Express N-1 vector.



Appendix 15

BovProt1/1 promoter construct from Raynaud et., al (2005) containing bovine calpastatin exon 1xa sequences and the short CA tandem repeat region



Appendix 16

TFSEARCH FOR CAST PROMOTER TYPE I (Settings: Verterbrate Score threshold:85)

ZsBovProt 1/1, 1/2 and 1/3

ZsBovProt 1/1

```

1  CTGTTCTTCG GTTTTGATTG GAACCCAAGA CTTGTCCCAC ATGAAACATC entry  score
    <-----> SRY 90.9
    -----> USF 88.6
    -----> SRY 87.3
    <-----> Pbx-1 87.3
    <--- CdxA 87.1

51 ATTGCACTTT CAAAACAAGT AAAGCCGCAC AAAACACACC CAGGCCCGTG entry  score
    -----> SRY 92.7
    -----> SRY 90.9
    --- CdxA 87.1
    <-----> C/EBPb 85.5

101 TGTGTACACA CACACACACA CACACACACG CACACACACA CACAATCACA entry  score
    <-----> GATA-1 89.0
    <-----> GATA-3 87.5

151 ACACACGGAG TCACACACAG ACCAATTTGT TTCTGCAATC CGCTTCCTCA entry  score
    <-----> SRY 100.0
    <-----> C/EBPa 90.3
    <-----> AP-1 88.7
    <-----> GATA-2 87.0
    <-----> GATA-1 85.7
    <-----> CP2 85.4

201 TCCAGAAGGT CCAGGCCCCG GGCCATCTGA GTTGGTAAAT TCTCCTCCTA entry  score
    <-----> GATA-2 88.5
    -----> C/EBP 86.2

```

ZsBovProt 1/2

```

251 GTTAACTCAG AGCAGATTGC AGAAATGCTG TCTAATTCTT GAGATTCTTA entry  score
    <-----> CdxA 93.6
    --- p300 91.1
    <-----> CdxA 89.7
    -----> C/EBPb 88.8
    -----> C/EBPa 87.3
    -----> GATA-1 86.1

301 GGGAGTGGGG CTTGAGATGT AGACGGGGCC ACGCCCCGGT CCCGCTCTCC entry  score
    -----> C/EBPb 93.6
    -----> p300 91.1
    -----> Sp1 89.0
    -----> C/EBPb 86.2
    -----> MZF1 85.2

351 TTGCACAAC TCGAGCCAAG TCTAGGCAAG TCGGGGGGAA AACCCCGCCA entry  score
    <-----> c-Rel 97.5
    <-----> GATA-1 95.1
    -----> C/EBPb 93.6
    -----> GATA-2 92.9
    -----> MZF1 92.2
    <-----> NF-kap 92.1
    <-----> NF-kap 90.7
    <-----> Sp1 87.7
    <-----> GATA-3 87.5
    -----> C/EBPb 86.2
    -----> NF-kap 86.0

```


401 TCTCCAGCCC CTCCTCTCTG CGACCCACTG GGGCACCAAG GCGACCTCGG entry score
 ----- GATA-1 95.1
 ----- GATA-2 92.9
 - Sp1 87.7
 ----- GATA-3 87.5
 <-- RREB-1 85.1

ZsBovProt 1/3

451 GTGGGGTGGG GTGTCCCTGG GAGAAGATAC GGGACCCAGG GTGTGAGTTG entry score
 -----> GATA-1 91.5
 -----> GATA-2 90.9
 -----> Sp1 90.4
 -----> GATA-1 89.0
 -----> GATA-3 88.8
 -----> HSF2 87.8
 -----> AML-1a 87.4
 -----> AML-1a 87.4
 -----> SREBP- 85.9
 -----> HSF1 85.5
 -----> RREB-1 85.1
 -----> GATA-1 85.1

501 CAAACAGGCA GCCCCGGGCT GCTGCCGCCC GGGCGCTGCC AACTGCAGGC entry score
 -----< c-Myb 86.7

551 AGGAAGGGGA GGGCCCTGCC CGGCGAAGGG GAGCTCTCGC GGGTCGGGGC entry score
 ----- Sp1 94.5
 -----> MZF1 87.0
 -----> MZF1 87.0
 --- c-Myb 86.7

601 TGGGTCGGAA AAGCTGCCTC ACAGGCGCGC CCGCCAGCCC CTCCGCGCCC entry score
 -----> Sp1 94.5

651 TCGCTCCCTC CCAGCGCTCC CCGGCTCCAG CCTCCCTTCC AGGCTCCGCC entry score
 -----> ZID 87.9
 -----> Lyf-1 85.7
 -----> MZF1 85.2

701 GCGCCCGCCC GGAGGCAGCG CTCGCACCGG entry score
 -----> c-Ets- 85.3

Appendix 17

TFSEARCH FOR CAST PROMOTER TYPE II (Settings: Vertebrate Score threshold: 85) CAST Promoter 2/1,2/2 and 2/3

ZsBovProt 2/1							
1	CTGCCCCGCA CACCCAGGAG GTGAGTGGCG CTCCTACCGC CGGGGTTGAG	entry	score				
51	CGCGGGGAGG ATCTCGGGGC CCCCCGAAGC CTCGGGTCCA CTTTCAGAGG	entry	score				
	----->					MZF1	90.4
				----->		HSF2	87.2
					----->	deltaE	85.8
	----->					GATA-2	85.4
101	AGGGTTAGCC TTCCTGGCCT TCTGTCCCTG GCGGCCACCC CTCCCAGGCC	entry	score				
151	CGGAGAAACA TCTGGAGAGA GACGGGTGTG GACTCAGGAC CTGCCTCTGG	entry	score				
	----->					HSF2	89.1
	----->					SRY	87.3
	<-----					GATA-1	86.9
						E47	86.5
201	GCAGGTGACC GGAGGGGCCC GCGGATGGCC CTGGGTCCCG GCCAAGTTCA	entry	score				
				----->		GATA-2	92.5
				----->		GATA-1	90.2
	<-----					USF	86.9
	----->					E47	86.5
251	CTGGGCGCGG CGCAAGCCAC ATTGGAGTGG GCTTCCCTGG CGGCTCAGCT	entry	score				
				-----		MyoD	86.0
				<-----		CP2	85.4
301	GGTGAAGAAT CCGCCTACAA TGCGGGAGAC CTGGGCTCGT TGGGTTGGGA	entry	score				
				<-----		CP2	89.6
				-----		Ik-2	88.2
				-----		Lyf-1	87.0
				<--		c-Rel	86.8
				<--		NF-kap	86.6
	---					MyoD	86.0
351	AGATCCCCTG GAGAAGGGAA CTGGCTCCCA GTATCCTGGC CTGGAGAATT	entry	score				
				<-----		GATA-2	91.3
					<-----	NF-kap	88.5
	---					Ik-2	88.2
				<-----		GATA-1	87.8
	-->					Lyf-1	87.0
				-----		NF-kap	86.9
	-----					c-Rel	86.8
	-----					NF-kap	86.6
				<-----		Ik-3	86.0
401	CCCTGCACTG GATAGTCCAT GGGTGGCGGA GTCAGGACAC GACTGAGCGA	entry	score				
	---					NF-kap	88.5
				----->		GATA-2	87.7
	-->					NF-kap	86.9
				----->		GATA-1	86.1
	-----					Ik-3	86.0
ZsBovProt 2/2							
451	CTTTTGCTCA CTTGTCCTC ATGCGTTGGG AAAAGTTGGT GGATGAGACA	entry	score				
	<-----					Nkx-2	90.7
				----->		Ik-2	89.5
				----->		C/EBPb	88.1
				----->		Lyf-1	85.7
				-----		AP-1	85.3
501	GGGGTGGGTG AGAAGCGGAT AAGGGTAAAA GGAGAATGAA ATTCATTTGG	entry	score				
	----->					GATA-1	92.8
	----->					GATA-2	91.7
	----->					GATA-1	91.5
	----->					GATA-X	89.9
				-----		Ik-2	88.6
				----->		GATA-1	87.8
				----->		GATA-3	86.6
	>					AP-1	85.3

```

551 GACCCCCCGG GATTCCGGAG TCTGTAAGAT CAGCAGAAAA GTCTGGTCTT entry  score
----->                                     Ik-2    88.6
      ----->                                     GATA-1  88.6
      ----->                                     GATA-2  87.0
                                     <----->      CdxA    86.4
                                     ----->      HSF2    85.9

601 CCGCCCAGTC AGGGCTGCAG GGCGTGGCTG CCTGGAGCAA TAACACTTCT entry  score
                                     ----->      Tst-1  89.6
                                     ----->      CdxA    86.4
                                     <--->      Ik-2    86.0

651 TCCCTAAGCA GCTTTGAGCC AAACCGGCAG GGCGGGGCGG GGAGGGGCGG entry  score
                                     ----->      Sp1     95.9
                                     ----->      Sp1     94.5
                                     ----->      MZF1    93.0
                                     ----->      Sp1     89.0
                                     -->      Sp1     87.7
                                     <----->      E2F     86.2
      ----->                                     Ik-2    86.0

                                     ZsBovProt 2/3
701 GGCAGGGGCG GGGAGGGGCG GGGAGAGGGC GGGGTGAGCC GGAGGGGCGG entry  score
                                     ----->      Sp1    100.0
      ----->                                     Sp1     95.9
      ----->                                     Sp1     95.9
-->                                     Sp1     94.5
      ----->                                     Sp1     94.5
      ----->      MZF1    93.0
      ----->      MZF1    93.0
      ----->      Sp1     87.7

751 GGTCTGGGTA GGCCCCGCCC AGCTGTCTTG CCCACCCCTT GACAGCCTAG entry  score
-->                                     Sp1    100.0
      <----->                                     Sp1     89.0

801 GTGCTTACAG AGTTAGTCCC AGTCAGGTCT GCGGCAGGTG GAGTGCGAAC entry  score
                                     <----->      USF     89.4
                                     ----->      MyoD    88.4
                                     ----->      E47     85.6
                                     <----->      deltaE   85.2
      ----->                                     Ik-2    85.1

851 CCGTGGCCCT TTGCTGCGCT GCACCCGTGT CCTCGCCGGG TCCCTCGGGT entry  score

901 CTCTCGCTGC GTCTCTCGGA ACACATC entry  score

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Appendix 18

TFSEARCH for CAST PROMOTER TYPE III (Settings: Vertebrate Score threshold:85)

CAST Promoter 3/1, 3/2 and 3/3

ZsBovProt 3/1							
1	TAGCATTCTA	TTGACTTCCC	AGGTGGCTCA	GACGGTAAAG	TGTCTGCCTC	entry	score
					----->	Nkx-2.	90.7
						AP-1	85.6
					<-----	Ik-2	85.5
					<-----	deltaE	85.2
51	CAATGTGGAA	GACCCAGGTT	CAATCCCTGG	GTCGGGAAGA	TCCTCTGGAG	entry	score
					<-----	c-Rel	86.8
101	AAGGAAATGG	CAACCCACTC	TAGTACTCTT	GCCTGGAAAA	TCCCATGGAT	entry	score
					<-----	NF-kap	95.0
					<-----	NF-kap	94.7
					<-----	c-Rel	94.2
					<-----	NF-kap	93.6
					<-----	c-Rel	86.0
151	GGAGGAGCCT	GGCAGGCTAC	AATCTATGGG	GTCGCAAAAA	GTCGGACACG	entry	score
					----->	AML-1a	87.4
201	ACTGAGCAAG	TTCAAGTGTA	AGTGTATTCT	TCCAGAAGGA	AAAGCATTTC	entry	score
					----->	Nkx-2.	100.0
						<-- GATA-X	95.6
						< GATA-3	95.0
						<-- GATA-1	90.3
						Nkx-2.	88.4
						<-- GATA-1	88.0
						<-- GATA-1	85.5
251	CTTATCTCTC	CTCAGTAACA	AAAGGATATT	TATTGGTGCG	AATACGGCGG	entry	score
						GATA-X	95.6
						GATA-3	95.0
						MZF1	93.0
						GATA-1	90.3
						Pbx-1	90.2
						CdxA	90.0
						Ik-2	89.9
						CdxA	89.7
						GATA-1	88.0
						c-Ets	87.7
						SRY	87.4
						Ik-3	87.2
						CdxA	87.1
						CDP CR	87.1
						Ik-1	86.5
						SRY	86.4
						GATA-1	85.5
301	GGAGAGATTG	CTACCTGTGT	AAGATTAAAA	CAGAATAGTG	ACATTTAGCA	entry	score
						MZF1	93.0
						SRY	90.0
						C/EBPb	85.5
						C/EBPa	85.3
						CdxA	85.0
351	AAAGCATTCC	AGGCTAGCCA	AGGCTACATC	TCCCAACTGA	TAGAGAAGGG	entry	score
						Lyf-1	90.9
						GATA-3	88.4
						GATA-1	87.2
						GATA-1	86.9
						GATA-2	86.2
						GATA-1	85.7
						C/EBPb	85.5
						C/EBPa	85.3

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401 GTAGCTGGCC AACAGAAGAA GGGGTAGGTG GCCAACGTTG TCTGCGTTTC entry score
451 AGTTCGCATG CTTTGAGAGA AGATTCTCAG GGCGGAGAGG AATTTATTAG entry score
      <-----
      ----->
      ----->
      -----> CdxA 90.0
      -----> CdxA 89.7
      <-----
      -----> HSF1 89.5
      -----> CdxA 87.1
      -----> Lyf-1 87.0
      -----> AML-1a 85.4
      -----> Pbx-1 85.3

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ZsBovProt 3/2

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501 TGGTCGATTA AGTTTCTTGA TTGCCCATGA ATTGGCTGAG TATATGTATA entry score
      - SRY 92.7
      Nkx-2. 88.2
      <--- CdxA 87.9
      <--- CdxA 87.2
      - CdxA 87.1
      CdxA 87.1
      -----> GATA-1 86.1
      <----- SRY 85.5
      ---> AML-1a 85.4
      - Pbx-1 85.3
      -----> Nkx-2. 85.3

```

```

551 AACACATTTA TTTATGGCAG TATGAATCA TGCATTCTTA TTTTATTACG entry score
      -----> CdxA 100.0
      -----> CdxA 99.3
      -----> SRY 92.7
      < SRY 90.9
      -----> CdxA 90.0
      -----> CdxA 89.7
      --- CdxA 87.9
      -----> CdxA 87.9
      --- CdxA 87.2
      <----- CdxA 87.1
      -----> CdxA 87.1
      <----- CdxA 86.4
      -----> Pbx-1 86.3

```

```

601 TAGTTTAAAA TCCATTCTTA TTTTGAAATG TTTAGTCATA ATGTATAATG entry score
      -----> C/EBPb 93.7
      -----> SRY 90.9
      -----> CdxA 90.7
      <----- C/EBPa 87.3
      -----> CdxA 86.4
      -----> CdxA 85.7
      -----> CdxA 85.7

```

```

651 TACAGTAATT CAGTAGAATT TTGGTTCTGG TAAACCACTC TTTAGAAAGC entry score
      <----- CdxA 93.6
      <----- CdxA 92.9
      -----> STATx 86.5
      -----> S8 86.5
      -----> SRY 86.4
      -----> SRY 85.5
      -----> AML-1a 85.4

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701 AAAATCTGTT ATGGGATATA AAATGTTAAT TTACTCTGTA GTCTAAAAAG entry score
      <----- CdxA 92.9
      -----> HFH-2 90.1
      -----> S8 90.0
      -----> CdxA 87.9
      -----> CdxA 87.2
      ---> SRY 86.4
      -----> Oct-1 86.0

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ZsBovProt 3/3

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751 CGACATGTGG GGGCGTGTGT TCGCACAGAG ACAGCCTTGG AGCGGTAGAC entry score
      <----- USF 88.8

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----->		MyoD	86.0
<-----		N-Myc	85.5
801 CTCTGCCATC AGCGGGTGAC CTGCCTCGCG TTGTTTTGTT AGCCAGCCCG	entry	score	
		SRY	96.4
<-----		GATA-1	91.4
<-----		GATA-2	87.0
	<-----	SRY	86.9
	<-----	SRY	86.4
851 GGGGATGCCA GCGGCGCAGT CAGAGCGGGC ATCGCCCCCT CCGCGAACCG	entry	score	
		GATA-1	89.4
----->		GATA-2	86.2
	<-----	GATA-2	86.2
----->		GATA-1	85.7
901 CGCGGGGCTC CGGGGGCGGT GCTGCCCGGG GCGGGCAGAG GCGGGCGGGG	entry	score	
		MZF1	93.0
	----->	Sp1	89.0
		Sp1	89.0
----->		Sp1	86.3
951 AGCGGCCTGC GTCGCGCGTC CCGGGACCGC CCGGCCCGGT CCCTCTCCTG	entry	score	
>		MZF1	93.0
>		Sp1	89.0
	<-----	Sp1	87.7
1001 GAGCTCGGCT GCGGCTGCAA ACTGAAACCG CGGGGCCGAG CGCAATCGGG	entry	score	
	<-----	GATA-1	93.5
	-----	Ik-2	92.1
	----->	IRF-1	92.0
	----->	IRF-2	90.6
	<-----	GATA-2	88.5
	-----	Ik-1	86.5
1051 AATGCAAACG TTCCGCAAGC	entry	score	
----->		Ik-2	92.1
----->		Ik-1	86.5

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